

CRANFIELD UNIVERSITY



YVONNE H. LANYON

**THE DEVELOPMENT OF A BACTERIAL BIOSENSOR FOR THE
ANALYSIS OF BENZENE IN WORKPLACE AIR**

SCHOOL OF INDUSTRIAL AND MANUFACTURING SCIENCE

Ph.D. THESIS

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Ph.D. THESIS

Academic Year 2004-05

YVONNE H. LANYON

**The Development of a Bacterial Biosensor for the
Analysis of Benzene in Workplace Air**

Supervisor: Dr. I.E. Tothill

December 2004

ABSTRACT

The presence of toxic volatile organic compounds (VOCs) such as benzene in workplace air has accounted for the death of many occupationally exposed workers over the last century. The conventional gas chromatographic method of monitoring benzene is known to be costly, complex and in most cases, laboratory-based. Therefore a need exists for the development of low-cost, easy to use, portable devices that can be used on-site for the rapid evaluation of airborne benzene.

In this thesis, the development of an amperometric bacterial biosensor based on *Pseudomonas putida* ML2 for the detection of airborne benzene is described. Benzene can be used by the bacteria as a sole carbon source, and its aerobic degradation can be measured using a dissolved oxygen electrode. In this work, *P. putida* ML2 cells were immobilised between two cellulose acetate membranes and fixed onto a Clark dissolved oxygen electrode. Biosensor responses were investigated in batch and kinetic (Flow Injection Analysis) mode, and also using screen-printed electrodes. In each case, the response characteristics, sensitivity, reproducibility and lifetime of the sensor were investigated, as well as construction techniques and operational parameters.

The applicability of the biosensor for the analysis of air samples containing benzene was investigated. Air samples were collected from an exposure room of controlled concentration using charcoal adsorption tubes, and benzene extracted with solvent desorption using dimethylformamide (DMF). DMF proved to be compatible for use with the biosensor, causing minimal interference with the sensor response and causing no toxic effects on the bacterial cells. The biosensor displayed a linear detection range between 0.025 – 0.15 mM benzene based on standard solutions containing a maximum of 2% DMF, with a response time of 6 minutes. This linear detection range allowed the analysis of air containing between 3 – 16 ppm benzene, based on a 60-minute sampling period. The inter-assay reproducibility of the sensor response to standard benzene calibration curves under such conditions gave a 3% variation coefficient based on 5 separate assays ($n = 17$) using the same bacterial membrane.

The FIA system was easily transported to an *in situ* location for the air sample analyses, and a correlation was obtained between the biosensor and gas chromatography (GC) results for the exposure room air samples investigated. Moreover, the biosensor displayed no interference to other benzene related compounds in the BTEX (benzene, toluene, ethylbenzene, xylene) range.

Overall, this thesis has described the development of an alternative method for the monitoring of benzene in workplace air, using a bacterial biosensor based on Flow Injection Analysis. Advantages over the conventional GC methods including ease of operation, cost-effective production and portability demonstrate that the *P. putida* ML2 biosensor has potential applications as an alternative means for the rapid analysis of workplace air containing benzene.

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Italy

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Appendix 1. Publication. Lanyon, Y.H., Marrazza, G., Tothill, I.E., Mascini, M. (2004). Flow injection analysis of benzene using an amperometric bacterial biosensor. *Analytical Letters* 37(8), 1515-1528.

Appendix 2. Publication. Lanyon, Y.H., Marrazza, G., Tothill, I.E., Mascini, M. (2005). Benzene analysis in workplace air using an FIA-based bacterial biosensor. *Biosensors and Bioelectronics* 20, 2089-2096.

Appendix 3. Poster presentation. XVIIth International Symposium of Bioelectrochemistry and Bioenergetics, Florence, 19-24 June 2003.

Appendix 4. Poster presentation. The Eighth World Congress on Biosensors, Granada, 24-26 May 2004.

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ABBREVIATIONS

Ag/AgCl: Silver/Silver Chloride.
ACGIH: American Conference of Governmental Industrial Hygienists
API: American Petroleum Institute.
Batch Mode: Biosensor used in a magnetically stirred solution of potassium phosphate buffer, in a temperature controlled reaction vessel.
BTEX: Benzene, Toluene, Ethylbenzene, Xylene.
CS₂: Carbon disulphide.
CV: Coefficient of Variation.
DEFRA: Department of the Environment, Food, and Rural Affairs.
DMF: N,N-Dimethylformamide
DMSO: Dimethylsulfoxide
DNA: Deoxyribose nucleic acid.
EEC: European Economic Community
EPA: Environment Protection agency (USA).
FIA: Flow Injection Analysis.
GC: Gas Chromatography or Gas Chromatogram.
GM: Genetically Modified.
Kinetic Mode: Biosensor used within the flow injection analysis system.
LB: Luria Bertani (bacterial medium).
MeOH: Methanol
NADH: Nicotinamide adenine dinucleotide.
NIOSH: National Institute of Occupational Safety and Health.
OSHA: Occupational Safety and Health Administration (USA).
ppm: parts per million.
Pt: Platinum.
RNA: Ribose nucleic acid.
SEPA: Scottish Environment Protection Agency.
SEPAC: Canadian Association of Petroleum Producers.
SD: Standard Deviation.
SPE: Screen-Printed Electrode.
STEL: Short-Term Exposure Limit.
TWA: Time-Weighted Average.
VOC: Volatile Organic Compound.
WHO: World Health Organisation.

CHAPTER 1

INTRODUCTION

CHAPTER 1: INTRODUCTION

1. Introduction

The presence of toxic volatile organic compounds (VOCs) such as benzene in workplace air has accounted for the death of many occupationally exposed workers over the last century (DEFRA, 2003). Benzene is included in the list of carcinogenic substances in Annex I of the European Directive 67/548/EEC. The current permissible exposure level to benzene in the workplace in Europe is 1 ppm for an eight-hour time weighted average, with a 5 ppm short-term exposure limit (Council Directive 90/394/EEC amended by Directive 97/42/EC).

To ensure that employees are not exposed to levels of volatile carcinogens higher than the legislative limit, accurate air tests must be relied upon. Conventional methods of air monitoring are known to be costly, time consuming, and require skilled personnel (Harper, 2000). The use of a bacterial biosensor is proposed in this thesis as an alternative method for the analysis of benzene in workplace air samples, with the added advantages over the conventional gas chromatographical (GC) methods of low operation costs, ease of use, and portability for *in situ* measurements.

Chapter 1 will review benzene and its associated toxicity and legislation, and describe the conventional methods of air sampling and analysis. The chapter will also review the use of bacterial biosensors for environmental monitoring, and describe in detail the biological component and transducer of the biosensor developed in this project.

1.1. Benzene

Benzene is a natural component of petroleum, and was first discovered by Faraday in compressed oil gas in 1825. It is obtained in the coking of coal and in the production of illuminating gas from coal. Benzene can also be manufactured by catalytic reforming

and separation of aromatic compounds, thermal or catalytic dealkylation of toluene, and toluene disproportionation; from pyrolysis gasoline (Budavari, 1996).

Benzene consists of a six-carbon ring structure, C_6H_6 , and is physically a clear, colourless, volatile, highly flammable liquid with a characteristic odour. It is slightly soluble in water (0.188% at 23.5°C) and miscible with alcohol, chloroform, ether, carbon disulphide, carbon tetrachloride, glacial acetic acid, acetone, and oils (Budavari, 1996).

The benzene molecule can be represented either by the Kekulé ring formula as shown in Figure 1.1.(1), or in a more electronically accurate way, which indicates that all carbon – carbon bonds are identical (Figure 1.1.(2)). The length of the carbon – carbon bonds in benzene are intermediate in length between single and double carbon – carbon bonds. X – ray diffraction has revealed that all 12 atoms of benzene lie in a single plain and all bond angles are exactly 120° (Fruscella, 2002). The chemical reactions which benzene can undergo are substitution, addition and cleavage of the ring. Various substitutions are the most important reactions for industrial applications (Fruscella, 2002). In the present work, the cleavage of the benzene ring during its aerobic degradation by *Pseudomonas putida* ML2, had been used as the basis of a biosensor for its detection in workplace air samples.

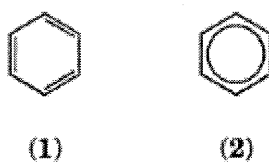


Figure 1.1. The Chemical Structure of Benzene, C_6H_6 ; (1) the Kekulé ring formula, (2) the electronically accurate version of the benzene ring.

Benzene, an industrial chemical, is a major raw material used extensively as a solvent in the chemical and pharmaceutical industries. It is made mostly from petroleum sources, and is used by various industries to make other chemicals, such as styrene, cumene (for various resins), and cyclohexane (for nylon and synthetic fibres). Benzene is also used

for the manufacturing of some types of rubbers, lubricants, dyes, detergents, drugs, and pesticides. Natural sources of benzene include volcanoes and forest fires, and it is also a natural part of crude oil, petrol, and cigarette smoke (NTP, 2002).

Industrial processes are the main sources of benzene in the environment (EPA, 2002). However, levels in the air can increase due to emissions from burning coal and oil, benzene waste and storage operations, motor vehicle exhaust, evaporation from petrol stations, and the use of industrial solvents. In addition, since tobacco contains high levels of benzene, tobacco smoke is another source of benzene in air. Industrial discharge, disposal of products containing benzene, and petrol leaks from underground storage tanks can release benzene into water and soil (EPA, 2002).

The Environment Protection Agency (EPA, 2002) also states that benzene can pass into air from water and soil surfaces – once in the air, it reacts with other chemicals and breaks down within a few days. Benzene can also attach to rain or snow and be carried back to the ground. In water and soil, benzene breaks down more slowly; it is slightly soluble in water and can pass through the soil into underground water. Benzene does not, however, accumulate in the environment (EPA, 2002).

1.1.1. Benzene Toxicity and Legislation

The primary routes of potential human exposure to benzene are inhalation and dermal contact, with the former being the dominant pathway, accounting for more than 99% of the total daily intake (WHO, 2000). At room temperature and atmospheric pressure, benzene is sufficiently vaporized to pose a significant health hazard (Fruscella, 2002). Individuals employed in industries that make or use benzene may be exposed to the highest levels of this hydrocarbon. These industries include benzene production (petrochemicals, petroleum refining, and coke and coal chemical manufacturing), rubber tyre manufacturing, and storage or transport of benzene and petroleum products containing benzene. Other workers who may be occupationally exposed to benzene

include steel workers, printers, rubber workers, shoemakers, laboratory technicians, and petrol station employees (EPA, 2002).

DEFRA (2002) states that benzene is readily absorbed into the body when breathed into the lungs with about half being retained. As it is more soluble in fat than in water, it is distributed in the body to fatty tissues including the brain and bone marrow where blood cells are made. Without further exposure, the benzene is eliminated by chemical breakdown in the body or by metabolic excretion in the urine, 80% being eliminated within about two days (DEFRA, 2002).

When benzene enters the body, it is transformed enzymatically into metabolites such as benzene oxide, phenol, hydroquinone, and catechol which are reported to be more toxic than benzene itself - they are considered to accumulate in bone marrow and play important roles in causing leukaemia (WHO, 2000). The mechanism of oxidative DNA damage induced by benzene metabolites was investigated by Kawanishi and Hiraku (1998). It was reported that hydroquinone is oxidised to the semiquinone radical and undergoes further oxidation to 1,4-benzoquinone, which in turn, is reduced to the semiquinone radical by NADH. During the auto-oxidation, oxygen is dismutated to hydrogen peroxide, which interacts with copper to generate active species capable of causing DNA damage. It was thus concluded that benzene metabolites would cause cancer through oxidative DNA damage (Kawanishi and Hiraku, 1998). The WHO (2000) also reviewed several studies of DNA damage caused by benzene metabolites.

The first case reports of benzene-induced blood diseases date from 1897 (EPA, 2002). The first report of benzene causing leukaemia was published in 1928, and later in 1948, the American Petroleum Institute published a toxicological review of benzene, noting that benzene causes leukaemia and that the only safe level of exposure to benzene was zero ppm (parts per million). The most thorough and well investigated study, which has also been the main study used for quantitative risk assessment, is on workers that used to be employed in the manufacture of rubber film, the so-called Pliofilm cohort (WHO, 2000). Table 1.1 details an epidemiological study of benzene among Pliofilm rubber

workers that revealed significantly increased risks of leukaemia, and how regulations for benzene exposure have developed since then (Infante, 2000).

Table 1.1.
Benzene: Early warnings and actions (Source: Infante, 2000).

Year	Action
1897	Santessen report on observed aplastic anaemia in Sweden and other reports show that benzene is a powerful bone marrow poison.
1926	Greenburg and colleagues observe abnormally low white blood cell counts in benzene workers.
1928	Dolore and Borgomano publish the first case of benzene induced-leukaemia.
1939	A number of investigations recommend the substitution of benzene with other solvents, but this was not implemented.
1946	American Conference of Governmental Industrial Hygienists (ACGIH) recommends a limit of 100 ppm for benzene exposure, even though some cases of benzene poisoning were associated with levels of 25 ppm and 10 ppm.
1947	Recommended value reduced to 50 ppm.
1948	Further reduced to 35 ppm.
1948	American Petroleum Institute (API) concludes that the only absolutely safe level is zero, but recommends 50 ppm or less.
1957	ACGIH lowers recommended exposure to 25 ppm.
1950s – 60s	Obvious lack of precaution for workers exposed to benzene in many parts of the world with fatal consequences.
1977	First cohort study published of workers linking benzene exposure directly to leukaemia.
1977	Based on these results, the US Department of Labor wants to reduce exposure to 1 ppm, but is challenged in the courts by API.
1978	Benzene was voluntarily withdrawn from consumer products in the United States.
1980	US Supreme Court issues the Benzene Decision, severely limiting regulatory actions.
1987	New benzene standard of 1 ppm. This 10 year delay caused more than 200 deaths in the United States.
1996	Studies showing benzene-related diseases from 1 ppm level of exposure.
2001	Petrol contains benzene, giving public exposure risk.

Since then, many epidemiological studies of benzene have been undertaken, which have established benzene as a cause of various human hematologic cancers and diseases. Table 1.2 shows a global study of the number of deaths of workers occupationally exposed to benzene.

Among the other associated health effects, acute (short-term) inhalation exposure to benzene may cause drowsiness, dizziness, headaches, and unconsciousness in humans (Budavari, 1996). These symptoms stop when the exposure ceases, however, death may result from exposure to very high levels of benzene. Ingestion of large amounts of benzene may result in vomiting, dizziness, convulsions, and death in humans. Exposure to benzene liquid and vapour may irritate the skin, eyes, and upper respiratory tract. Animals acutely exposed show anaemia, bone marrow cell depression, and immunological effects from inhalation and oral exposure (EPA, 2002).

The EPA (2002) states that chronic (long-term) inhalation exposure has caused various disorders in the blood, including reduced numbers of red blood cells and aplastic anaemia, in occupational settings. Toxicity to the humoral and cellular immune systems has also been seen. In addition to these effects, menstrual disorders and a decreased size of ovaries have been observed in women occupationally exposed to high levels of benzene. Adverse effects on the foetus, including low birth weight, delayed bone formation, and bone marrow damage, have also been observed from exposure of pregnant animals to benzene by inhalation (EPA, 2002). WHO (2000) has reviewed several studies on the exposure effects of benzene including; haematotoxicity (bone marrow depression) and immunological, reproductive, genotoxic and carcinogenic effects.

Among the diseases that have been associated with benzene are acute myelogenous leukaemia (AML), acute lymphocytic leukaemia (ALL), chronic myelogenous leukaemia (CML), chronic lymphocytic leukaemia (CLL), hairy cell leukaemia (HCL), non-Hodgkin's lymphoma (NHL), Hogkin's disease, multiple myeloma, myelodysplastic syndrome (MDS), aplastic anemia, pancytopenia, other cytopenias, myelofibrosis, and polycythemia vera (Metzger, 2001).

Table 1.2.
Leukaemia deaths in workers occupationally exposed to benzene (Source: DEFRA, 2002)

Study	Number of Subjects	Number of Deaths	Exposure Estimates (ppb-years unless otherwise indicated)	SMR †	(95% CI)‡
Goodyear Pliofilm Plant Workers employed in a department where benzene was used for at least one day during 1940-1965. Deaths occurring after 1950 counted.	1165	2 2 2 3	1-39,990 40,000-199,990 200,000-399,990 >400,000	109 322 1186 6637	(12-394) (36-1165) (133-4285) (1334-19,393)
Chemical Workers Workers exposed to benzene for at least 6 months between 1946 and 1975 (only the cumulative exposure group is included here).	3536	5* 5* 5*	500 2000 >2000 Estimates for 30 year equivalent levels of exposure.	91* 147* 175*	(29-213) (48-343) (57-409)
Dow Chemical Workers Workers exposed in organic and resin synthesis (excludes confounding exposures).	888	3	1500 25,400 351,000 Estimates for individual cases.	162	(33-461)
Coke Oven and other Coal Product Workers (National Smokeless Fuels) (British Steel Corporation)	3812 2708	3 2	310-1320ppb** Estimated exposure concentrations.	42 41	(9-123) (5-147)
Chinese Factory Workers Workers exposed to benzene in painting, shoe-making, rubber synthesis, leather, and adhesive and organic synthesis factories.	15,643 12,187	17 8	2000- 345,000ppb** Concentrations where leukaemias occurred.	501 830	(292-802) (358-1635)
* All lymphopoietic and haematopoietic cancers.					
** No data on duration of exposure given.					
† Standardised Mortality Ratio (The ratio of the number of deaths observed in the study group or population to the number that would be expected if the study population had the same specific rates as the standard population, multiplied by 100).					
‡ CI: Confidence Interval, the 95% CI is the range in which, allowing for variability in study populations, there is a 95% chance of the true result falling. If the value 100 is not included in the 95% CI shown in the Table then there is only a 1 in 40 chance that the increase in leukaemia deaths in the benzene-exposed group is an erroneous result (p<0.025).					

The preceding and present sections have highlighted the widespread use and existence of benzene both in the workplace and in the environment, and the associated toxicity of employee exposure. For this reason, levels of benzene in various fields are strictly regulated under several legislative Acts. Releases of the benzene part of BTEX (Benzene, Toluene, Ethylbenzene, Xylene) compounds are controlled in the UK through the National Air Quality Strategy; regulations on pollution of surface waters (SI 1997/2560); and Pollution, Prevention and Control (PPC) regulations. European Directives controlling emissions of BTEX compounds include those concerned with pollution of aquatic environments (76/464); assessment and management of ambient air quality (96/62/EC); control of solvents (99/12/EC); protection of health and safety at work (98/24/EC); and benzene is also listed as a "priority substance" for the proposed Water Framework Directive (SEPA, 2004).

The exposure of employees to benzene vapours in the workplace in Europe is regulated by Council Directive 90/394/EEC, which protects workers from the risks related to exposure to carcinogens at work. This directive was updated in 1997 with the introduction of the European Air Quality Directive 97/42/EC. In both Europe and The United States, the current permissible exposure level to benzene in the workplace is 1 ppm in air (3.25 mg/m^3 of air) for an eight-hour time-weighted average (TWA) with a short-term exposure limit (STEL) of 5 ppm for any 15-minute period (OSHA 2002, and European Directive (97/42/EC).

1.2. Benzene Monitoring in the Workplace

Section 1.1 has highlighted the health effects of benzene in a range of occupational settings. European legislation states that occupational exposure limit values must be regarded as an important component of the general arrangements for the protection of workers (Directive 97/42/EC). Such guidelines rely on accurate analytical techniques for the measurement of airborne carcinogens, such as benzene, in the workplace.

Most of the analyses performed for the monitoring of organic pollutants in the environment cannot be done without the application of pre-concentration and sample

pretreatment techniques (Matisová and Škrabáková, 1995). Samples are most often not compatible with direct injection into the analytical instrumentation; analytes can be present in matrices incompatible with the gas and/or liquid phases of such systems (such as soil or sediments), or the concentration of the analytes is lower than the detection limit. Thus, it is usually necessary to perform sample preparation steps prior to analysis. This mainly consists of pre-concentration of the analytes, isolation of the analytes from the matrix and removal of interfering compounds (Matisová and Škrabáková, 1995). Methods for determining the concentration of benzene and its homologues in air comprise stages of analyte enrichment using adsorption methods, desorption of the compounds, and subsequent quantitative determinations with the use of gas chromatography (Czaplicka and Klejnowski, 2002).

For any type of air sampling exercise, it is possible to make choices in the type of sorbent material used, the method of sampling (e.g. active or diffusive), the method by which the trapped vapours are removed (or not) from the sorbent for analysis, and the method of analysis (Harper, 2000). Different techniques may be more or less applicable depending on whether the measurement period is short or long-term, the concentrations are high or low, and whether the sample is meant to be personal or site-specific (Harper, 2000). The following 3 subsections will respectively discuss the collection, adsorption, and desorption methods commonly used for the determination of airborne levels of benzene.

1.2.1. Air Sample Collection

Decisions regarding the most appropriate sampling techniques for assessing benzene exposure should be made through consultation with a qualified occupational hygiene professional. Table 1.3 summarises the range of monitoring methods available for the determination of benzene in ambient air, although the principles are the same for indoor measurements (Skov *et al.*, 2001).

Table 1.3.

Summary of monitoring methods for the determination of benzene in ambient air (Skov *et al.*, 2001)

No.	Method	Average sampling time	Detection limit ($\mu\text{g m}^{-3}$)	Sample flow/uptake rate (ml min^{-1})	Advantage	Disadvantage
1	Differential optical absorption spectroscopy (DOAS)	30 s	1.0	Not relevant, measurements are in open path	High time resolution, real-time data, no chemical interference, multi-component measurements.	High cost, zero point offset problems, optical interference from O_3 and toluene.
2	BTEX monitors	15-30 min	0.16	10-30 min	Flexible time resolution, real-time data, analysis of BTEX simultaneously.	High cost, need of a monitoring hut with air condition, need of a carrier gas supply.
3	On-line GC instruments	$\frac{1}{2}$ to 1 h	0.1	10-30 min	High time resolution, real-time data, analysis of C2-C12 simultaneously.	High cost, need of a monitoring hut with air condition, need of a carrier gas supply, some instruments need supply of liquid N_2 .
4	Canisters with thermal desorption	Grab sampling	0.3	Grab samples	No electrical power is needed, same comments as for no. 2 and 3 depending on the analysis technique.	Only grab samples can be taken (discontinuous measurements), difficult to clean.
5	Pumped sampling on tubes with thermal desorption-GC	15 min-24 h	0.14-4	50-150	Low cost, many sampling sites with one analytical instrument, work with toxic species is minimised, flexible time resolution.	Expensive thermal desorption system is needed, strongly labour-demanding.
6	Pumped sampling on tubes with solvent extraction ^a -GC	3-24 h	0.14-4	50-750	Low cost, many sampling sites with one analytical instrument, analysis is made on a cheap GC, flexible time resolution.	Toxic solvents are needed, strongly labour-demanding.
7	Diffusive sampling with thermal desorption-GC	1-4 weeks	0.5	~ 1	No electrical power is needed, long sampling time possible, same comments as for no. 3.	Long sampling is needed, expensive thermal desorption system is needed.
8	Diffusive sampling with solvent extraction ^a - GC	1-4 weeks	0.5	$\sim 1^b$	No electrical power is needed, long sampling time possible, same comments as for no. 4.	Long sampling is needed, toxic solvents are needed.

^aThese sampling methods have been used in combination with the biosensor for air sample analysis, Chapter 6.

^bRadiello diffusion tubes have an uptake rate of 80 ml min^{-1} (Radiello®, 2004).

In summary, there are four general approaches used to sample benzene from air in the workplace (SEPAC, 1997), including:

- **Active sampling:** pumping air through a benzene collection medium.
- **Passive sampling:** collecting benzene through diffusion onto a collection medium.
- **Grab sampling:** Pulling a sample of contaminated air into a containment device.
- **Real-time sampling:** Read-out generated from an electronic measuring device.

Measurement of benzene in an individual's breath or blood or the measurement of breakdown products in the urine (phenol) can estimate personal exposure. However, the tests must be done shortly after exposure and are not helpful for measuring low levels of benzene (EPA, 2002).

Measurements taken for the purpose of determining employee exposure to benzene are best taken so that the representative average 8-hour exposure may be determined from a single 8-hour sample or two 4-hour samples. Short-time interval samples (or grab samples) may also be used to determine average exposure level if a minimum of five measurements are taken in a random manner over the 8-hour work shift. Random sampling means that any portion of the work shift has the same chance of being sampled as any other. The arithmetic average of all such random samples taken on one work shift is an estimate of an employee's average level of exposure for that work shift.

Air samples should be taken in the employee's breathing zone (air that would most nearly represent that inhaled by the employee). Sampling and analysis must be performed with procedures meeting the requirements of the standard (OSHA, 2002). Moreover, the concentration of benzene reported from workplace samples must be translated into an expression of worker exposure. For example, a worker may spend two hours in a location where area measures are high and six hours in an environment where area measures are low; the final expression of exposure would be averaged accordingly (SEPAC, 1997). Personal exposure measurements have been used in the present work for the assessment of airborne levels of benzene in a Prague factory, Czech Republic, as described in Chapter 6.

The standard method utilised by the United States' Occupational Safety and Health Administration (OSHA) for monitoring employee exposures to benzene involves the collection of benzene vapour on charcoal absorption tubes by active sampling, using pre-calibrated air pumps (OSHA, 2002). This method is also used in Europe (WHO, 2000), and allows the sampling of a large volume of air in a relatively short period of time (Table 1.3), hence a greater collection of benzene vapour on the solid sorbent.

The less widely used passive air sampling method is an alternative to the pumped-tube method, but requires a much longer sampling period due to the slow uptake rate of benzene on the sorbent (Skov *et al.*, 2001). The principle of diffusive sampling is the uptake of compounds on a sorbent cartridge described by Fick's 1st law ($F = -DdC/dx$, where F is the flux, D is the diffusion coefficient, C is the concentration, and x is the diffusion path length). Radiello[®] diffusion tubes have been used for air sample analysis in the present work as described in Chapters 2 and 6, which have an uptake rate of 80 ml min⁻¹ (Radiello[®] 2004).

Solid phase micro extraction (SPME) has also been compared to the standard charcoal/solvent desorption active sampling technique for solvent vapour monitoring by Li *et al.* (2001). For VOCs, this method combined sample collection and concentration in one step using a mixed phase carboxen/polydimethylsiloxane (PDMS) fibre, with thermal desorption directly into the GC instrument. A greater sensitivity and shorter sampling time was reported using SPME with respect to the active sampling technique. However, some problems existed for the calibration of the fibre, and the overall procedure is expensive (Li *et al.*, 2001).

1.2.2. Sample Pre-concentration

Solid sorbents are used to sample hydrocarbon gases by trapping the species on the active sites of the surface of the sorbent (Boubel *et al.*, 1994). Activated charcoal, Carbotrap B, Tenax, Carbopak B and Carbopak B HT, Carbosita B and Amborsorb XE-340 are the sorbents most frequently used in analyte enrichment (Czaplicka and Klejnowski, 2002). Adsorption by means of activated charcoal is the most widely employed sampling method for volatile airborne pollutants in the field of industrial hygiene, both by active and passive sampling devices (Matisová and Škrabáková, 1995).

Coconut shells are the most abundant and cheapest starting material, and are used in the majority of NIOSH and OSHA methods, and widely in official methods in European countries (Harper, 2000).

Activated carbon has a very complex surface structure containing a wide range of functional groups including phenolic, carboxylic, carbonylic, aldehydic, etheric, peroxidic, quinone and lactone groups (Matisová and Škrabáková, 1995). The principal binding mechanisms include hydrophobic interactions, charge-transfer complexation, hydrogen bonding, cation exchange and other specific interactions. The surface of such carbons possesses a high sorption capacity, but it is very heterogeneous. Low recovery of many organic solutes is often associated with the strength of multiple binding interactions, which provide an efficient adsorption mechanism accompanied by inefficient solvent elution, leading to a low overall recovery. Irreversible adsorption can occur in some cases as well as catalytic transformations to different products (Matisová and Škrabáková, 1995).

During active air sampling, it is critical that the breakthrough sampling volume, the amount of air passing through the tube that saturates its absorptive capacity, not be exceeded. The breakthrough volume is dependent on the concentration of the gas being sampled and the absorptive capacity of the sorbent. This means that the user must have an estimate of the upper limit of concentration for the gas being sampled (Boubel *et al.*, 1994).

1.2.3. Sample Desorption

Once the sample has been collected on the solid sorbent, the tube is sealed and transported to the analytical laboratory. To recover the sorbed gas, two techniques may be used. The tube may be heated while an inert gas is flowing through it. At a sufficiently high temperature, the absorbed molecules are desorbed and carried out of the tube with the inert gas stream (thermal desorption). The gas stream may then be passed through a pre-concentration trap for injection into a gas chromatograph for chemical analysis. The second technique is liquid extraction of the sorbent (solvent desorption), and subsequent gas or liquid chromatography (with sample evaporation

upon injection into the analytical instrument in the former case). Sometimes a derivitisation step is necessary to convert the collected material chemically into compounds which will pass through the column more easily (Boubel *et al.*, 1994).

The conventional method for monitoring employee exposure to benzene incorporates the collection of benzene vapour on charcoal adsorption tubes, which are desorbed using carbon disulphide (CS₂), and subsequently analysed by gas chromatography (GC) (OSHA, 2003). For solvent desorption, the solvent to analyte ratio is usually around 1000:1 or more for effective recovery so it is important to choose a solvent that will not interfere with the detection of the analyte. The solvent must also be capable of stripping the analyte(s) from the sorbent with a high degree of efficiency and reproducibility (Harper, 2000). However, the recovery is known to be influenced by several other thermodynamic and kinetic factors: the amount of analyte, the presence of other adsorbed substances, the humidity of the sampled air, the ratio between the amount of charcoal and the desorbing liquid, the time and the temperature of contact, and the particular batch of charcoal (Matisová and Škrabáková, 1995).

CS₂ is the most common solvent used for the desorption of benzene from a charcoal sorbent (Harper, 2000). It has a high heat of adsorption on activated carbon, displacing other molecules, and is a good solvent for non-polar compounds (Harper, 2000). However, it is also highly toxic with a high vapour pressure (275 mm Hg at 20°C), hence it is very difficult to avoid human contact with the vapours. Moreover, possible evaporation of CS₂ during and after addition of precise amounts to the charcoal constitutes a possible source of error (Johansen & Wendelboe, 1981). Further solvents used for desorption of VOCs from solid sorbents include; methylene chloride, methanol, dimethylsulfoxide, and acetonitrile. However, certain combinations of these are usually required for effective benzene recovery from charcoal (Harper, 2000).

The solvent chosen for sample desorption and analysis with the biosensor used in the present work was N,N-Dimethylformamide (DMF). DMF has been tested and compared to CS₂ as an alternative solvent for the desorption of organic vapours from charcoal (Johansen & Wendelboe, 1981). Although DMF is in the same toxicity range as CS₂, its

low vapour pressure (2.65 mm Hg at 20°C) makes it easier to handle both in safety and analytical senses (though it can be absorbed through the skin). DMF was found to be less efficient than CS₂ for the desorption of hydrocarbons from charcoal, resulting in an 80% recovery for benzene (Johansen & Wendelboe, 1981). However, it was reported that a desorption efficiency close to 100% does not necessarily result in a better quantification than a less efficient desorption, such as 80%, apart from a gain in sensitivity. NIOSH air sampling guidelines state that analyte recovery should be 75% or greater (Kennedy *et al.*, 1995). Johansen & Wendelboe (1981) also reported that DMF provided a greater sample stability after desorption than CS₂, which is important if samples are left for several days prior to analysis.

Section 1.2 has detailed the conventional methods of benzene monitoring in air, and the various methods of sample collection, pre-concentration and desorption, with analysis most commonly by gas chromatography. Table 1.3 highlighted the main advantages and disadvantages of these techniques, and in most cases, the disadvantages have been the high costs and labour demand associated with such methods. Consequently there is a need for alternative lower cost, and easier to use devices for the routine analysis of workplace air, to comply with legislative exposure levels. A bacterial biosensor has been proposed in this thesis, to offer an alternative means of benzene monitoring in the workplace, offering the aforementioned advantages. Section 1.3 will therefore review microbial biosensors including their function and practical use for environmental monitoring.

1.3. Biosensors

A biosensor is a device that detects, transmits and records information regarding a physiological or biochemical change. Technically, it is a probe that integrates a biological component with an electronic transducer thereby converting a biochemical signal into a quantifiable electrical response (Tothill and Turner, 2003). According to Turner (2002), the term biosensor has been used historically for a number of devices either used to monitor living systems or incorporating biotic elements. He further states that the current consensus is that the term should be reserved for sensors, which

have more rapid response times with respect to measurements in a closed chamber (Buerk, 1993). A batch system has also been used in the present work (Chapter 4).

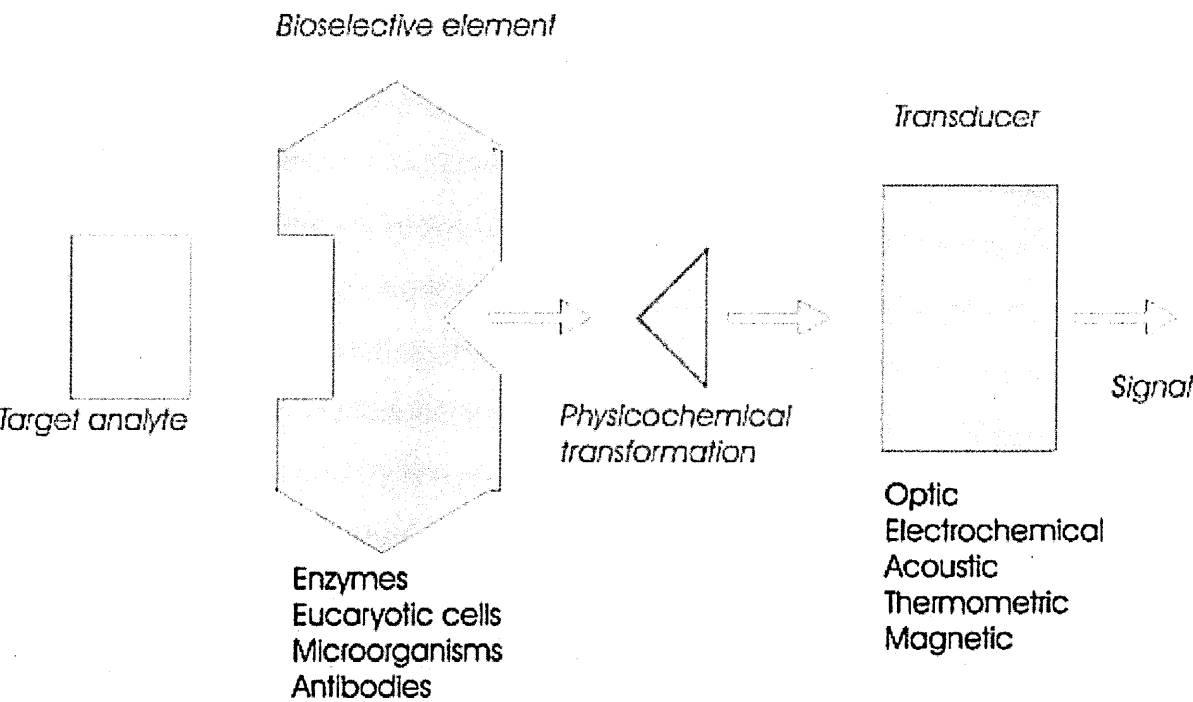


Figure 1.2. Scheme of a generic biosensor (Turner *et al.*, 2000)

Major attributes of a good biosensing system are its specificity, sensitivity, reliability, portability (in most cases), ability to function even in optically opaque solutions, real time analysis and simplicity of operation (D’Souza, 2001). Since the method and instrumentation finally chosen has to be respectively simple and of reasonable size, and the system inexpensive and easy to use, potentiometric and amperometric electrodes have been extensively adopted (Tran, 1993).

1.3.1. Microbial Biosensors

Microbial biosensors are devices incorporating a biological sensing element (a microorganism) that can specifically recognise species of interest, either intimately connected to or integrated within a suitable transducing system. The transducer

functions to quantitatively convert the biochemical signal into an electronic signal that can be suitably processed and outputted (D'Souza, 2001).

Microbial Biosensors can be based on respiration activity, or on the measurement of electrochemically active metabolites. In case of the respiration activity, consumption of dissolved oxygen by aerobic microorganisms, caused by the assimilation of specific compounds of interest, can be detected by an oxygen electrode. These changes can then be employed to estimate substrate concentrations in the sample (Karube and Suzuki, 1990). The second type of microbial biosensor detects electrochemically active metabolites such as H_2 , CO_2 , NH_3 and organic acids, which are secreted from microorganisms. Transducers utilized in this kind of sensor are photodetectors, potentiometric or amperometric electrodes, and thermistors (Blum and Coulet, 1991). The most widely used types of transducers are electrochemical and optical; with the favoured electrochemical method being amperometry (Bentley *et al.*, 2001).

Microbes have a number of advantages as biological sensing materials in the fabrication of biosensors. They are present ubiquitously and are able to metabolise a wide range of chemical compounds. Furthermore, microorganisms have a great capacity to adapt to adverse conditions and to develop the ability to degrade new molecules with time. Microbes are also amenable for genetic modifications through mutation or through recombinant DNA technology and serve as an economical source of intracellular enzymes (D'Souza, 2001). Non-viable cells obtained after permeabilisation or whole cells containing periplasmic enzymes have mostly been used as an economical substitute for enzymes. Viable cells make use of the respiratory and metabolic functions of the cell, the analyte to be monitored being either a substrate or an inhibitor of these processes (D'Souza, 2001).

Microbial biosensors offer the following advantages over pure enzymatic biosensors (Racek, 1995):

- Isolation, purification, and immobilisation of enzymes is often difficult.

- Some enzymes can lose their activity during isolation or immobilisation if this process leads to the damage of the active centre or to the disintegration of enzyme complexes.
- Enzymes in the cell's natural environment are usually extremely stable.
- Multi-step enzyme reactions in intact cells can be used, making it possible to avoid the preparation of complicated artificial multi-enzyme systems.
- Coenzymes and activators are often present in cells, and thus, it is not necessary to add them into the system; the cell itself usually cares for their effective regeneration.
- Cultivation of microbial cells is easy and cheap when compared with the preparation of a pure enzyme.

It can be said that the cell represents the most suitable milieu for enzymes – they are “naturally immobilised” within the cell, and is why they are marked by great stability.

Nevertheless, as with all aspects of research, there are also some disadvantages and difficulties associated with a biosensor such as this. Reaction and recovery times can run more slowly because the substrate must pass through the cell membrane to the specific enzyme. Solutes can pass only via specific translocation systems, either with an active transport system or by facilitated diffusion; passive transport by diffusion is of minor importance. Active transport, which allows accumulation of substrates against a concentration gradient, requires highly specific carrier proteins and consumes metabolic energy (Mulchandani and Rogers, 1998).

Other variable parameters of microbial biosensors include cell death on the immobilisation surface (caused by immobilisation or storage techniques). Furthermore, other metabolic pathways in the cells can be the source of side products that are also registered by a transducer (Racek, 1995).

1.3.2. Immobilisation of Microorganisms

Early work on microbial biosensors utilised mainly gel entrapment methods of immobilisation (e.g. polyacrylamide and collagen). Nowadays most microbial biosensors are constructed using membrane entrapment methods (Karube and Suzuki,

1990). Membrane entrapment is probably the mildest and simplest immobilisation technique. Passive trapping of cells into the pores or adhesion onto the surfaces of cellulose or synthetic membranes has been well documented (Mulchandani and Rogers, 1998). Membrane immobilisation techniques offer the major advantage that cells immobilised via adhesion are in direct contact with the liquid. The associated drawback, however, is the possibility of cell wash out during continuous use (D'Souza, 2001).

Many methods have been employed for immobilising microorganisms (Chiabata and Wingrand, 1983). The immobilisation technology plays a major role in the construction of a microbial biosensor because it does not only help to form the required close proximity between the biological material and the transducer, but also helps to stabilise it for re-use (D'Souza, 2001). Biomaterials can be immobilized either through adsorption, entrapment, covalent binding, cross-linking or a combination of all these techniques (Bikerstaff, 1997). Selection of a technique and/or a support would depend on the nature of the biological element and the substrate as well as on the configuration of the transducer used (Kumar *et al.*, 1992). Additionally, the following points must also be considered when choosing an immobilisation technique (D'Souza, 2001): (i) if an oxygen electrode-based sensor is used, gas permeability through the microorganism-immobilized membrane is crucial; and (ii) if the biosensor system is based on the function of viable cells, gentle immobilisation techniques should be employed.

1.3.3. Microbial Biosensor Construction and Modes of Operation

To construct a microbial biosensor, it is important to understand the mechanism of the sensing system and consider the factors that affect the stability, dynamic range, and response time to optimise performances. The function of a microbial respiratory sensor can be described as follows (Mulchandani and Rogers, 1998):

1. Oxygen diffuses from the air-saturated solution through the dialysis membrane (the membrane containing the microorganisms) and the gas-permeable membrane, and is then reduced at the cathode. A small proportion of the oxygen is consumed by the microorganisms. The steady state current represents the

oxygen diffusion through the composed membrane and reflects the endogenous respiration of the microorganisms.

2. If an assimilable substrate is added to the measuring solution, the substrate permeates through the dialysis membrane, is taken up by the microbial cells, and subsequently degraded. These processes cause an increase of respiration rate resulting in a decrease in the dissolved oxygen concentration, and hence the current decreases until a new steady state is reached.

Figure 1.3 shows a schematic representation of the action of an oxygen electrode microbial sensor, and a typical concentration profile through the layers of the microbial sensor (Hall, 1990).

In principle there are two possibilities of measurement for respiratory-based microbial biosensors (Figure 1.4): (a) endpoint measurement, in which the difference in current reflects the respiration rate of the substrates, and (b) the kinetic measurement, first derivative of the current-time response corresponding to the acceleration of respiration. The first method had been most frequently used in microbial sensors, where relatively high concentrations of biomass on the membranes are required. However, because of the diffusional resistance imposed by the microbial cell membranes, the response times for microbial sensors are longer than for enzyme electrodes (Mulchandani and Rogers, 1998).

Two principle measurement systems employed for respiration activity microbial biosensors include batch mode and Flow Injection Analysis (FIA), kinetic mode. For batch mode measurements, the sensor is placed in a thermostatically-controlled buffer solution, which is saturated with air and magnetically stirred during the measurement. The current output is measured, and when the current becomes stable the sample is injected. The substrate is metabolised by the microbes causing a decrease in the dissolved oxygen concentration near the electrode surface. Consequently, a corresponding decrease in current is observed until a new steady state is reached. The sensor can then be removed from the sample solution and placed in fresh buffer, and the current gradually increases again returning to its initial level.

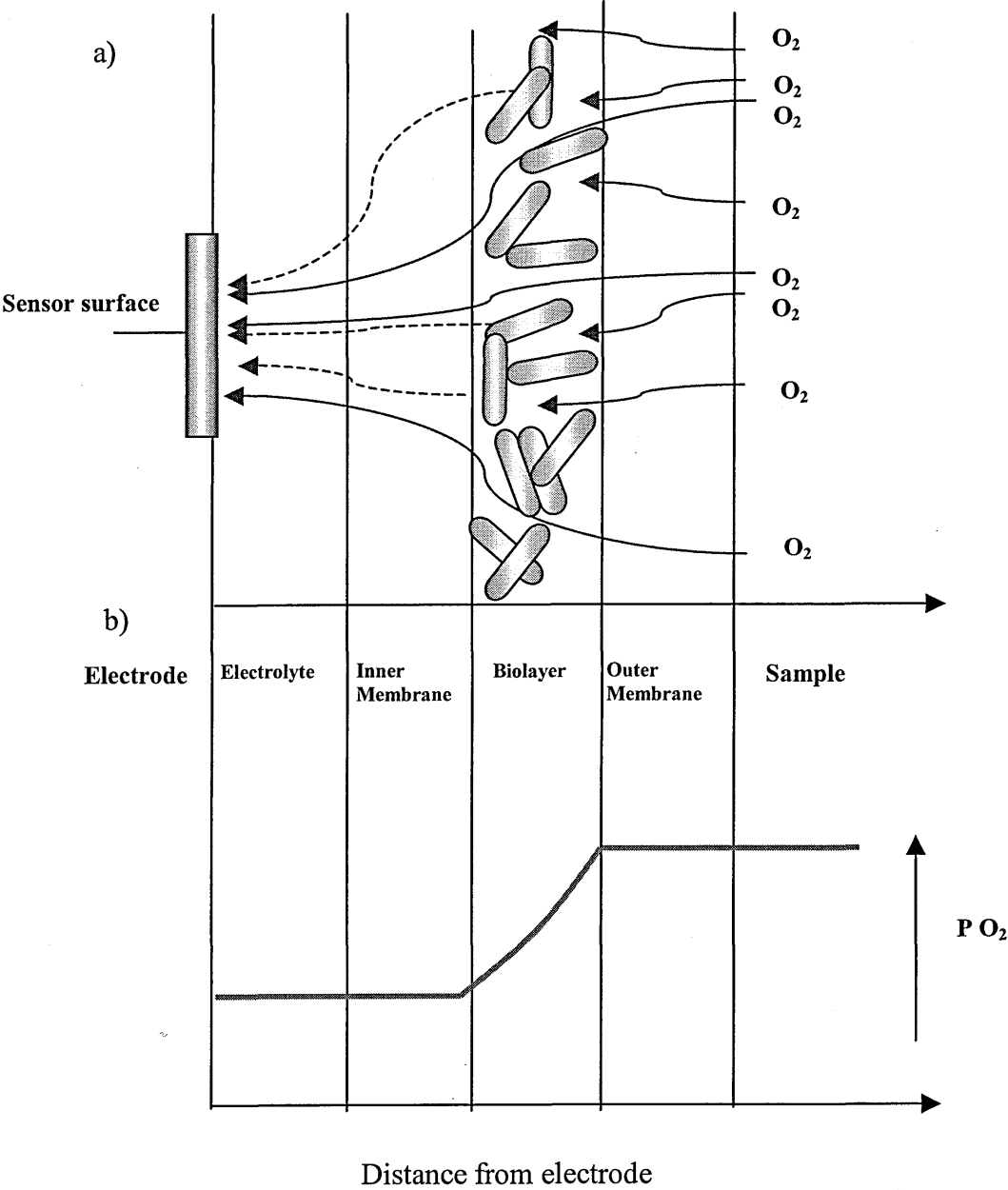


Figure 1.3. (a) Schematic representation of the action of an oxygen electrode microbial sensor. (b) Typical profile through the layers of the microbial sensor. (Adapted from Hall, 1990).

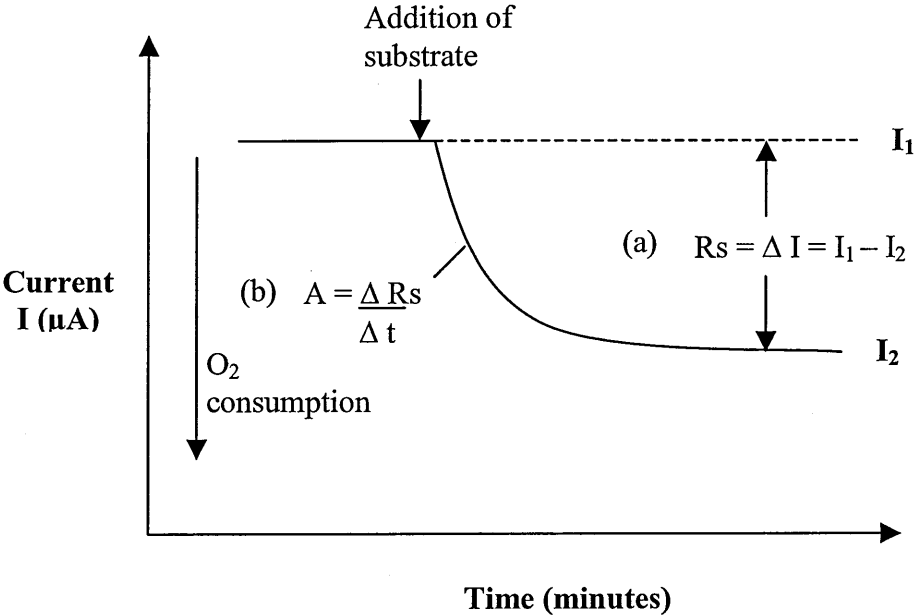


Figure 1.4. Typical response curve of a microbial sensor. Principles of measurement: (a) endpoint measurement (substrate respiration rate, R_s), and (b) kinetic measurement (acceleration of respiration rate, A). (Adapted from Mulchandani and Rogers, 1998).

FIA is a continuous flow technique in which the concentration of an analyte is measured uninterruptedly in a stream of fluid. One of the aims of employing a FIA system in combination with a biosensor is to automate the various steps and procedures, thus minimising labour costs and increasing precision and accuracy. FIA usually involves the injection of a liquid sample into a flowing, non-segmented carrier stream. The injected sample forms a zone in the carrier liquid, which is transported to the biosensor inside a detection chamber. The carrier stream is permanently monitored by the sensor which records the analyte zone (peak) as it passes through its flow cell (Freitag, 1996).

The FIA system consists of a water-jacketed flow cell containing the sensor, a peristaltic pump, a sampler injector, and a recorder. Similar to the batch system, the sample is injected when the electrode current output is in a steady state (representing the endogenous respiration rate of the bacteria). The injected sample causes an increase in bacterial respiration rate and oxygen consumption, which results in a decrease in the concentration of dissolved oxygen near the electrode surface. This is accompanied by a corresponding decrease in the output sensor signal until the injected sample starts to leave the flow cell and at which point the sensor signal starts to recover back to the original baseline.

In contrast to the batch mode, FIA operating procedures are largely automated, and the system can provide the following advantages: (i) more rapid sensor response and baseline recovery allowing the analysis of a greater number of samples over a given period; (ii) increased simplicity of operation which enables the development of an automated system without requiring the constant presence of trained personnel and hence, saving costs; (iii) more accurate sample analysis due to the constant flow rate and precise injection volume (Karl and Pacey, 1989).

1.3.4. The Transducer of the Microbial Biosensor: The Clark Dissolved Oxygen Electrode

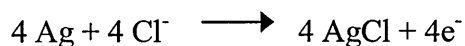
The Clark oxygen electrode is the most common transducer type used in the construction of cell-based biosensors. It is mostly used for the registration of microbe respiration, that is, metabolic processes during which oxygen is consumed, no matter which substrate is metabolised (D'Souza, 2001).

Amperometric sensors are based on the detection of electroactive species involved in the chemical or biological recognition process. The signal transduction process is accomplished by controlling the potential of the working electrode at a fixed value (relative to a reference electrode) and monitoring the current as a function of time. The applied potential serves as the driving force for the electron transfer reaction of the electroactive species. The resulting current is a direct measure of the rate of the electron transfer reaction. It is thus reflecting the rate of the recognition event, and is proportional to the concentration of the target analyte (Wang, 2002).

A Clark dissolved oxygen electrode consists of a platinum (Pt) cathode and a silver (Ag) anode both immersed in a saturated potassium chloride (KCl) electrolyte solution (providing the necessary chloride ions for the Ag/AgCl reference electrode), encased in glass and formed into a probe structure. The electrolyte solution is separated from the test solution by an oxygen permeable membrane (this membrane also serves to eliminate the passage of interfering substances which may be reduced at the same potential). When a potential difference of -600 mV is applied across the electrodes such that the platinum electrode is made negative with respect to the silver anode, electrons are generated at the anode and are then used to reduce oxygen at the cathode. The oxygen tension at the cathode then drops and this acts as a sink so that more oxygen diffuses towards it to make up the deficit. Since the rate of diffusion of oxygen through the membrane is the limiting step in the reduction process, the current produced by the electrode is proportional to the oxygen tension in the sample (Wilson and Walker, 1994).

These electrode reactions may be summarised as follows (Skoog *et al.* 2004):

At the anode (reference electrode):



At the cathode (working electrode):



Overall:



When the oxygen sensor is immersed in a flowing or stirred solution of the analyte, oxygen diffuses through the membrane into the thin layer of electrolyte immediately adjacent to the disk cathode, where it diffuses to the electrode and is immediately reduced to water. Thus 2 diffusion processes are involved – one through the membrane, and the other through the solution between the membrane and the electrode surface. For a steady state condition to be reached in a reasonable time (10 – 20 s), the thickness of the membrane and the electrolyte film must be 20 μm or less. Under these conditions, it is the rate of equilibrium of O_2 transfer across the membrane that determines the steady state current that is reached (Skoog *et al.* 2004). This ensures good linearity of response and a low background current (Blum & Coulet, 1991).

It is important that the contents of the reaction vessel, into which the oxygen probe is immersed, are stirred as the platinum cathode reduces oxygen to produce the electrical current. A correctly constructed electrode will show a reduction in current (oxygen tension) when the stirrer is switched off due to depletion of oxygen in the potassium chloride-filled electrode chamber (Wilson and Walker, 1994). Similarly, when using the dissolved oxygen electrode within a Flow Injection Analysis (FIA) system, the required oxygen tension relies on the flow rate of the carrier stream; a very low flow rate was found to cause a reduction in the current in the present work (Chapter 5).

Aside from pressure, both the solubility and the rate of diffusion of oxygen are affected by temperature. Therefore it is necessary to have some form of temperature control during experimentation. Care should also be taken to ensure that all reagents reach the

required temperature before experimentation commences; if insufficient time is allowed for reagents to reach the required temperature, incorrect results will be obtained (Wilson and Walker, 1994). This may create difficulties when investigating the temperature effects on microbial biosensor responses.

1.3.5. The Biological Component of the Microbial Biosensor

It is necessary to obtain a suspension of suitable microorganisms with sufficient activity of a particular enzyme, or enzymatic system, for microbial biosensor construction. This is usually achieved by cultivating microbial cells in a liquid medium containing the enzyme substrate that will be the measured substance for the cell-based biosensor at the same time. The substrate induces enzyme synthesis within the cells.

If microbial cells do not convert the substrate to a suitable product that could be measured with an ion-selective electrode, the microorganisms can be immobilised on the surface of an oxygen electrode. The electrode registers the respiratory activity of the cells as a fall in oxygen concentration near the cells. This type of biosensor, often called a respiratory electrode, must use living cells exclusively. However, in order for quantitative results to be obtained, the constant concentration of the cells in a suspension or in a membrane on the surface of a transducer must be guaranteed. In addition, the cells must be in the same metabolic status; various phases of the microbe cultivation differ in the cells' respiration intensity. Therefore, the standard density and metabolic status of the microbial cells must be strictly kept (Racek, 1995).

Most bacterial biosensors have been constructed using cells taken from the late exponential or stationary phases of growth (D'Souza, 2001), typically from an overnight culture in the latter case. *P. rathonis* T was tested in various stages of growth for the development of biosensor receptor elements by Taranova *et al.* (2002). It was established that the maximal response to sodium dodecyl sulphate (SDS) at a concentration of 0.2 kg m^{-3} could only be obtained using 5- to 6-hour cells that corresponded to the exponential phase of growth. It was also reported that the degradation of SDS and some other anionic surfactants by this culture occurred much

more quickly in the exponential phase of growth than in the stationary one (Taranova *et al.*, 2002).

1.3.5.1. Bacterial Growth

Bacterial growth leads to a rise in cell number when microorganisms reproduce by processes like budding or binary fission. In the latter, individual cells enlarge and divide to yield two progeny of approximately equal size. Increases in the total cell mass, as well as in cell numbers, accompany population growth. Bacterial growth is usually measured using a spectrophotometer, based on the fact that microbial cells scatter light striking them. Because the cells in a population are of roughly constant size, the amount of scattering is proportional to the concentration of cells present.

When the concentration of bacteria reaches about 10 million (10^7) cells per ml, the medium appears slightly cloudy or turbid. Further increases in concentration result in greater turbidity and less light is transmitted through the medium. The extent of light scattering can be measured by a spectrophotometer and is almost linearly related to the bacterial concentration at low concentration levels (Prescott *et al.*, 1993). Thus, population growth can be easily measured spectrophotometrically as long as the population is high enough to give detectable turbidity.

The exact relationship of optical density at a particular wavelength to cell number varies with the particular strain used and the growth conditions, because the mass/cell number ratio varies with the medium. It also varies with the brand of spectrophotometer being used since the parameter being measured is scattering, not absorbance (which all spectrophotometers measure essentially the same). The degree to which light is scattered in any given instrument depends on the distance from the cuvette to the phototube which differs among different instruments. Thus, it should not be assumed that if a protocol calls for growing the cells to A_{550} 0.6, then 0.6 will be the optimal absorbance on a different model spectrophotometer. If the exact concentration of cells is critical for the success of a method, it is always better to report the concentration as 4

$\times 10^8$ cells ml^{-1} rather than A_{550} 0.6. As a rough estimation, $1 \text{ OD}_{550} = 8 \times 10^8$ cells ml^{-1} (UNC-CH, 2002).

Population growth is studied by analysing the growth curve of a microbial culture. When microorganisms are cultivated in a liquid medium, they are usually grown in a batch culture or closed system; that is, they are incubated in a closed culture vessel with a single batch of medium. Because no fresh medium is provided during incubation, nutrient concentrations decline and concentrations of wastes increase. The growth of microorganisms reproducing by binary fission, such as *P. putida*, can be plotted as the logarithm of cell number versus the incubation time (Prescott *et al.* 1993). The resulting curve has four distinct phases (Figure 1.5):

1. **Lag Phase:** No immediate increase in cell numbers or mass, possibly due to old cells requiring re-synthesis of essential co-factors, and ribosomes; or due to a different medium.
2. **Exponential Phase:** Microorganisms are growing and dividing at the maximum rate possible. Growth is constant, with the microorganisms dividing and doubling in number at regular intervals. The population is most uniform in terms of chemical and physiological properties during this phase; therefore exponential phase cultures are usually used in biochemical and physiological studies.
3. **Stationary Phase:** Eventually population growth ceases and growth becomes horizontal. This phase is usually attained by bacteria at a population level of around 10^9 cells per ml. The total number of viable cells remains constant in this phase. Reasons for entering a stationary phase include: nutrient limitation, limitation of O_2 availability, or toxic waste accumulation.
4. **Death Phase:** Detrimental environmental changes like nutrient deprivation and the build up of toxic wastes lead to the decline in the number of viable cells characteristic of this phase.

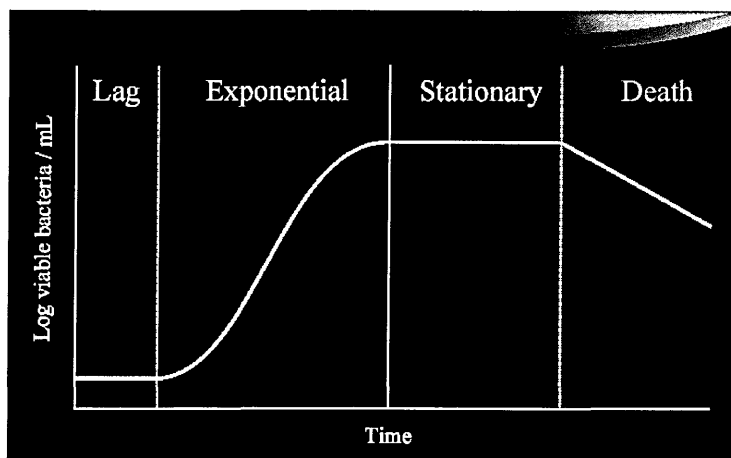


Figure 1.5. The four phases of the bacterial growth curve.

The four phases of the bacterial growth curve vary depending on the microorganism, the medium used, and the growth conditions. It is therefore necessary to measure these phases, in order to investigate the best time in which to take the cells for use on the biosensor. Once established, cells taken at the same time from each liquid culture should be in the same metabolic status, cell numbers per ml of medium should be within a comparable range, and thus, variation between biosensor responses can be minimised. However, the stability of cells during the immobilisation process and their survival on the immobilisation surface cannot be guaranteed.

As previously outlined, microbial biosensors based on respiratory activity, must use living cells exclusively. However, spectrophotometrical measurements will measure living and dead cells in the same way; they both exhibit light scattering regardless of viable status. It is therefore necessary to measure the number of living cells per ml of the liquid medium using the standard viable cell count method (Prescott *et al*, 1993). This is usually achieved by conducting serial dilutions of 1 ml of a liquid culture (to obtain countable cell numbers), and spreading a known volume onto a rich medium agar plate. These plates are incubated to permit cell growth, and the resulting colonies are counted. Each colony is the progeny of a single cell, thus viable cell numbers per ml of medium can be calculated by multiplication of the total number of colonies (or Colony Forming Units, CFUs) by the overall dilution factor. This method is, however, subject

to errors caused by: cells left in pipette tips, cells left on the spreader, and cell clumping on the agar surface. Some microbial biosensors reported in the literature have therefore measured the dry weights of the liquid cultures used for immobilisation, as a means of comparison between assays (Im Lee & Karube, 1996; D'Souza, 2001).

1.3.5.2. The Genus *Pseudomonas*

The genus *Pseudomonas* contains straight or slightly curved gram-negative rods, 0.5 to 1.0 μm by 1.5 to 5.0 μm in length, that are motile by one or several polar flagella and lack prosthecae or sheaths (Figure 1.6). These chemoheterotrophs are aerobic and carry out respiratory metabolism with oxygen (and sometimes nitrate) as the electron acceptor. All pseudomonads have a functional tricarboxylic acid cycle and can oxidise substrates to CO_2 (Prescott *et al.*, 1993).

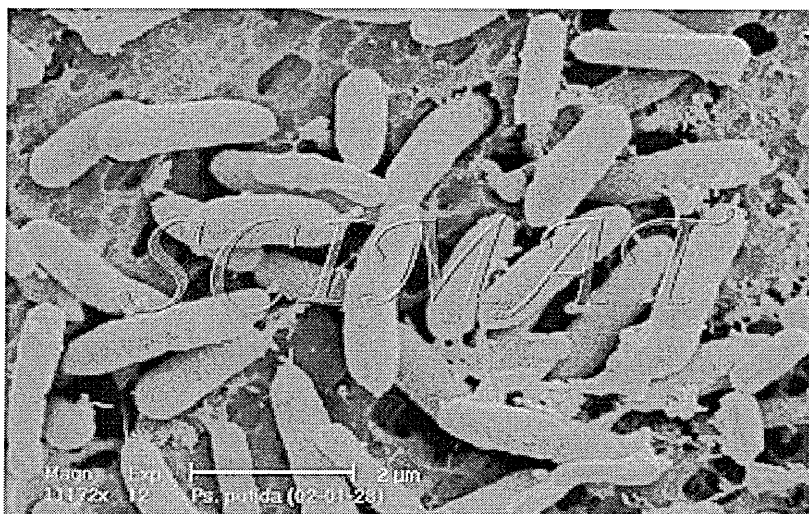


Figure 1.6. *Pseudomonas putida* cells on a cellulose nitrate filter
(Source: UNC-CH, 2002).

The genus is an exceptionally heterogeneous taxon composed of 30 species distributed among five rRNA homology groups and over 60 less well examined species. The three best characterised groups, RNA groups I-III, are subdivided according to properties such as the presence of poly-B-hydroxybutyrate (PHB), the production of a fluorescent pigment, pathogenicity, the presence of arginine dihydrolase, and glucose utilisation. For example, the fluorescent subgroup does not accumulate PHB and produces a diffusible, water-soluble, yellow-green pigment that fluoresces under UV radiation. *Pseudomonas aeruginosa*, *P. fluorescens*, *P. putida*, and *P. syringae* are members of this group (Prescott *et al.*, 1993).

Many of the pseudomonads can degrade an exceptionally wide variety of organic molecules. Thus, they are very important in the microbial breakdown of organic materials. The fluorescent pseudomonads can use approximately 80 different substances as their carbon and energy sources (Prescott *et al.*, 1993). It is characteristics such as these that make microorganisms such an attractive biological component of biosensors for environmental monitoring.

1.3.5.3. Benzene Catabolism by *Pseudomonas putida* ML2

Pseudomonas putida ML2 is a naturally occurring microorganism, originally isolated from refinery soil, and is able to aerobically biodegrade benzene and, more importantly, utilise it as its sole carbon and energy source (Axcell and Geary, 1975; Mason, 1994). Figure 1.7 shows the biochemical pathway utilised by the microorganism in the catabolism of benzene. In the microbial cell, benzene is first oxidised by the multicomponent benzene dioxygenase enzyme system to *cis* 1,2-dihydroxycyclohexa-3,5-diene or *cis*-benzene glycol in the presence of NADH and ferrous ions. The *cis*-benzene is subsequently dehydrogenated to catechol and finally undergoes ring cleavage to *cis-cis* muconic acid. Degradation of this acid then occurs via the 3-oxoadipate pathway to give tricarboxylic acid (TCA) cycle intermediates (Mason, 1994).

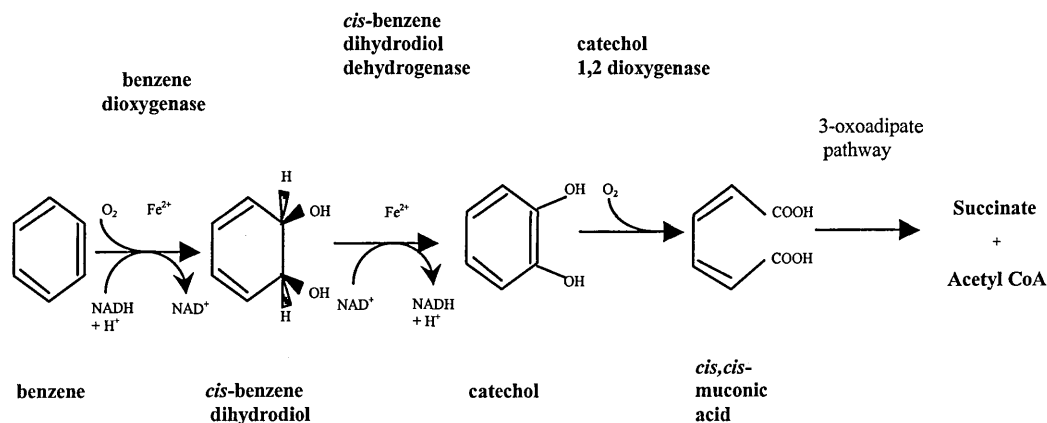


Figure 1.7. The biochemical pathway utilised by *P. putida* ML2 in the catabolism of benzene (Mason, 1994).

The genes encoding the dioxygenase (*bedC1C2BA*) and dehydrogenase (*bedD*) are localised on a 112-kb (kilo-base) catabolic, pHMT112, in *P. putida* ML2. The 1098-bp (base-pair) *bedD* is found to lie upstream of *bedC1C2BA* and flanked by two 42-bp direct repeats, each containing a 14-bp sequence identical to the inverted repeat of IS26. In addition, two directly repeated sequences of the IS elements ISI489v1 and ISI489v2 are found to flank the *bed* gene cluster forming a composite transposon Tn5542. A comparison of the *bedD* and *bedC1C2BA* genes reveals differences in the guanine and cytosine content and codon usage. This together with their organisation on a composite transposon suggests that both genes have been separately acquired and placed in juxtaposition to one another. The formation of such a hybrid “upper” pathway in *P. putida* ML2 with the help of IS elements, would confer upon the bacterium the ability to metabolise benzene (Fong *et al.*, 2000).

Section 1.3 detailed the various components of a microbial biosensor based on the respiratory activity of a particular bacterium. In the next section, the practical use of such biosensors in environmental monitoring is reviewed.

1.4. Review of Microbial Biosensors in Environmental Monitoring

The most common transducer type used to monitor the microbial biocatalyst is the Clark oxygen sensor (Gaisford *et al.*, 1991). It is primarily used for the registration of microbe respiration (a process during which oxygen is consumed, regardless the substrate being metabolised). The first developed whole cell-based biosensor for determination of a particular analyte was the microbial electrode with *Acetobacter xylinum* cells immobilised on a cellulose membrane on the surface of an oxygen electrode. It served for the determination of ethanol concentrations on the basis of oxygen consumption during ethanol assimilation in bacterial cells (Racek, 1995). Since then, various bacterial biosensors based on the Clark oxygen electrode have been developed.

This principle is most commonly employed in biosensors for determining biological oxygen demand (BOD) and in devices with immobilised bacteria specific to a particular analyte (Racek, 1995). BOD estimation serves as a criterion of water pollution with organic compounds. Standard BOD assays typically require 5 days, in contrast to biosensor-based analyses which take around 15 minutes (Marty *et al.*, 1997). Since the BOD biosensor first appeared (Karube *et al.*, 1977), it has been thoroughly investigated and is the most extensively used microbial biosensor today (D'Souza, 2001).

Microbial biosensors offer distinct advantages in the field of pollution monitoring: for example, test samples do not need to be optically clear and can be measured over a wide range of concentrations without pre-treatment. Furthermore, they offer the possibility of real-time analysis, which is particularly important for the rapid measurement of analytes in industry. Microbial biosensors based on respiratory activity have had several applications in environmental monitoring in recent years, using a range of different bacteria, including BOD biosensors for the determination of organic pollution in wastewater (Liu *et al.*, 2000; Tothill and Turner, 1996; Marty *et al.*, 1997; Yang *et al.*, 1996, and those based on specific analytes of interest. Such analytes have included benzene (Tan *et al.*, 1994; and Campanella *et al.*, 1996); cyanide (Im Lee & Karube, 1996); naphthalene (Reshetilov *et al.*, 1997a); trichloroethylene (Han *et al.*, 2002); and phenols (Timur *et al.*, 2003).

Table 1.4 shows a range of whole-cell (including many bacterial) biosensors that have been developed in recent years for the detection of a wide range of environmental analytes.

1.4.1. Bacterial biosensors based on the Clark dissolved oxygen electrode.

The oxygen electrode is the most common transducer type used in the construction of cell-based biosensors (D'Souza, 2001). It is mostly used for the registration of microbe respiration, that is, metabolic processes during which oxygen is consumed, no matter which substrate is metabolised. The principle is used in biosensors such as those for biological oxygen demand estimation as a criterion of water pollution with organic substances. It is also used in biosensors with immobilised bacteria specific to one particular analyte.

Pseudomonas putida ML2 has been studied previously for the analysis of benzene catabolism and related enzymes (Geary *et al.*, 1990; Zamanian and Mason, 1987; and Mason, 1994). The use of this strain as a biosensor for the monitoring of benzene has been demonstrated by Tan *et al.* (1994), and Campanella *et al.* (1996), the ox1 strain in the latter case. Tan *et al.* (1994) described the biosensor as an excellent microbial system for the fabrication of a benzene biosensor of high specificity and sensitivity towards benzene, with negligible responses to related aromatic hydrocarbons. It was reported that the sensor showed excellent stability and reproducibility with a linear range from 0.07 to 1.35 mM of benzene. Campanella *et al.* (1996) reported a linear range between 0.02 and 0.7 mM benzene, however this biosensor also displayed higher responses to toluene, chlorobenzene, and nitrobenzene. Response times were between 2 – 10 minutes, and 1 minute, from Tan *et al.* (1994) and Campanella *et al.* (1996), respectively. In both cases, the biosensors were operated at a temperature of 25°C, and a pH of 6.8. The immobilisation methods utilised, involved filtration through a cellulose acetate membrane, and entrapment in an agar gel by Tan *et al.* (1994), and Campanella *et al.* (1996), respectively.

Table 1.4.

Whole-cell biosensors for environmental applications (Source: D'Souza, 2001).

Analyte	Microorganism	Transducer/Immobilisation	Detection Limit	Reference
BOD	<i>Trichosporum cutaneum</i>	Miniature oxygen electrode (UV cross-linking resin) (ENT-3400)	0.2-18 mg l ⁻¹	Yang <i>et al.</i> (1996)
BOD	<i>T. cutaneum</i>	Miniature oxygen electrode array (photo cross-linkable Resin)	<32 mg l ⁻¹	Yang <i>et al.</i> (1997)
BOD	<i>T. cutaneum</i>	Oxygen electrode (entrapment)	10-70 mg l ⁻¹	Marty <i>et al.</i> (1997)
BOD	<i>Ps. putida</i>	Oxygen electrode (adsorption on porous nitro cellulose membrane)	>0.5 mg l ⁻¹	Chee <i>et al.</i> (1999)
BOD	Activated sludge (mixed microbial consortium)	Oxygen electrode/flow injection system (entrapped in dialysis membrane)	>3.5 mg l ⁻¹	Liu <i>et al.</i> (2000)
BOD	Salt tolerant mycelial yeast <i>A. adenivorans</i> LS3	Oxygen electrode (PVA†)	2.61-524 mg l ⁻¹	Tag <i>et al.</i> (2000)
Bioavailable organic carbon in oxic sediments	Yeast cells	Oxygen electrode (PVA)	Microscale	Neudoerfer and Meyer (1997)
Anionic surfactants (linear alkyl benzene sulfonates (LAS))	LAS degrading bacteria isolated from activated sludge	Oxygen electrode (reactor type sensor, ca-alginate)	<6 mg l ⁻¹	Nomura <i>et al.</i> (1994)
Acrylamide: acrylic acid	<i>Brevibacterium</i> sp.	Oxygen electrode (free cells)	0.01-0.075 and 0.01-0.1 g l ⁻¹	Ignatov <i>et al.</i> (1997)
Phenolic compounds	<i>Ps. putida</i>	Oxygen electrode (reactor with cells adsorbed on PEI glass)	100 µM	Nandakumar and Mattiasson (1999a)
Nitrite	<i>Nitrobacter vulgaris</i> DSM10236	Oxygen electrode (adsorption on Whatman paper)	>10 µM	Reshetilov <i>et al.</i> (2000)
Cyanide	<i>S. cerevisiae</i>	Oxygen electrode (PVA)	0.15-15 nM	Ikebukuro <i>et al.</i> (1996)
Chlorophenols	<i>Rhodococcus</i> sp. <i>Trichosporon beigellii</i>	Oxygen electrode (PVA)	0.004-0.04 and 0.002-0.04 mM	Riedel <i>et al.</i> (1993, 1995)
3-Chloro-benzoate	<i>Ps. putida</i>	Oxygen electrode (PVA)	40-200 µM	Riedel <i>et al.</i> (1991)
Chlorinated and brominated hydrocarbons (1-chlorobutane and ethylenebromide)	<i>Rhodococcus</i> sp. DSM6344	Ion selective electrodes (alginate)	0.22 and 0.04 mg l ⁻¹	Peter <i>et al.</i> (1996)
Polycyclic aromatic hydrocarbons (Naphthalene)	<i>Sphingomonas yanoikuyae</i> B1 or <i>Ps. Fluorescens</i> WW4	Oxygen electrode (polyurethane based hydrogel)	0.01-3.0 mg l ⁻¹	Koenig <i>et al.</i> (1996, 1997a)
Organophosphate nerve agents (paraxon, methyl parathion, diazinon)	GEM†† <i>E. coli</i> (organophosphorous hydrolase)	Potentiometric (adsorption on electrode surface)	0.055-1.8, 0.06-0.91 and 0.46-8.5 mM	Mulchandani <i>et al.</i> (1998a)
Organophosphate nerve agents (paraxon, parathion, coumaphos)	GEM†† <i>E. coli</i> (organophosphorous hydrolase)	Fibre-optic (agarose)	0.0-0.6, 0.0-0.03 and 0.0-0.075 mM	Mulchandani <i>et al.</i> (1998b)
Pollutants such as diuron and mercuric chloride	<i>Synechococcus</i> sp. PCC7942	Photoelectrochemical (photo cross linkable PVA bearing styrylpyridium group)	0.2 and 0.06 µM	Rouillon <i>et al.</i> (1999)
Herbicides* (diuron and atrazine)	Chloroplast/thylakoid membranes	Pt-electrode in microelectrochemical cell (photo cross linkable PVA bearing styrylpyridium group)	2x10 ⁻⁵ and 2x10 ⁻⁴ mM	Rouillon <i>et al.</i> (1995)
Mono and polyphenols* (atrazine)	Potato (<i>S. tuberosum</i>) slices (polyphenol oxidase inhibition)	Oxygen electrode (tissue slice sandwiched between membranes)	20-130 µM	Mazzei <i>et al.</i> (1995)

† Polyvinyl alcohol

†† Genetically engineered microbes

* Tissues or cellular organelle based

Pseudomonas putida BS283, carrying the naphthalene degradative plasmid was used for the development of a sensor based on the respiratory activity of the cells in response to naphthalene. Cells were immobilised by adsorption onto chromatographic paper, and fixed onto a Clark oxygen electrode. A lower detection limit of 0.9 μM naphthalene was reported, with maximum sensor responses observed at pH 6 – 6.2, at a temperature of 20 – 25°C. At a concentration of 10 μM naphthalene, the baseline recovery time did not exceed 30 minutes, however the response time of the sensor was not reported. For the lifetime of the bacterial membrane, the average time for the response to decrease by half was about 5 days, followed by a total loss of cellular activity after 6 – 8 days (Reshetilov *et al.*, 1997a).

Pseudomonas fluorescens (NCIMB 11764) was immobilised onto a cellulose nitrate filter for the development of a biosensor for cyanide. The bacteria consume oxygen upon degradation of cyanide, which was measured using a Clark oxygen electrode. This particular sensor was designed to operate in the gas phase, and measurements were performed in a batch reaction cell filled with distilled water (pH 2) at a temperature of 30°C. A linear range between 0.1 – 1 mg l^{-1} cyanide was reported, with a response time of less than 3 minutes, and a baseline recovery time of 15 minutes. A 73% decline in the response from that initially was reported within the first 4 days of use of the bacterial membrane; however this was followed by a stable response from then up to 1 month (Im Lee & Karube, 1996).

Gluconobacter oxydans cells were immobilised by adsorption onto chromatographic paper and fixed onto a dissolved oxygen probe for the detection of xylose. A xylose detection limit between 0.5 mM and 20 mM was reported, with a response time of 3 minutes, and a baseline recovery time of 7 - 15 minutes (depending on the substrate concentration). Bacterial membranes were stored under dry conditions at +4 and –10°C, and in both cases, a gradual loss of 30% from the initial sensitivity was reported within 20 days, with little further loss over the next 15 days (Reshetilov *et al.* 1997b).

The key factor for the application of microorganisms in biosensors is the narrow substrate specificity, which allows elimination of the influence of the accompanying

compounds when measuring environmental samples and detection accuracy (Taranova *et al.*, 2002). An amperometric bacterial biosensor for the detection of anionic surfactants was developed using several bacterial strains belonging to the genera *Pseudomonas* and *Achromobacter*. The sensitivity and selectivity of the biosensors based on these strains to degrade anionic surfactants was investigated using an oxygen electrode. Cells were immobilised in a 2% agar gel and fixed onto the oxygen probe. Bacterial strains belonging to both the *Pseudomonas* and *Achromobacter* genera showed significant similarity of their substrate specificity. A linear response for sodium alkansulfonate detection was found between 0.15 – 3.13 mM for the *Pseudomonas* sp. 2T/1. The biosensor recovery times for all substrates were reported to be 40-60 minutes for 0.2 kg/m³; 5-10 minutes for 0.05 kg/m³; and 2-3 minutes for 0.001 kg/m³ (Taranova *et al.*, 2002).

Oxygen consumption was also used as the basis for an amperometric microbial biosensor for microbial corrosion monitoring. *Pseudomonas* species, isolated from a corroded metal surface, were immobilised onto an acetylcellulose membrane and fixed onto a Clark oxygen electrode. The system was based on the sensing of sulphur oxidising bacterial activity, and a linear response was reported up to 0.7 mM sulphuric acid, with a 5 minute response time (Dubey and Upadhyay, 2001).

1.4.2. Bacterial biosensors in combination with Flow Injection Analysis

A Flow Injection Analysis (FIA) system, in which the sample is injected into a carrier stream of buffer, and pumped through a flow cell (containing the transducer and immobilised microorganisms), is an alternative method to the batch stirred solution measurements reviewed in Section 1.4.1. The FIA system allows a faster sample analysis time (sensor response and recovery times), and a more automated operation, which is particularly advantageous for on-line *in situ* measurements, or for analysing a large number of samples.

Biosensors in combination with an FIA system have been widely associated with enzyme sensors, for example, the monitoring of glucose and lactate (Haouz and Stieg,

2002), glucose during fermentation (White *et al.*, 1996), and for the monitoring of galactoside conjugates and glycerol (Vega *et al.*, 1998).

Few reports of microbial biosensors in combination with an FIA system can be found in the literature, among them include a BOD biosensor, based on an immobilised mixed culture of microorganisms on a Clark oxygen electrode for the on-line monitoring of waste and wastewater. The BOD biosensor had a response time of 25 seconds for one measurement, followed by a 4-8 minute recovery time. FIA conditions incorporated a flow rate of 0.6 ml min^{-1} , and an injection loop volume of 250 μl . In batch mode, this sensor had a response time of 20 minutes, with a 15-40 minute recovery time. However, there was a good agreement between the sensitivity in both batch and kinetic modes, with added advantages in the latter case such as less time consumption, easier operation, and a greater suitability for use in on-line monitoring (Liu *et al.*, 2000).

A microbial biosensor based on FIA was also developed for the direct measurement of trichloroethylene (TCE). *Pseudomonas aeruginosa* JI104 was used as the TCE degrader, and the chloride ion released during its degradation was detected using an ion selective electrode. FIA conditions incorporated a flow rate of 0.09 ml min^{-1} , and an injection loop volume of 1 ml. The chloride ions released were linearly related to the TCE concentration in the range $0.03\text{-}2 \text{ mg l}^{-1}$ (Han *et al.*, 2002).

Respiratory activity was also used as the basis of measurement for the development of an automated whole-cell biosensor in a flow-through system, using screen-printed electrodes as the transducers. Thick-film platinum electrodes polarised at -900 mV were used to determine the oxygen consumption of the metabolising bacteria, with fructose as the substrate. The cells were immobilised onto cellulose acetate membranes and integrated into a reaction chamber consisting of a screen-printed Pt-sandwich electrode. The reaction chamber was connected to an FIA system, and the device was reported to offer the possibility of on-line estimation of wastewater BOD (Heim *et al.*, 1999).

1.4.3. Bacterial biosensors based on screen-printed electrodes and other transducer types for environmental monitoring

The Clark dissolved oxygen probe has been the most common transducer type used in the development of bacterial biosensors, based on microbial respiration. However, other transducer types have been used, allowing the exploitation of different cellular functions in response to specific analytes of interest.

A comparison of transducer types was made, based on the respiratory activity of *Saccharomyces cerevisiae* yeast cells, for the development of toxicity cell biosensors. The different types of transducer employed were: a Clark oxygen electrode, a CO₂ electrode, a glass pH electrode, and a solid-state electrode (field-effect transistor on a silica chip). The biosensors were used to determine several typical toxic agents, including; metal ions, and cationic and anionic surfactants. The results obtained demonstrated that the CO₂, pH, and solid-state electrodes were no less suitable a transducer as the classical Clark oxygen electrode (Campanella *et al.*, 1997).

A whole-cell benzene biosensor was developed by Cheol Gil *et al.* (2000), using a recombinant bioluminescent bacterium (*Escherichia coli* harbouring a lac::lux CDABE fusion). Expression of the lux genes from the lac promoter resulted in constitutive light emission (bioluminescence) by *E. coli* RFM443 carrying the recombinant lux plasmid. The bioluminescence level decreased with increasing inhibition on the metabolism of cells by toxic chemicals such as benzene. This biosensor was fabricated with an immobilisation technique utilising a solid agar medium for the measurement of toxicity through direct contact with the gas. The supplement of nutrients through the agar medium could maintain the activity for a prolonged period and allow the cells, embedded in the solid medium, to directly detect gaseous toxicity. The minimum detectable concentration of benzene vapour was 0.2% volume, which was approximately equal to 48 ppm of benzene vapour, and a response time of 10 minutes was reported. The biosensor could also be produced in a compact size for use as a portable monitoring device. Limitations included the diffusion of the gas through the immobilised-cell agar medium. Furthermore, this bacterial strain did not permit the

classification of toxicity, and was not sensitive to benzene concentrations below the regulation levels (Cheol Gil *et al.*, 2000).

A more recent transducer used in combination with whole bacterial cells was developed by Held *et al.* (2002), in which *Escherichia coli* K12 cells were immobilised onto gold screen-printed electrodes (SPEs) for the determination of various mono- and disaccharides. The selectivity of the individually addressable sensors of the array was achieved by the combination of the metabolic response, measured as the oxygen consumption of the mutated bacterial cells lacking different transport systems for individual carbohydrates. The local consumption of molecular oxygen caused by the metabolic activity of the immobilised cells was amperometrically determined on the gold screen-printed surface of the electrode at a working potential of -600 mV versus Ag/AgCl (Held *et al.*, 2002).

Oxygen detection was further used as the measurement basis of free fatty acids based on the immobilisation of *Arthrobacter nicotianae* in calcium alginate on surface of platinum SPEs. A linear range between 9.5 - 165.5 μ M butyric acid was obtained, with a response time of 3 minutes, and the sensors could be used for up to one hour (allowing a total of four assays per day). A gas-permeable membrane was reported to be unnecessary for the dissolved oxygen measurements (Schmidt *et al.*, 1996).

A further respiratory-based SPE biosensor for the detection of phenol was recently developed by Timur *et al.* (2003). The sensor was based on the immobilisation of a *Pseudomonas putida* strain of bacteria in a gelatin membrane cross-linked with glutaraldehyde directly on the gold working electrode. This sensor was also used without the presence of a gas-permeable membrane for wastewater sample analysis, and no interference from the sample matrices was reported. The sensor was used for up to 25 measurements over a 6-h period, without a decrease in the response.

Screen-printed electrodes were also used as the transducer for the development of another phenol sensor based on immobilised *Pseudomonas* strains. Whole cells were immobilised by absorption onto the graphite surface of mediator-modified screen-

printed electrodes using a cellulose acetate solution. The sensor was based on the bacterial oxidation of phenol, and the transfer of resulting electrons to the electrode by means of insoluble mediators. This method proved to have a lower limit of detection with respect to a similar phenol biosensor based on the Clark oxygen electrode, 1 μM and 2.5 μM phenol, respectively. Insufficient substrate selectivity was also reported for the latter method (Skládal *et al.*, 2002). However, the mediators were not suitable for some strains of bacteria, thought to be related to the inability of the degradation pathways in the cells to exchange electrons with the mediators (Skládal *et al.*, 2002).

1.5. PROJECT AIMS

The presence of toxic volatile organic compounds (VOCs) such as benzene in workplace air has accounted for the death of many occupationally exposed workers over the last century (DEFRA, 2003). Benzene is included in the list of carcinogenic substances in Annex I of European Directive 67/548/EEC. The current permissible exposure level to benzene in the workplace in Europe is 1 ppm for an eight-hour time weighted average, with a 5 ppm short-term exposure limit (Council Directive 90/394/EEC amended by Directive 97/42/EC). The project aims are described in the following points:

1. Conventional chromatographical methods for the monitoring of airborne benzene are often complex, costly, require skilled personnel and are in most cases, laboratory-based.
2. The development of low cost, easy to use, and portable methods for the rapid detection of benzene in the workplace is desirable for occupational risk assessment.
3. The main objective of this work was to develop a bacterial biosensor able to achieve the above objectives. The developed biosensor should have applications for the measurement of airborne benzene levels in the workplace, and prove an alternative means to conventional methods.

In this work, *Pseudomonas putida* ML2, originally isolated from refinery soil as a benzene degrader, was immobilised between two cellulose acetate membranes, and fixed onto an amperometric Clark-type dissolved oxygen electrode. This thesis describes the development of the biosensor in batch mode and in a flow injection analysis (FIA) system, towards its application for air sample analysis. A range of experimental parameters has been investigated in order to optimise the best working conditions for the bacterial biosensor, and several air sampling techniques and their associated methodologies have been investigated. All of the research for this thesis was conducted at Florence University, Italy, with additional air sampling research conducted at the Fondazione Salvatore Maugeri (FSM) Institute of Pavia, Italy.

CHAPTER 2

MATERIALS AND METHODS

CHAPTER 2: MATERIALS & METHODS

2.1. Reagents

Bacterial culture constituents: KH_2PO_4 and K_2HPO_4 were purchased from Acros Organics (Fisher Scientific UK Ltd, Loughborough); tryptone was purchased from Oxoid S.p.A. (Milan, Italy); $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ and $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ were purchased from Fluka (Milan, Italy). $(\text{NH}_4)_2\text{SO}_4$ and benzene were purchased from Sigma (Milan, Italy).

Buffer components (KH_2PO_4 and $\text{K}_2\text{HPO}_4 \cdot 3\text{H}_2\text{O}$), KCl (used for the electrolyte solution), and desorption solvents (carbon disulphide, dimethylsulfoxide, methanol, acetonitrile and N,N-dimethylformamide) were purchased from Merck (Milan, Italy).

Standard benzene solutions were prepared daily in potassium phosphate buffer (50 mM, pH 7) from pure benzene (11.2 M). Benzene is toxic and highly volatile, and all procedures were undertaken to minimise benzene evaporation, and hence variation among solutions of the same concentration. All solutions were therefore prepared inside a fume cupboard, and all vials sealed with a Teflon cap liner. Benzene was injected into solutions using a Hamilton syringe.

2.2. Bacterial Cultivation

Pseudomonas putida ML2 (kindly provided by Prof. F. Briganti, University of Florence, Italy) were grown in a minimal liquid medium according to previous methodologies by Geary *et al.* (1990) and by Prof. F. Briganti, University of Florence. The minimal liquid medium contained the following constituents per litre: KH_2PO_4 (1.02 g), K_2HPO_4 (3.06 g), $(\text{NH}_4)_2\text{SO}_4$ (1 g), Tryptone (0.2 g), $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ (0.4 g), and $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ (0.04 g) (dissolved in deionised water and adjusted to pH 7 - 7.2). Minimal medium solid agar plates contained the same constituents with the addition of 15 g l^{-1} agar (after autoclaving, these were poured into sterile Petri dishes and stored at 4°C until required). It was necessary to prepare the $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ and $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ separately from the other components, as they precipitated when adjusting the pH (they were mixed

together after autoclaving). The advantage of using a minimal medium such as this one, with respect to a very rich medium, is that there is less chance of contaminating microorganisms growing in the same medium. Furthermore, *Pseudomonas putida* ML2 requires the presence of benzene during growth to activate the enzymes of the benzene deoxygenase pathway (Geary *et al.*, 1990).

The liquid medium was sterilised by autoclaving at 120°C, pressure 1 bar, for 20 minutes prior to the inoculation with the bacteria. When the medium had cooled, a single bacterial colony was transferred from a solid minimal medium agar plate (stored at 4°C) to 50 ml of liquid medium in a 500 ml culture flask, using a flamed wire loop. 50 µl of benzene was added to the culture flask to give a 0.1% v/v (10 mM) of the hydrocarbon. The culture was incubated overnight for 18 hours in an incubator shaker, at 30°C and 150 rpm (Gerhardt Thermoshake, Königswinter, Germany).

All materials in direct contact with the bacteria were sterilised in the autoclave prior to use (e.g. pipette tips, culture flasks, measuring cylinders, glass wear), and all bacterial work was undertaken in a sterile laminar flow cabinet, next to a Bunsen burner (except when working with benzene). All procedures were undertaken to ensure minimal contamination (e.g. flaming bottle necks and lids, wire loops, and spreaders).

To measure the bacterial growth, 1 ml of an 18-hour liquid culture was transferred into a cuvette and the bacterial growth measured using a Cary 3 UV-VIS Spectrophotometer (Varian Instruments, Italy). An average absorbance ($A_{660\text{nm}}$) of 1.3 was obtained after 18 h of growth.

For maintenance, *P. putida* ML2 colonies were sub-cultured monthly by transferring one colony from a minimal medium agar plate and spreading it onto a fresh minimal medium agar plate. Benzene (20 µl) was added onto a sterile glass disk and placed inside the Petri dish lid during incubation, to allow the bacteria to grow in the presence of the benzene vapours and hence keep the benzene deoxygenase enzymes activated. The agar plates were incubated for 2-3 days at 30°C. Direct addition of benzene directly onto the Petri dish lid was found to cause some degradation of the plastic, hence the

glass disks were used thereafter. Most importantly, the benzene cannot be added directly onto the bacteria, as this will cause cell death.

Stock solutions of *Pseudomonas putida* ML2 were prepared for long-term storage at -80°C . Sterilised storage vials were filled with 1 ml of an 18-hour liquid culture (absorbance typically 1.3 units), and sterilised glycerol added to give 8% v/v. The vials were then transferred to -80°C , where they can be stored for several years. Glycerol prevents the formation of crystals during freezing which may rupture the cells. To test the viability of the frozen culture, cells were taken with a sterilised wire loop and streaked onto minimal medium agar plates (benzene added on the glass disk as before), and incubated at 30°C for 2-3 days.

2.2.1. Viable Cell Counts and Dry Weight Measurements

Viable cell counts were conducted by serial dilutions of 1 ml of a liquid medium culture, spread onto Luria Bertani (LB) medium agar plates, containing the following constituents per litre: bacto-tryptone (10g), yeast extract (5g), sodium chloride (10g), bacteriological agar (15g). (This medium was also used for the culture of *Escherichia coli* JM109 (provided by Prof. F. Briganti, University of Florence) described in Sections 3.4 and 4.6).

An overnight liquid culture was grown and the growth measured with a spectrophotometer after 18 hours. An absorbance after 18 hours of growth was typically around 1.3 and with this value, it was estimated that the culture contained around 8×10^8 cells ml^{-1} as previously reported (UNC-CH, 2002). A 10^{-6} dilution was carried out using sterilised 1 ml Eppendorf tubes, vortexing between each dilution, to give a final concentration of 8×10^2 cells ml^{-1} (a 10^{-6} dilution could be achieved by conducting three 10^{-2} dilutions). From the final 1 ml dilution, 0.25 ml of this was pipetted onto an LB agar plate and distributed over the agar surface using a sterilised L-shaped spreader. It was estimated that approximately 200 cells would therefore be distributed onto the agar surface. The diluting medium used was the previously outlined minimal liquid medium used for cell culture.

Agar plates were incubated at 30°C over-night (*P. putida* growth occurs faster on the richer LB agar, with respect to the minimal agar), and the resulting colonies on the agar were counted (a grid drawn on the bottom of the dish to enable easier counting). Each colony is the progeny of a single bacterial cell, therefore, the number of cells ml⁻¹ in the original culture is then the number of cells on the agar plate multiplied by the overall dilution factor. However, it is important to note that cell losses may occur during the serial dilution (in pipette tips, for example – although this was minimised by flushing with the diluting medium) or left on the L-shaped spreader. Cell clumping can also occur on the agar surface.

Dry weight measurements of bacterial culture volumes after centrifugation were used as a means of investigating cell loading on the immobilisation membrane. This was achieved by transferring differing volumes from an 18-hour bacterial culture into pre-weighed Eppendorf® tubes and centrifuging for 10 minutes at 6000 rpm (Eppendorf Minispin Plus). The bacterial pellet was re-suspended in 1 ml of sterile phosphate buffer (50 mM, pH 7) as a washing step, and re-centrifuged under the same conditions as aforementioned. The supernatant was then discarded, the pellet dried overnight at 30°C and subsequently weighed.

2.3. Immobilisation

Initial experiments were conducted using the following immobilisation method: A 10 or 5 ml volume of *Pseudomonas putida* ML2 cells, taken from a liquid culture grown for 18 hours, was immobilised onto a 47 mm diameter cellulose acetate membrane (0.2 µm porosity) (Sartorius, Italy), by filtration using a vacuum pump. The membrane was washed twice with 10 ml sterile potassium phosphate buffer (50 mM, pH 7), to remove any medium left on the membrane which may permit contamination. Another identical membrane was then placed on top to sandwich the cells in between. The membrane was either used immediately on the biosensor or placed in an empty sterile Petri dish and stored at 4°C until required. For use on the biosensor, a 5 mm² piece of this membrane was cut and placed between a gas permeable membrane (provided by Florence

University, Italy) and nylon net, and fixed onto the dissolved oxygen probe with a rubber O-ring.

However, using this method significant variation was found between pieces from the same membrane, perhaps due to a non-homogenous distribution of the cells over the surface. It was therefore necessary to use a membrane with a smaller diameter (one which could be entirely fixed onto the probe and thus eliminate the need to cut smaller pieces). It was also necessary, therefore, to concentrate the cells into a smaller volume.

Subsequent immobilisations were conducted using the following method: cells were harvested by centrifuging 2 ml of an 18 hour culture at 6000 rpm for 10 minutes at room temperature. The supernatant was discarded, and the pellet re-suspended in 1 ml of sterile potassium phosphate buffer (50 mM, pH 7). The re-suspended pellet was filtered drop-wise through the centre of a sterile 13 mm diameter cellulose acetate membrane (0.2 micron) using a vacuum pump. The membrane was washed drop-wise with the same volume of sterile potassium phosphate buffer, and another cellulose acetate membrane (25 mm diameter, 0.2 micron) was placed on top to sandwich the cells in between. The gas-permeable polyethylene membrane, and the two cellulose acetate membranes (containing the immobilised bacteria) were fixed onto the dissolved oxygen probe using a rubber o-ring as shown in Figure 2.1.

Final modifications to the latter immobilisation method incorporated the re-suspension of the bacterial pellet after centrifugation into a smaller volume of buffer (100 μ l). This enabled the bacterial solution to be pipetted directly onto the cellulose acetate membrane, allowing the buffer to filter through naturally, without the use of the vacuum pump. This was thought to be a gentler immobilisation technique, and permitted the construction of the biosensor in a faster time.

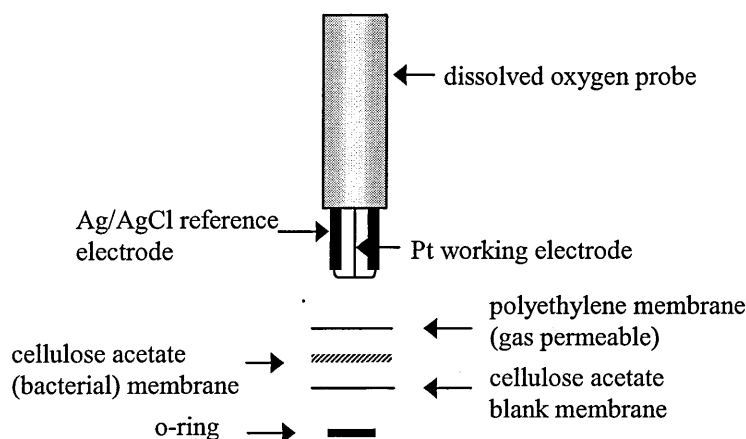


Figure 2.1. The fixation of the bacterial membrane onto the dissolved oxygen probe.

2.4. Instrumentation

2.4.1. Batch Mode

A battery operated portable Idronaut Oxyliquid model 23 dissolved oxygen probe and meter (Idronaut s.r.l., Italy) were used as the transducer, and measuring device, respectively. All biosensor tests in batch mode were conducted in 10 ml of 50 mM potassium phosphate buffer (pH 7) in a water-jacketed beaker held at a constant temperature of 25°C. The tip of the oxygen probe (containing the immobilised bacteria) was immersed in the buffer solution through a rubber bung (to prevent benzene evaporation from the reaction vessel). A syringe needle was also inserted through the rubber bung and left in place to permit the passage of atmospheric air into the reaction vessel, and through which, benzene could be injected with a Hamilton syringe.

The beaker was magnetically stirred to create oxygen tension in the potassium chloride electrolyte solution, allowing the reduction of oxygen at the platinum working electrode, and thus the production of electrical current. The passage of oxygen through the membranes was also essential for the aerobic degradation of benzene by the immobilised bacteria. The sensor response was continuously monitored on a chart recorder (Linseis Inc., Italy).

2.4.2. Kinetic Mode (Flow Injection Analysis)

For the Flow Injection Analysis (FIA) system (kinetic mode), a potassium phosphate buffer (50 mM, pH 7) carrier solution (saturated with atmospheric air) was pumped using a peristaltic pump (Gilson Minipuls3, Gilson Medical Electronics, France) through a custom-made water-jacketed flow cell containing the dissolved oxygen probe (Figure 2.2). A Rheodyne model 7125 syringe loading sample injector (Rheodyne, CA, USA) was used to introduce standard solutions into the carrier stream. The oxygen probe and meter were the same as those used in batch mode, and the sensor response was continuously monitored on a chart recorder (Linseis Inc., Italy). The oxygen meter could also be replaced by a PalmSens[®] portable potentiostat and pocket PC for *in situ* measurements (as shown in Figure 2.2).

Initial experiments were conducted at 30°C in the kinetic mode with respect to 25°C in batch mode, to compensate for the buffer cooling through the FIA system, though this was later found to be unnecessary as no difference in the sensor response occurred between room temperature (23°C), and 30°C when connected with the water bath (as discussed in Section 5.4).

2.5. Measurement Scheme

The steady state baseline of the oxygen meter prior to the injection of standard solutions, represents the endogenous respiration rate of the immobilised bacteria. Dissolved oxygen diffuses continuously through the dialysis membranes from the aerated potassium phosphate buffer to the surface of the platinum working electrode of the oxygen probe, where it is reduced at a fixed potential of -600 mV versus a Ag/AgCl reference electrode, as shown in Figure 2.3. After injection, benzene diffuses through the cellulose acetate membrane and is assimilated by the immobilised bacteria, resulting in an increase of bacterial respiration rate and oxygen consumption. This causes a decrease in dissolved oxygen near the electrode surface, resulting in a decrease in the output sensor signal until a new steady state is obtained, in the case of batch mode experiments. This decrease in dissolved oxygen, and corresponding decrease in the

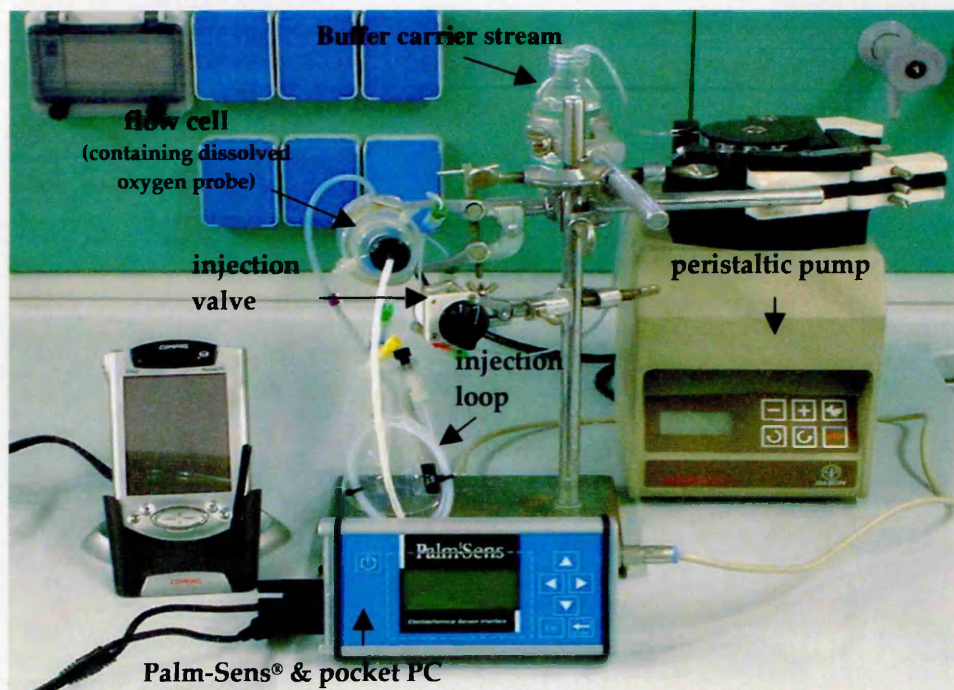


Figure 2.2. Scheme of the bacterial biosensor within the flow injection analysis system, pictured here connected to the PalmSens® portable potentiostat.

sensor signal is proportional to the concentration of benzene injected. With the FIA system, a similar decrease in the output sensor signal is obtained until the injected sample starts to leave the flow cell and at which point the sensor signal starts to recover back to the original baseline. Thus the sensor response is determined by the height of the peak, which corresponds to the amount of dissolved oxygen consumed by the bacteria, and hence to the concentration of benzene injected.

The output reading from the oxygen meter is displayed in arbitrary units, not current readings, hence all results are presented as a percentage decrease from the original steady state baseline; $Bx/Bo \times 100$, where: Bx is the secondary sample baseline, and Bo is the original baseline. Similarly where potentiostats have been used, some current readings have also been presented as a percentage decrease from the original baseline for comparison.

The lower detection limit of the biosensor was defined as the mean of the blank response plus 3 times the standard deviation, which incorporated responses equal to or greater than a 5% decrease from the original baseline based on benzene in potassium phosphate buffer (50 mM, pH 7). For air sample analysis with the inclusion of 2% DMF in the buffer (Section 2.7), responses equal to or greater than an 8% decrease from the original baseline was used to define the lower detection limit of the biosensor. Responses succeeding a 60% decrease from the baseline (resulting from the degradation of a high concentration of benzene) were generally avoided due to the irreproducibility of the biosensor response at higher levels of oxygen consumption.

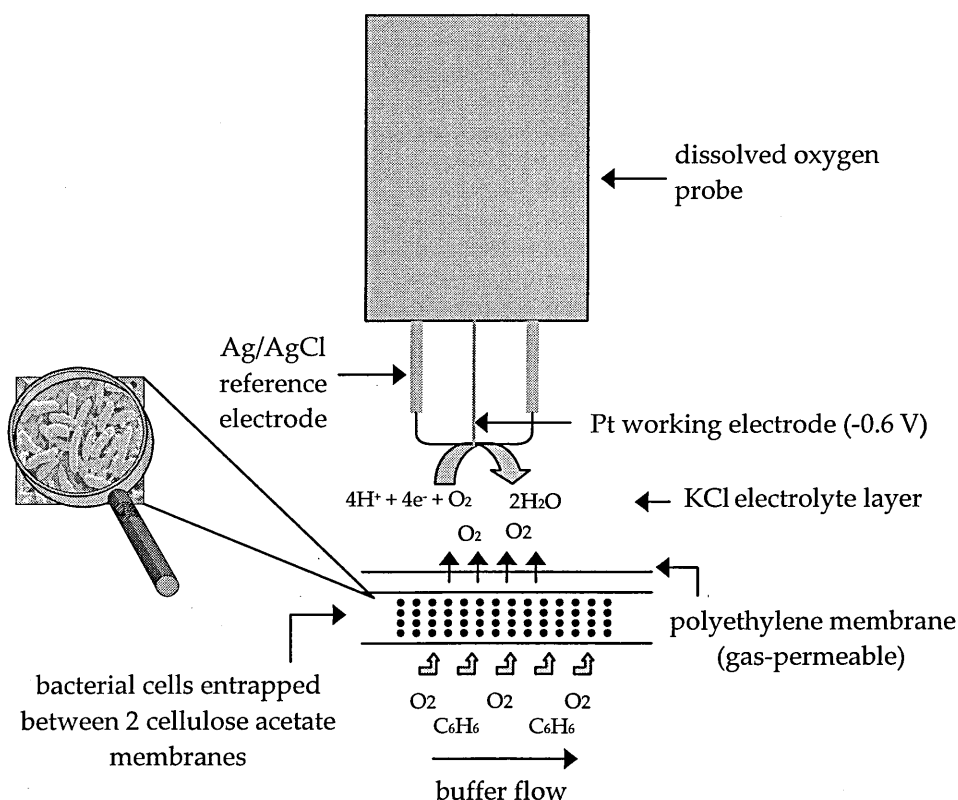


Figure 2.3. Scheme of the bacterial biosensor showing the passage of oxygen through the bacterial membranes to the surface of the working electrode, as the basis of measurement.

2.6 Experimental Procedures

2.6.1. Batch Mode

For the creation of benzene calibration curves in batch mode, for each concentration, the probe was immersed in a fresh 10 ml of potassium phosphate buffer (to allow a faster baseline recovery), the baseline allowed to stabilise, and injections of benzene made using a Hamilton syringe. The required volume of benzene from a 10 mM solution was injected to create the desired concentration within the 10 ml of buffer in the reaction vessel. Calibration curves could also be performed in the same 10 ml of buffer, by adding increasing volumes of 10 mM stock solution to the desired concentrations. The change in the sensor output reading from the original baseline to the secondary baseline (the sensor response) was noted for each concentration over time.

For examining the optimal buffer pH (between values of 5 and 9), individual 10 ml volumes of potassium phosphate buffer (50 mM, pH 7) were adjusted to the desired pH using 1 M potassium hydroxide and hydrochloric acid where necessary. Each of those 10 ml volumes were then transferred into the reaction vessel, the baseline allowed to stabilise, and a concentration of benzene injected. The change in the sensor output reading from the original baseline to the secondary baseline (the sensor response) was noted for each pH over time. The concentration of benzene injected for the assay depended on the sensor response under normal conditions at pH 7 prior to testing. The sensor response could sometimes vary between different bacterial membranes, and with the age of a particular bacterial membrane. Therefore, the concentration yielding a significant decrease from the original baseline (for example, a 40% decrease) prior to testing under normal conditions, was used throughout the assay.

For the investigation of temperature effects up to 35°C, above room temperature the water bath was adjusted to the desired temperature, and the temperature inside the reaction vessel (of the 10 ml potassium phosphate buffer, 50 mM, pH 7) was measured using a probe thermometer. Measurements made below room temperature were not easily achieved. 10 ml volumes of potassium phosphate buffer (50 mM, pH 7) taken

from cold storage at 4°C, were transferred into the reaction vessel and the sensor baseline allowed to stabilise. However, by the time taken to reach a steady state output reading, the buffer had heated quite considerably. A starting temperature of 16°C was the lowest that could be achieved under those conditions. For each temperature, a concentration of benzene was injected into the reaction vessel, and the change in the sensor output reading from the original baseline to the secondary baseline (the sensor response) was noted over time. The concentration of benzene injected depended on the sensor response prior to the assay as described for the pH test.

To investigate the effect of interfering compounds such as glucose, toluene, and catechol; these compounds were diluted to an intermediary concentration and injected into the reaction vessel using the same method as for benzene.

2.6.2. Kinetic Mode (Flow Injection Analysis)

For all measurements in the kinetic mode, potassium phosphate buffer (50 mM, pH 7) in an open bottle (saturated with atmospheric air) was pumped through the flow cell and the baseline of the sensor output was allowed to stabilise. For the creation of calibration curves, the desired concentration of benzene was made and then injected into the FIA carrier stream using the manual sample injector. For each concentration of benzene, the decrease in sensor response was noted over time, in the same way as for the batch mode.

For examining the optimal buffer pH (between values of 5 and 9), individual 100 ml volumes of potassium phosphate buffer (50 mM, pH 7) were adjusted to the desired pH in the same way as described for the batch mode. For each pH, the 100 ml volume was pumped through the flow cell, and the baseline of the oxygen meter allowed to stabilise. A concentration of benzene was injected and the sensor response over time noted. The concentration of benzene injected depended on the sensor response prior to the assay as described for batch mode.

Temperature testing was undertaken in the same way as described for the batch mode. The potassium phosphate buffer (50 mM, pH 7) was immersed in the water bath and

allowed to heat up to the desired pre-set temperature, and pumped through the water-jacketed flow cell. Below room temperature, potassium phosphate buffer taken from cold storage at a starting temperature of 4°C was pumped through the flow cell and the sensor baseline allowed to stabilise. However, under those conditions, the buffer heated quite considerably during the time taken to reach a steady sensor output. For each temperature, a concentration of benzene was injected and the sensor response was noted over time. The concentration of benzene injected depended on the sensor response prior to the assay as described for the batch mode.

The interfering compounds tested with the FIA were prepared as for batch mode in Section 2.6.1.

2.7. Air Sampling Methodology

2.7.1. Dimethylformamide (DMF) experiments

Calibration curves conducted for benzene for use in air sample analysis were prepared from a stock solution of 10 mM benzene in pure DMF. This was diluted in potassium phosphate buffer (50 mM, pH 7) to concentrations between 0.025 and 0.2 mM prior to injection into the FIA system, thus creating DMF concentrations no greater than 2%.

For the DMF desorption efficiency experiments, this involved injections of liquid benzene directly onto the primary sections of charcoal air sampling tubes (see Section 2.7.2) followed by the transfer of the charcoal into gas-tight vials (see Section 2.7.2) for solvent desorption (following NIOSH air sample desorption procedures, Kennedy *et al.* 1995). For each experiment, 4.5 µl of benzene was injected into the primary charcoal section, and DMF volumes between 2 and 10 ml were used to investigate desorption efficiency (benzene recovery). Each eluent was diluted in potassium phosphate buffer (between 1:250 - 1:50), prior to injection into the FIA system in order to obtain a theoretical 0.1 mM benzene concentration for each eluent (in the case of 100% desorption). Corresponding reference solutions containing 4.5 µl of benzene over the same DMF volume range (without charcoal) were also made to represent 100% benzene

recovery solutions (diluted in the same way prior to injection into the FIA system). The reference results were then compared with the results obtained from the charcoal eluents after desorption (calculated from the biosensor standard calibration curves).

2.7.2. Active Sampling

Benzene air samples for the active sampling investigations were taken from an exposure room of controlled benzene concentration at the Fondazione Salvatore Maugeri (FSM) Institute of Pavia, Italy. Periodical “grab samples” were taken from the exposure room by syringe and analysed by gas chromatography (GC) (Hewlett Packard model HP5890) to monitor the benzene concentration for comparison with biosensor analyses. Air samples were collected using 400/200 mg charcoal adsorption tubes (Supelco – Sigma, Milan, Italy) attached to pre-calibrated air pumps (provided by the FSM Institute of Pavia, Italy) at a flow rate of 1 l min^{-1} inside the exposure room (Figure 2.4). The 400 and 200 mg amounts of charcoal represent the primary and backup sections, respectively (Figure 2.4). After the sampling period, the charcoal was transferred into a gas-tight vial (Supelco – Sigma, Milan, Italy) and 5 ml of DMF added for solvent desorption. Benzene extraction was undertaken for a period of 30 minutes at room temperature with regular agitation. All sample eluents were diluted in potassium phosphate buffer (50 mM, pH 7) prior to injection into the FIA system for biosensor analyses. All sample eluents were cross-analysed by GC, and all the GC measurements were conducted by Paola Zadra at the FSM Institute.

The original exposure room concentration was calculated from the biosensor results by firstly using the linear equation from the calibration curves to calculate the sample eluent concentration; then the exposure room concentration was calculated based on the DMF desorption volume and the number of litres of air sampled.

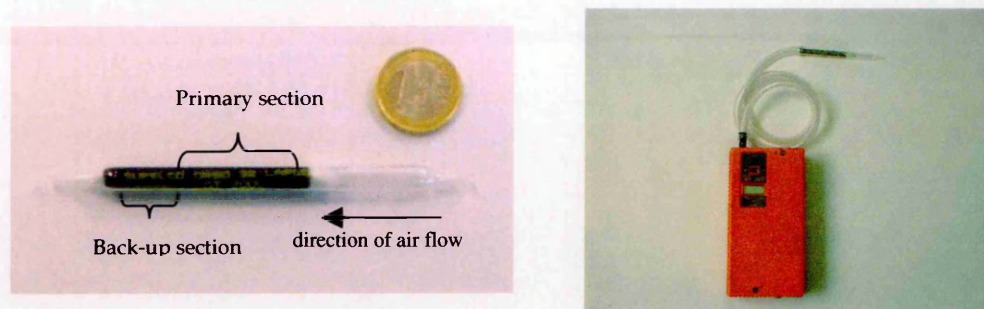


Figure 2.4. The charcoal tube and air pump used for active sampling.

2.7.3. Passive Sampling

Real industrial air samples (from a factory in Prague, Czech Republic) were taken using Radiello[®] diffusion tubes (Centro di Ricerche Ambientali, Padova, Italy) (Figure 2.5). 20 factory employees wore a diffusion tube containing a charcoal sorbent, for a sampling period of 7 hours. After the sampling period, benzene was extracted from 10 of the charcoal sorbents using DMF, and the other 10 using carbon disulphide (CS₂). For all samples, 2 ml of desorption solvent was placed inside the glass tube containing the charcoal cartridge, and left for a period of 2 hours at room temperature, with occasional shaking (following manufacturer guidelines (Radiello[®] 2004)). The concentration of benzene in the extraction eluents was determined by gas chromatography at the FSM Institute of Pavia, Italy, and the DMF extracted samples were also cross-analysed by the biosensor. These results were then used to calculate the original concentration of airborne benzene in the corresponding part of the factory in which the employees worked.

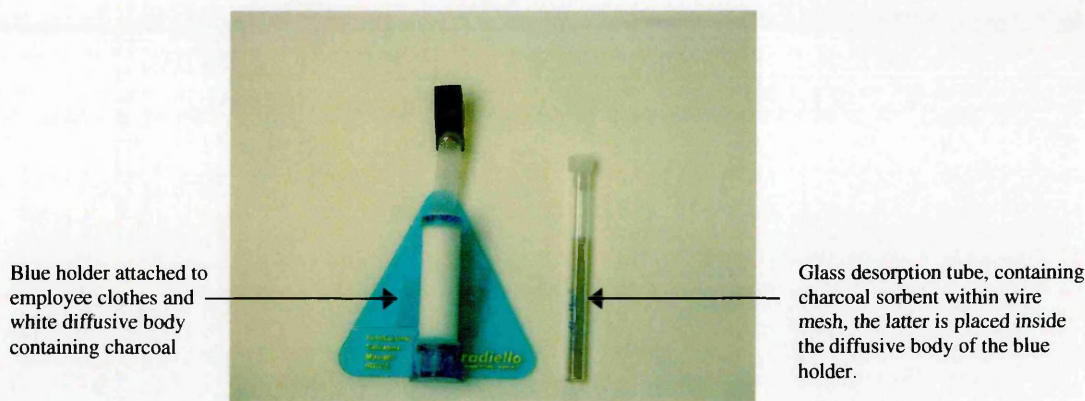


Figure 2.5. Passive air sampling diffusion tubes.

2.8. Screen Printed Electrodes

The screen-printed electrodes (SPEs) consisted of a gold working electrode, a silver pseudo-reference electrode, and graphite counter electrode (University of Florence, Italy). For immobilisation, a 1 ml volume from an 18-hour bacterial culture was centrifuged, and the pellet re-suspended in 30 μ l of potassium phosphate buffer (50 mM, pH 7) (the same cell loading as used for the Clark electrode but pre-concentrated into a smaller volume). The cell paste was then pipetted drop-wise onto the centre of a pre-cut 9 mm diameter cellulose acetate membrane to form a small circle and allowed to dry for 5 minutes. A small ring of double-sided adhesive (outer diameter 9 mm, thickness 2 mm) was placed around the working electrode. The bacterial cellulose acetate membrane was then stuck onto the adhesive ring, with bacteria sandwiched between the electrode surface and the cellulose acetate membrane (Figure 2.6).

For all measurements, the bacterially-modified SPE was immersed in a stirred solution of 10 ml 0.1 M KCl solution (dissolved in 50 mM potassium phosphate buffer, pH 7), to provide the necessary chloride ions for the Ag/AgCl reference electrode. All amperometric measurements were conducted with the PalmSens[®] portable potentiostat

at a fixed potential of -0.7 V versus Ag/AgCl , for the reduction of dissolved oxygen. Measurements of benzene were conducted as for the batch mode using the Clark electrode (Section 2.6.1).

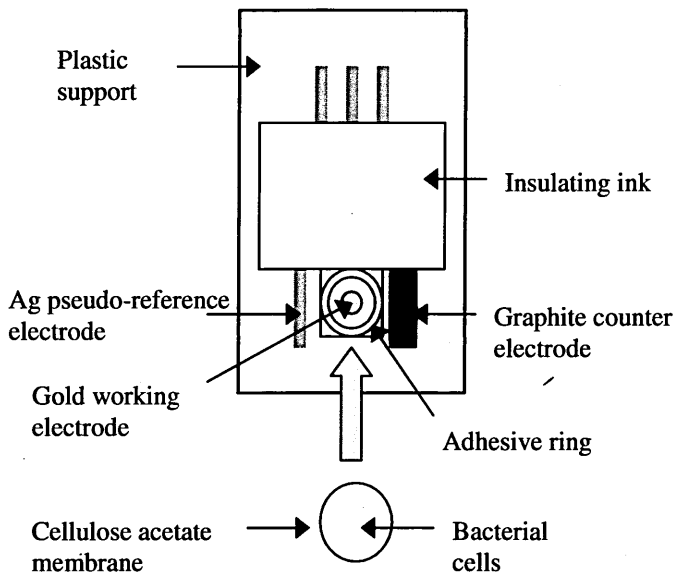


Figure 2.6. The construction of the bacterially-modified screen-printed electrode.

CHAPTER 3

RESULTS

BACTERIAL GROWTH

CHAPTER 3: BACTERIAL GROWTH

This chapter focuses on the growth of *Pseudomonas putida* ML2 cells, including the measurement of the different phases of growth in closed batch culture, and the measurement and calculation of cell numbers. This part of the project represents the preparation of the biological component of the biosensor for the analysis of benzene, and the optimisation of growth conditions and measurement techniques for reproducible cell numbers.

3.1. Growth of *Pseudomonas putida* ML2

The four phases of a bacterial growth curve vary depending on the bacterial strain, type of medium, and growth conditions. For the construction of a bacterial biosensor based on viable cells, it is therefore necessary to measure the growth curve of the selected strain, in order to harvest the cells in the correct phase. Most bacterial biosensors have been constructed using cells from the late exponential or stationary growth phases (D'Souza, 2001). This section will present the results obtained from measurements of growth and viability of *Pseudomonas putida* ML2.

Initial growth curves for this particular microorganism revealed that under the growth conditions described in Chapter 2, the strain had a long lag phase, reflected by no increase in spectrophotometrical scattering after 8 hours following incubation. The exponential and stationary phases were thus seen to occur overnight, reflected by maximum spectrophotometrical scattering after 18 hours. This behaviour can be attributed to the inoculation of dormant cells into the liquid medium, taken from an agar plate at 4°C. This long lag phase occurs because the cells require more time to regenerate the necessary enzymes, co-factors, and ribosomes in order to utilise benzene in the minimal medium. In order to monitor the growth phases, it was therefore necessary to take a sub-culture from an 18-h culture, which was transferred into a fresh liquid medium the following morning. In this way, actively growing cells are being transferred, which grow in an exponential manner immediately upon inoculation into a

fresh medium. Thus, the growth phases can be measured in one day as shown in Figure 3.1.

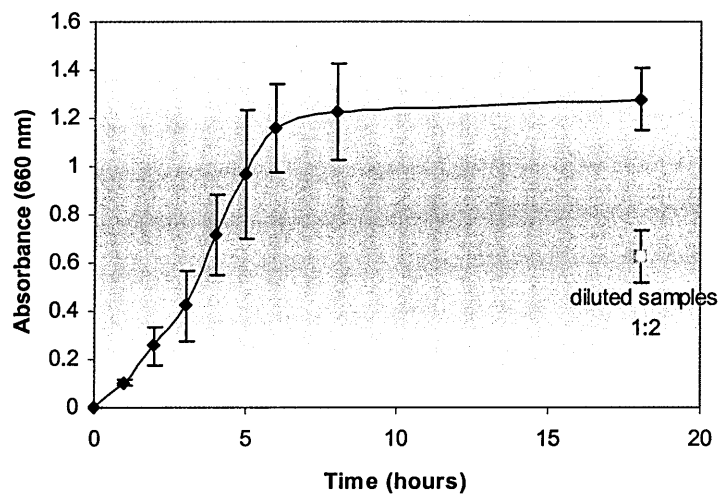


Figure 3.1. A typical growth curve for *Pseudomonas putida* ML2. The error bars for the values between 0 and 10 hours show the standard deviation obtained from 7 separate cultures grown on different days. The error bars of the value for the 18-h overnight cultures are based on 12 different cultures. The diluted samples conducted at 18 hours, were to demonstrate that the spectrophotometer had not reached its saturation point.

3.2. Measuring Cell Numbers

As described in Chapter 1, there are various means of calculating bacterial culture cell numbers. Spectrophotometrical measurements allow an estimation of cell numbers per ml of liquid medium; however, they cannot distinguish between living and dead cells. For a biosensor based on bacterial respiration, it is therefore necessary to measure the numbers of viable cells in the medium, to enable a calculation of viable cells immobilised on the membrane.

Viable cell counts were conducted by growing an overnight culture and measuring the absorbance after 18 hours. An average absorbance (A_{660}) after 18 hours of growth was typically around 1.3 when grown under the same conditions. With an absorbance value of around 1, it is estimated that a culture contains around 8×10^8 cells ml^{-1} , as previously reported (UNC-CH, 2002). A 10^{-6} dilution of 1 ml of an 18-hour culture was therefore carried out to give an estimated final concentration of 8×10^2 cells ml^{-1} . From the final 1 ml dilution, 0.25 ml of this was transferred onto an LB agar plate and distributed over the agar surface using a sterilised L-shaped spreader. It was hoped that approximately 200 cells would therefore be distributed onto the agar surface. Agar plates were incubated at 30°C overnight. The diluting medium used was the previously outlined minimal medium used for cell culture described in Chapter 2. Nutrient rich LB agar plates were used here as very poor growth occurred on the minimal medium agar plates for the viable cell counts.

After overnight incubation, the colonies on the agar were counted using a grid drawn on the bottom of the plate to enable easier counting. Each colony is the progeny of a single bacterial cell; therefore, the number of cells ml^{-1} in the original culture is then the number of cells on the plate multiplied by the overall dilution factor. However, it is important to note that cell losses may occur during the serial dilution (in pipette tips, for example – though this was minimised by flushing with the diluting medium) or left on the L-shaped spreader.

The results obtained from 3 viable cell counts conducted on the same 18-hour culture are shown in Table 3.1. In this case, 3 dilutions of 10^{-6} were conducted on 1 ml of the same overnight culture. A 31% coefficient of variation from the mean was obtained for the 3 counts, demonstrating the variation that can arise due to experimental error. A typical mean of around 15×10^8 cells ml^{-1} was obtained using a 10^{-6} dilution.

Further tests based on serial dilutions of 10^{-7} , resulted in a higher mean number between $50 - 60 \times 10^8$ cells ml^{-1} . This could be attributed to a lower concentration of cells transferred onto the agar plate, resulting in less cell competition and clumping, hence better growth on the agar surface. Table 3.2 shows the results obtained from three assays based on serial dilutions of 10^{-7} of three separate cultures grown for 18 hours. The results show reproducible viable cell counts ml^{-1} with a 4% variation coefficient in this case. These counts were then used as the basis of subsequent viable cell numbers per ml of 18-hour cultures. This enabled comparisons between different filtration volumes of the culture during immobilisation for cell loading investigations described in Chapter 4.

Figures 3.2 and 3.3 show the results obtained from plotting a viable cell count against the corresponding absorbance growth curve from the spectrophotometer. The viable cell counts were based on 10^{-6} and 10^{-7} dilutions, respectively for the two figures, resulting in a higher count in the latter case as described previously. Both Figures show a corresponding increase in viable cell numbers with increasing absorbance. However, viable cell counts conducted in the mid – late exponential phase (around 6 – 8 hours) were often difficult to achieve. At such a time, it is necessary to estimate the numbers of cells in the medium in order to conduct a serial dilution which will give a countable number of cells on the agar surface. In both Figures shown here, the concentration of cells on the agar surface was too high to permit an accurate cell count at 6 hours on Figure 3.2, and 8 hours in Figure 3.3.

Table 3.1.
Viable cell counts conducted over 3 assays based on 10^{-6} dilutions of the same 18-hour culture. Growth conditions: 30°C, 150 rpm, minimal medium.

Viable Cells ml ⁻¹ (x 10 ⁸)	Mean (x 10 ⁸ cells ml ⁻¹)	CV (%)
14	15	31
11		
20		

Table 3.2.
Viable cell counts conducted over 3 assays based on 10^{-7} dilutions of 3 separate 18-hour cultures. Growth conditions: 30°C, 150 rpm, minimal medium.

Absorbance (660 nm) after 18 hours of growth	Viable Cells ml ⁻¹ (x 10 ⁸)
1.19	55
1.27	57
1.24	59

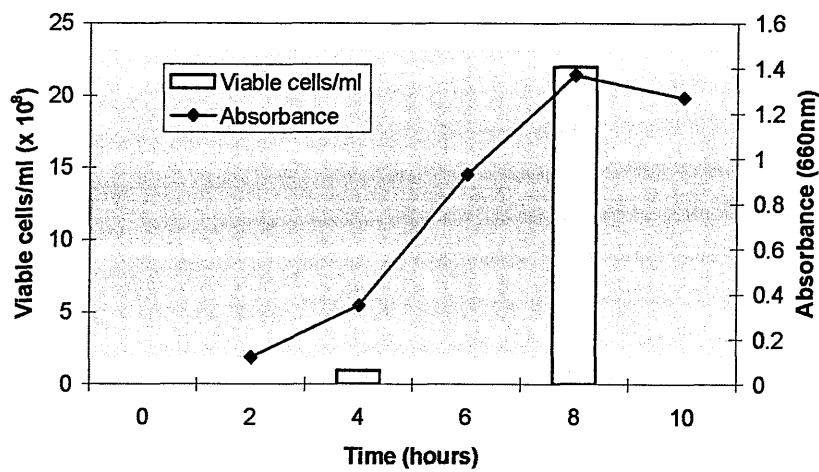


Figure 3.2. Absorbance growth curve and viable cell count for *Pseudomonas putida* ML2. Growth conditions: ML2 medium, 30°C, 150 rpm. Viable cell counts based on 10⁻⁶ dilutions.

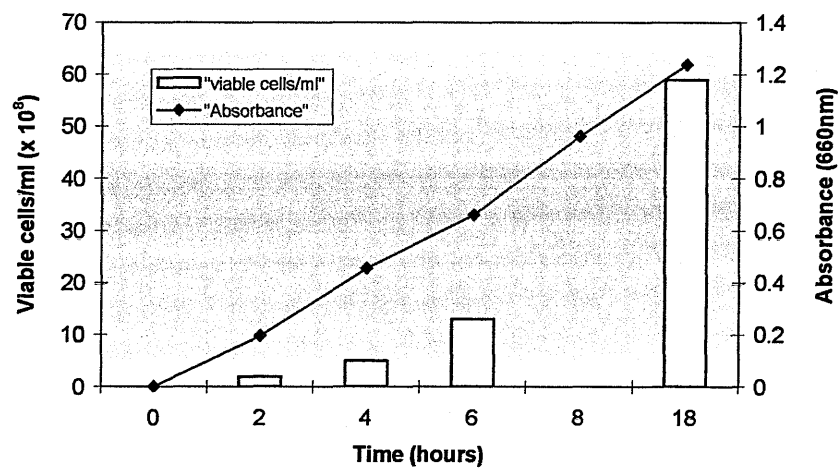


Figure 3.3. Absorbance growth curve and viable cell count for *Pseudomonas putida* ML2 Growth conditions: ML2 medium, 30°C, 150 rpm. Viable cell counts based on 10^{-7} dilutions.

Due to the error that can occur when conducting viable cell counts, a further means of investigating cell loading on the immobilisation membrane was based on the dry weights of culture volumes after centrifugation. For this investigation, increasing volumes between 1 and 4 ml of 18-hour bacterial cultures were centrifuged for 10 minutes at 6000 rpm in pre-weighed Eppendorf[®] tubes. The supernatant was discarded and the pellet re-suspended in sterile potassium phosphate buffer (50 mM, pH 7) as a washing step, before re-centrifugation under the same conditions. The supernatants were again discarded and the pellets dried overnight at 30°C. Figure 3.4 shows an increasing dry weight of the bacterial pellet with increasing culture volume. These results have been used for the investigation of cell loading on the immobilisation membrane, detailed in Chapter 5.

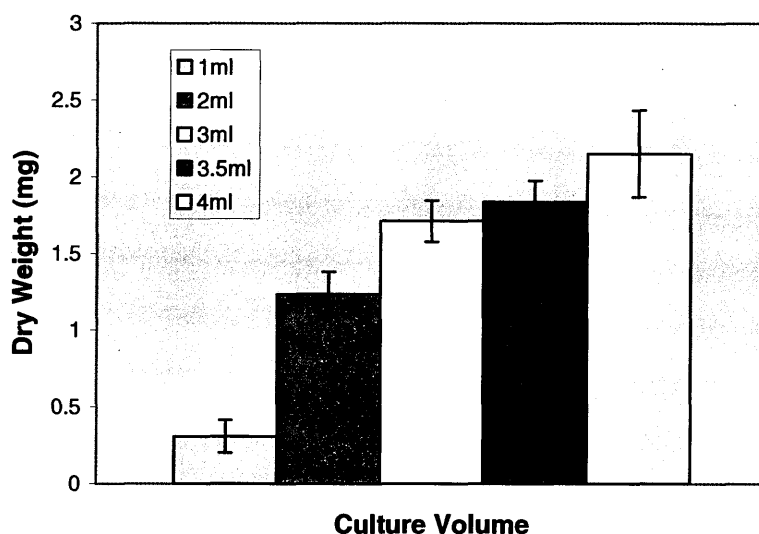


Figure 3.4. The dry weights of various volumes from 18-hour bacterial cultures after centrifugation. Error bars based on 5 measurements.

3.3. *Escherichia Coli* JM109

Chapter 1 reviewed the range of bacteria used in some types of biosensor for environmental monitoring (Table 1.3). Such strains of bacteria and their wide utilisation of organic compounds allow the analysis of many different types of target analyte. Furthermore, some strains may prove to be more robust, or degrade compounds in a faster time, with respect to others. In the present work, *Escherichia coli* (*E. coli*) JM109 cells, genetically modified with the genes encoding the benzene deoxygenase enzymatic pathway from *P. putida* ML2, were tested for their performance as the recognition layer of the biosensor. The sensitivity and stability of the cells for use on the biosensor was compared to the typical response characteristics of *P. putida* ML2 (Section 4.6).

Prior to the biosensor investigations, cell cultures of *E. Coli* were prepared and the dry weights of various culture volumes after centrifugation were measured for investigations of cell loading on the immobilisation membrane. One of the advantages stated on the use the genetically modified (GM) *E. coli* cells, was that the genes encoding the benzene deoxygenase enzymes remain continually expressed in *E. coli*, meaning that the cells do not have to be grown in the presence of benzene in order to activate the enzymes (Briganti, 2003). This is in contrast to *P. putida* ML2, which requires growth in the presence of benzene in order to degrade it when used on the biosensor. No *P. putida* growth occurred in the minimal medium without the presence of benzene. Thus the GM *E. coli* cells could be grown in a simple LB medium without the presence of benzene. From an 18-hour culture in this medium, increasing volumes from 0.25 to 2 ml were centrifuged, and the bacterial pellets dried to measure their dry weights. Figure 3.5 compares the dry weights of various culture volumes of *P. putida* ML2 grown in minimal medium with benzene, and *E. coli* grown in LB medium without benzene. Significantly higher cell numbers were obtained from the *E. coli* culture, due to the nutrient rich nature of the LB medium. The dry weight measurements were used as a guide for the cell loading of different volumes of the *E. coli* culture during immobilisation for the biosensor. The performance of the cells, including their sensitivity to benzene and their stability with respect to *P. putida*, is discussed in Section 4.6, Chapter 4.

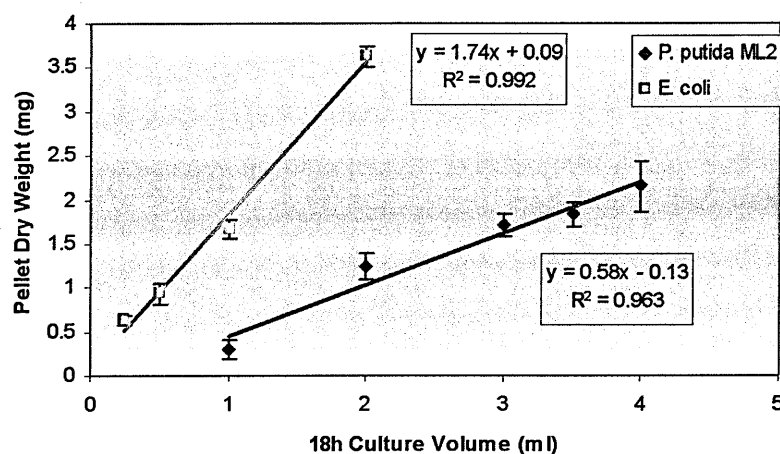


Figure 3.5. A comparison of bacterial pellet dry weights after centrifugation of volumes taken from 18-hour cultures of *E. Coli* JM109 grown in LB medium without benzene, and *P. putida* ML2 grown in minimal medium with benzene. Error bars based on triplicate assays.

3.4. Discussions

The preceding sections of Chapter 3 have presented the results obtained from the measurements of *P. putida* ML2 growth and viability, based on spectrophotometry, viable cell counts and dry weight measurements. Sub-culturing of overnight cultures has enabled the measurement of the lag, exponential and stationary growth phases over one day. After 18 hours of growth (overnight), an average absorbance (600 nm) of around 1.3, with a viable cell count of between $50 - 60 \times 10^8$ cells ml^{-1} , has been obtained. Tan *et al.* (1994) reported a *P. putida* ML2 cell concentration of 79×10^8 cells ml^{-1} , taken from the exponential growth phase, and used for membrane immobilisation for a benzene biosensor. The growth time of the culture was not reported. Under the growth conditions used in the present work, viable cell numbers of $50 - 60 \times 10^8$ cells ml^{-1} obtained after 18 hours of growth overnight, have enabled a lower limit of detection with respect to that obtained by Tan *et al.* (1994) (Chapter 4).

As aforementioned, most bacterial biosensors have been constructed using cells taken from the late exponential or stationary phases of growth (D'Souza, 2001), typically from an over-night culture in the latter case. *P. rathonis* T was tested in various stages of growth for the development of biosensor receptor elements by Taranova *et al.* (2002). It was established that the maximal response to sodium dodecyl sulphate (SDS) at a concentration of 0.2 kg/m^3 could only be obtained using 5- to 6-hour cells that corresponded to the exponential phase of growth. It was also reported that the degradation of SDS and some other anionic surfactants by this culture occurred much more quickly in the exponential phase of growth than in the stationary one (Taranova *et al.*, 2002). This highlights the importance of measuring the growth phases of a particular bacterium, in order to harvest the cells in the best phase or physiological condition for use on the biosensor. Chapter 5 includes the biosensor results obtained from measurements using exponential and stationary phase cells.

Section 3.2 has shown the results obtained from viable cell counts and dry weight measurements after centrifugation of *pseudomonas putida* ML2 cultures. These measurements have been used for the construction of the biosensor, in order to optimise the performance of the biosensor in terms of sensitivity, response characteristics, and reproducibility as described in Chapters 4 and 5.

Over the course of the project, *P. putida* ML2 cells were maintained on agar plates by the monthly transfer of cells to fresh agar, and stored at 4°C . Stock solutions of the original cell culture grown at the start of the project were also stored at -80°C in 8% glycerol. Throughout the project, bacterial cultures for the biosensor were grown using cells taken from the agar plates. Since it is possible that natural mutations can occur over time with the continual growth of bacteria, the biosensor response using cells taken from the original culture stored at -80°C were compared with the cells that were maintained monthly over the last 2 years. Chapter 5 shows the results obtained from the investigation, to determine the stability of the bacterial ability to degrade benzene over repeated growth.

Chapter 3 has described the optimisation of growth conditions and cell number measurements for the biological component of the biosensor, *Pseudomonas putida* ML2. The succeeding results chapters will focus on the construction and development of the biosensor, based on the optimised growth conditions thus far described, for the analysis of benzene.

CHAPTER 4

RESULTS

BIOSENSOR FUNCTION IN BATCH MODE

CHAPTER 4: BIOSENSOR FUNCTION IN BATCH MODE

In this Chapter, the response of the bacterial biosensor in batch mode is investigated. Experiments were conducted in a magnetically stirred solution of potassium phosphate buffer (10 ml, 50 mM, pH 7) in a water-jacketed beaker held at a constant temperature of 25°C. The beaker was closed with a rubber bung to prevent benzene evaporation, and benzene was introduced into the buffer by injection with a Hamilton syringe to the concentration desired. The sensitivity and reproducibility of the biosensor response to varying concentrations of benzene was investigated over a variety of parameters; including pH, temperature, time, cell loading and storage of the bacterial membrane. This part of the thesis represents the initial stages in the construction and development of the biosensor.

Before the investigation of the bacterial biosensor response to benzene, the response of the oxygen probe was tested during the elimination of dissolved oxygen. This was achieved by bubbling nitrogen through a beaker of potassium phosphate buffer (50 mM, pH 7) for a period of 5 minutes. The oxygen meter readings are arbitrary units, not amperometric current readings, hence the meter can be adjusted to 100 to enable biosensor responses to be expressed in terms of a percentage decrease from this original baseline value. Figure 4.1 shows the response of the dissolved oxygen probe during the elimination of dissolved oxygen using nitrogen. With only the gas permeable polyethylene membrane attached, a 90% decrease from the original baseline occurred within 1 minute, after which a secondary baseline was reached.

The responses with 2 blank cellulose acetate membranes, and with the bacterial membranes were also investigated; and in both cases, a slightly slower response can be seen due to the longer diffusion time of the oxygen through the cellulose acetate membranes. However, the secondary baseline value reached was the same (Figure 4.1).

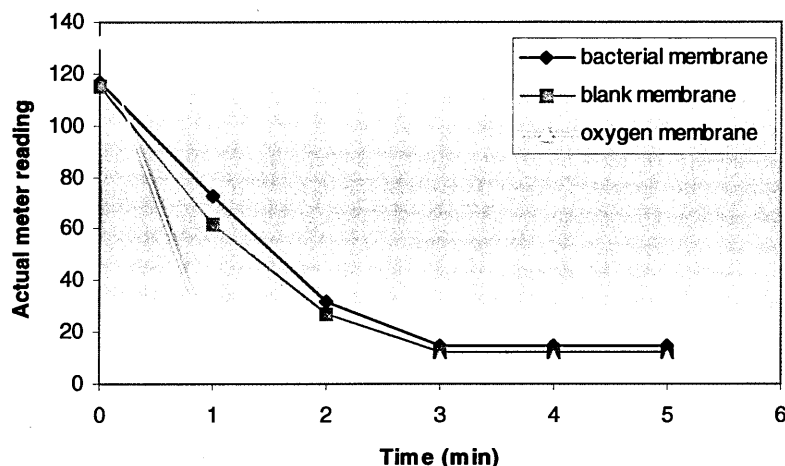


Figure 4.1. The response of the dissolved oxygen probe during the elimination of dissolved oxygen using nitrogen.

Being a battery operated portable device, it was necessary to check the oxygen probe response during the elimination of oxygen on a regular basis as the response was found to decrease with the age of the battery. The battery had to be renewed at least once every 6 weeks, as a general increase in the baseline of the deoxygenised buffer from around 10 to 50 units on the meter was evident from 9 V (a fully charged battery) to around 5 V (after 5-6 weeks of use), respectively. This may cause inaccuracies in the results, particularly if the working electrode is not held at -600 mV versus the Ag/AgCl reference electrode (the optimal potential for the reduction of oxygen).

4.1. Sensitivity and Reproducibility

Prior to the testing of the bacterial membranes, the Clark oxygen electrode was tested with blank membranes, and benzene concentrations injected. This was to ensure that the benzene itself did not cause a decrease in the dissolved oxygen concentration in the reaction vessel. No response was detected over a concentration range up to 1 mM benzene (results not shown). A blank membrane response has been included in Figure 4.4.

Figure 4.2 shows a typical response of the bacterial biosensor over time, after injection of a standard solution of benzene to give a 0.1 mM concentration in the reaction vessel. The response time of the biosensor in batch mode, the time taken to reach a secondary stable baseline, was between 8 – 10 minutes, depending on the concentration of benzene injected. Higher concentrations, and thus higher sensor responses, took longer to reach the secondary baseline. Calibration curves could be conducted in the same 10 ml of buffer (without changing the buffer between concentrations), by injection of increasing volumes of a 10 mM benzene stock solution as shown in Figure 4.3. After each calibration curve, a fresh 10 ml of buffer was used, with a baseline recovery time of 30 – 40 minutes.

The biosensor response in batch mode displayed a linear range between 0.025 – 0.1 mM benzene (Figure 4.4), with a lower detection limit of 0.01 mM. This was based on the immobilisation of 1 ml of an 18-hour bacterial culture, of 1.2 mg dry weight (after centrifugation). These results are comparable with those obtained by Tan *et al.* (1994), and Campanella *et al.* (1996), in which a lower detection limit of 0.07 mM and 0.01 mM benzene was obtained, respectively, for a benzene biosensor based on *P. putida*. Similar response and baseline recovery times were also obtained.

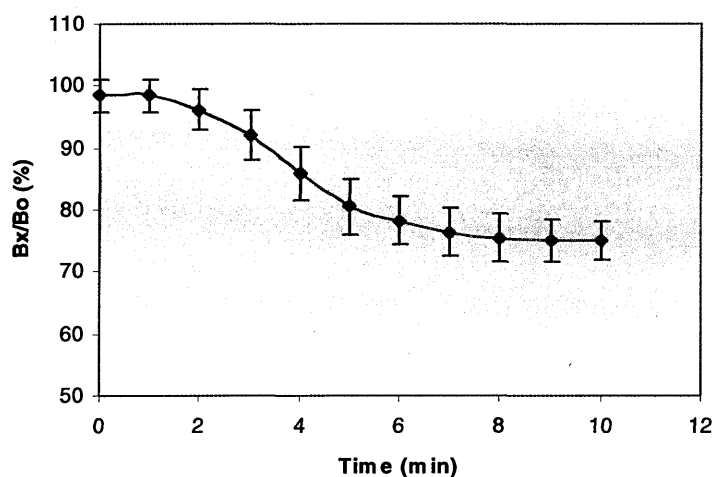


Figure 4.2. Typical biosensor response to 0.05 mM benzene in batch mode. B_x/B_o (%): the percentage decrease from the original baseline where, B_x = sample/standard baseline, and B_o = original baseline. Error bars are the standard deviation from the mean, based on three repetitions of the assay.

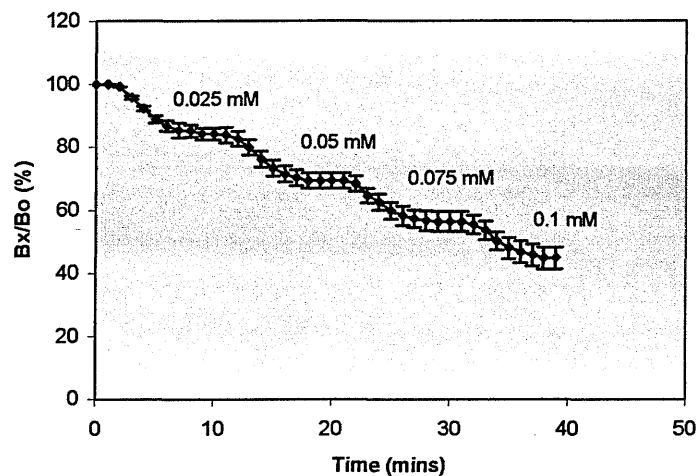


Figure 4.3. Typical response characteristics to calibration curves for benzene in batch mode. B_x/B_o (%): the percentage decrease from the original baseline where, B_x = sample/standard baseline, and B_o = original baseline. Error bars are the standard deviation from the mean based on three repetitions of the assay.

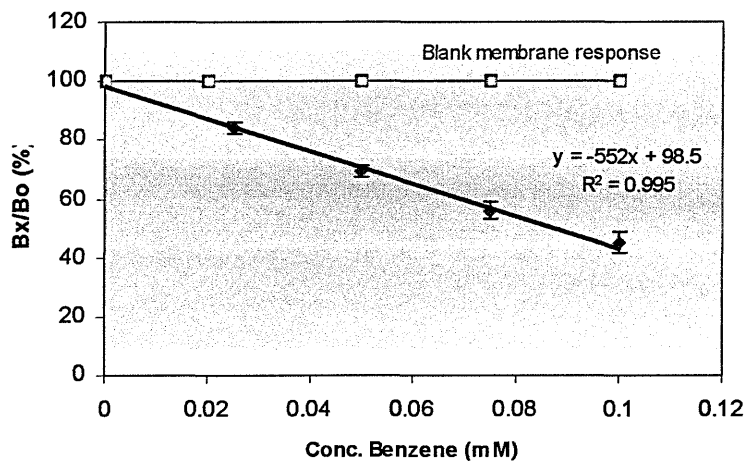


Figure 4.4. Calibration curve for benzene, and the response of a blank membrane (without immobilised bacteria). B_x/B_o (%): the percentage decrease from the original baseline where, B_x = sample/standard baseline, and B_o = original baseline. Error bars are the standard deviation from the mean based on three repetitions.

The reproducibility of the bacterial biosensor was tested over one day of continuous use. Figure 4.5 shows the results obtained from six calibration curves conducted in one day, leaving 30 minutes between each (the time taken for the original baseline to recover) at the start of the project. A decrease of 41% can be seen from the first to the sixth calibration curve. This could have been attributed to cellular metabolic control mechanisms, or feedback inhibition mechanisms, which prevent energy wastage in the cells. As the product of a particular enzymatic reaction accumulates, less of that product is needed by the cell, until the particular enzyme product actually inhibits the first enzyme of that metabolic pathway (Prescott *et al.*, 1993). This was also reported by Liu *et al.* (2000) in which a decrease in sensor response was found over repeated measurement of the same substrate or sample. Liu *et al.* (2000), however, suggested that the decrease in sensor response could have been a result of the frequency of measurements causing incomplete sensor recovery, or a slight increase in the bacterial endogenous respiration rate. However, in the present work, this decrease in sensitivity over one day was also evident when fewer measurements were taken.

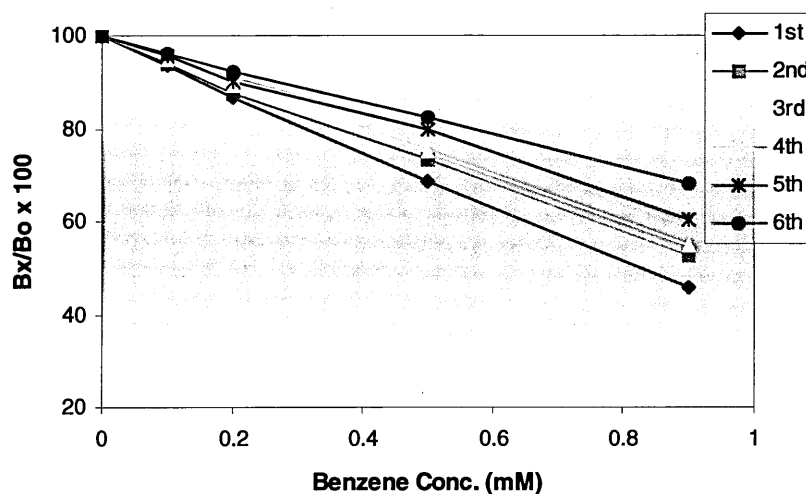


Figure 4.5. Testing the reproducibility of the biosensor. 6 calibration curves conducted in one day, leaving 30 minutes between each. B_x/B_o (%): the percentage decrease from the original baseline where, B_x = sample/standard baseline, and B_o = original baseline.

Over the course of the project, as the immobilisation method and operating conditions were optimised, a stable biosensor response was obtainable throughout a day of measurements, as shown in Figure 4.6. Further examples of this stability are also included in Chapters 5 and 6, as the biosensor has been developed towards air sample analyses. The improvement in reproducibility could be attributed to an improved immobilisation technique, in which bacterial cells from 1 ml of an 18-hour culture were centrifuged and re-suspended in a 100 µl volume of buffer, before being pipetted onto the surface of the cellulose acetate membrane, allowing the buffer to filter through naturally. The immobilisation method used in the initial stages of the project did not involve centrifugation, but the filtering of differing culture volumes (from 1 ml to 5 ml) through the cellulose acetate membrane using a vacuum pump. This is quite a forceful method, and may have caused some cell damage during the immobilisation process. This was also reported by Campanella *et al.* (1996) in which no response was obtained using this bacterial immobilisation technique. Following optimised immobilisation and operating procedures, the stability of the membrane over one day of use in the present work was not found to be influenced by the number of measurements made, as suggested previously.

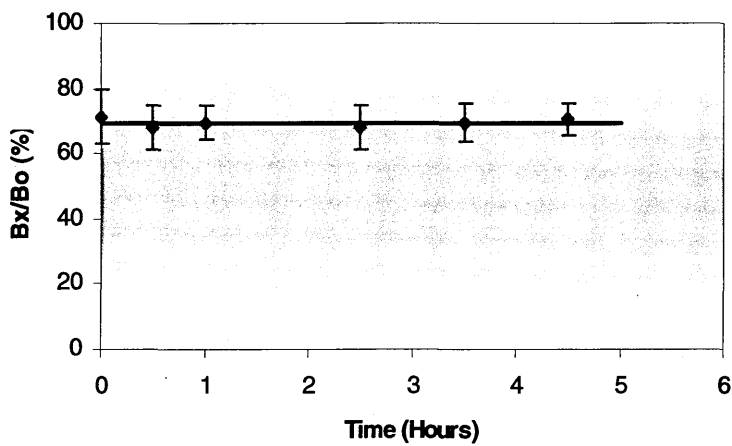


Figure 4.6. Reproducibility of the biosensor response to 0.05 mM benzene over one day of continuous use, based on 6 measurements. B_x/B_o (%): the percentage decrease from the original baseline where, B_x = sample/standard baseline, and B_o = original baseline. Error bars are the standard deviation from the mean, based on 4 repetitions of the assay using different bacterial membranes.

The reproducibility of the biosensor was also tested over one week of use, based on the same bacterial membrane. Figure 4.7 shows an initial decrease in sensitivity to 0.05 mM benzene from the first to the second day of use, and a stable response from then to the end of the 6-day assay period. The response of the bacterial membrane immediately following immobilisation, could have been due to the bacteria stabilising and adapting to their new environment on the membrane surface, hence some of the cells may be lost during this adjustment period. The lifetime of the bacterial membrane and its storage when not in use are discussed in more detail in Section 4.4.

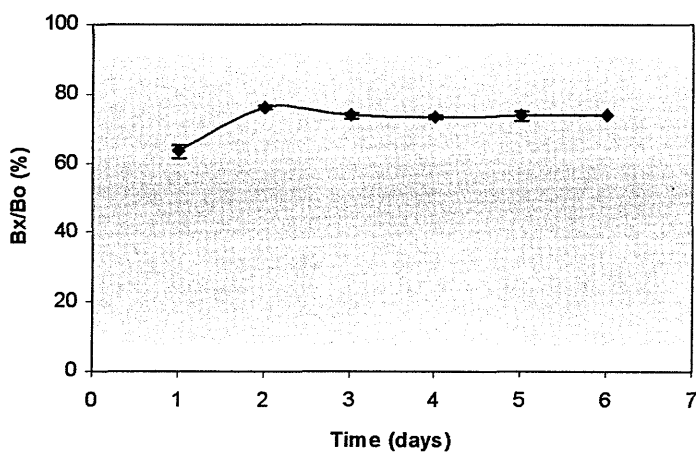


Figure 4.7. Reproducibility of the biosensor response to 0.05 mM benzene over 6 days of continuous use. Error bars based on 5 daily measurements. B_x/B_o (%): the percentage decrease from the original baseline where, B_x = sample/standard baseline, and B_o = original baseline.

The reproducibility of the optimised immobilisation method was investigated over 5 separate immobilisations from different 18-hour bacterial cultures. Immobilisations were based on centrifugations of 1 ml culture volumes re-suspended in 100 μ l buffer as described in Chapter 2. Table 4.1 shows the results from this investigation, which yielded a variation coefficient of 11% over the 5 immobilisations conducted, hence demonstrating the stability of the immobilisation method, and also the reproducibility of the cell numbers generated from the bacterial cultures.

Table 4.1.
Reproducibility of the immobilisation technique. Sensor response of different membranes to 0.05 mM benzene. Sensitivity displayed in percentage decrease from the original baseline. Mean based on triplicate measurements.

Immobilisation	Mean percentage decrease from the baseline (%)
1	37 ± 2
2	39 ± 2
3	39 ± 6
4	30 ± 4
5	33 ± 4

Variation coefficient = 11% (n = 5).

4.2. Effect of Cell Loading

Since the biosensor is based on bacterial respiration, the response is dependent on the number of viable cells immobilised on the membrane. Although spectrophotometrical measurements enable an estimation of cell numbers per ml of a liquid medium, it cannot differentiate between living and dead cells. Viable cell counts were therefore conducted to estimate more accurately the numbers of living cells per ml of liquid medium (Section 3.2). An average of around 50 - 60 x 10⁸ viable cells ml⁻¹ was calculated after 18 hours of growth. For the initial cell loading investigations conducted, a decrease in sensitivity was found using a membrane immobilised with 10 ml of a culture containing 7 x 10⁸ viable cells ml⁻¹, with respect to a membrane immobilised with 10 ml of a culture containing 20 x 10⁸ viable cells ml⁻¹ (Figure 4.8). However, as mentioned in Chapter 3, viable cell counts are subject to a large amount of error, hence are not an accurate means of assessing cell loading. Dry weight measurements after centrifugation of culture volumes were therefore selected as a more accurate means of cell loading assessment, after the establishment of the viability of the bacterial cells after 18-hours of growth (i.e. the cells were calculated to be in the stationary phase of growth and not in the death phase). This was also established by Axcell & Geary (1975) when *P. putida*

ML2 cells were grown for 18 hours under the same conditions. The results from the dry weight cell loading investigations are presented in Section 5.2.

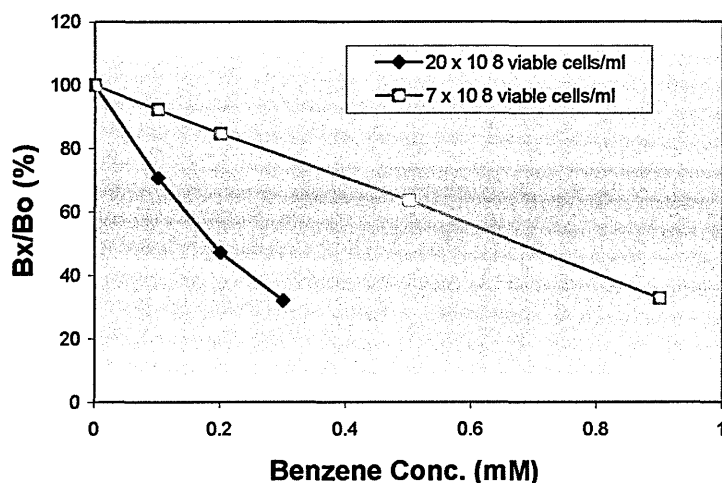


Figure 4.8. The effect of viable cell numbers on the bacterial membrane on the biosensor response in batch mode. B_x/B_o (%): the percentage decrease from the original baseline where, B_x = sample/standard baseline, and B_o = original baseline.

4.3. Effect of pH and Temperature

Optimal pH and temperature conditions are known to be essential for the functioning of enzymatic reactions, and thus for the function of enzyme-based biosensors. The effects of pH and temperature on the biosensor response to 0.1 mM benzene were therefore investigated in batch mode. This was achieved by increasing the temperature of the water bath, to which the water-jacketed reaction cell was attached. Figure 4.9 shows a 16% increase in sensor response from 16 – 35°C, accompanied by a corresponding increase in response time of around 4 minutes, respectively. A temperature of 30 – 35°C is closer to the optimum temperature for the growth of *P. putida*, and similar increases in sensor response were also reported by Tan *et al.* (1994), using the same

bacteria over this temperature range in batch mode. However, Figure 4.9 also shows that the sensor operated almost equally as well at a room temperature of 25°C, reflecting indoor workplace measurement conditions, hence eliminating the need for a water bath (provided the room temperature is controlled). A temperature of 30°C was chosen for overnight growth of the bacteria in liquid culture to prevent the cells from reaching the death phase during the 18-h period.

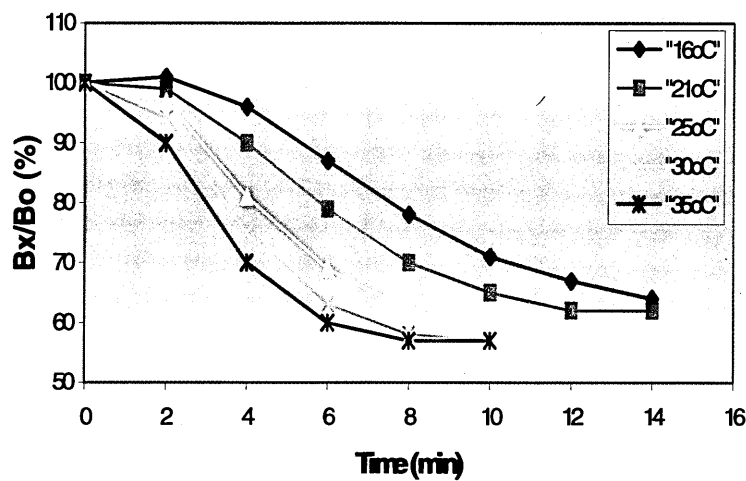


Figure 4.9. The effect of temperature on the sensor response in batch mode to 0.1 mM benzene. B_x/B_o (%): the percentage decrease from the original baseline where, B_x = sample/standard baseline, and B_o = original baseline.

The measurement of temperatures below room temperature ($23^{\circ}\text{C} \pm 1$) was not easily achieved as the buffer was taken from cold storage at a starting temperature of 4°C , and during the time taken for the sensor baseline to stabilise, the solution had heated quite considerably. It is important to note however, that changes in temperature during the measurement of dissolved oxygen, will cause inaccuracies in the results as the amount of dissolved oxygen will vary depending on the temperature of the buffer in the reaction vessel (Wilson and Walker, 1994). However, the biosensor is based on the measurement of the oxygen consumed by the bacteria, and not on direct measurements of dissolved oxygen concentration.

For a pH range between 5 and 9, volumes of 50 mM potassium phosphate buffer were adjusted to the desired pH and used to fill the reaction vessel into which the oxygen probe containing the immobilised bacteria was immersed. However, no significant differences in sensitivity over the pH range investigated were found in batch mode (results not shown). This could be due to the bacterial ability to naturally buffer the intracellular enzymes by the active transport of ions inside and outside of the cell, thus maintaining a neutral pH within the cell itself. This is contrary to the results obtained by Tan *et al.* (1994), in which the highest sensor responses were obtained between a pH of 7 and 8, which is the optimum pH for the growth of this bacteria.

4.4. Lifetime of the Bacterial Membrane

Due to the time taken and consumption of materials used for growth of an 18-hour bacterial culture, it is more beneficial to prepare one bacterial membrane and use it for as long as the sensitivity and reproducibility of the sensor response persists. Initial investigations of the lifetime of the bacterial membrane were conducted using two methods. The first method involved the storage of the bacterial membrane on the dissolved oxygen probe in a 1 mM benzene solution (in 50 mM potassium phosphate buffer, pH 7) at room temperature when not in use, following that utilised by Tan *et al.* (1994). However, this method caused a decreasing trend in sensitivity over 3 days using this storage method in batch mode such that no response to benzene was obtained on the 3rd day of use (results not shown). It can therefore be concluded that the decrease in response was caused by cell death on the membrane over time. Reshetilov *et al.* (1997a) and Liu *et al.* (2000) reported similar decreases in response utilising this storage method. However, Tan *et al.* (1994), found stable and reproducible responses for 3 months with storage at room temperature in a 1.35 mM benzene-buffer solution, also using *P. putida* ML2.

The second storage method involved the storage of the bacterial membrane at 4°C in a 1 mM benzene-buffer solution when not in use. This method proved to be much more effective in pro-longing the lifetime of the membrane. Figure 4.10 shows that over 22

days of continuous use in batch mode, this storage method maintained a high sensor response with no significant decrease over the assay period. Similar findings were also reported by Mulchandi *et al.* (2002), where membrane storage at 4°C maintained 90% of the original response for a two-week period in which the sensor was used 20 times.

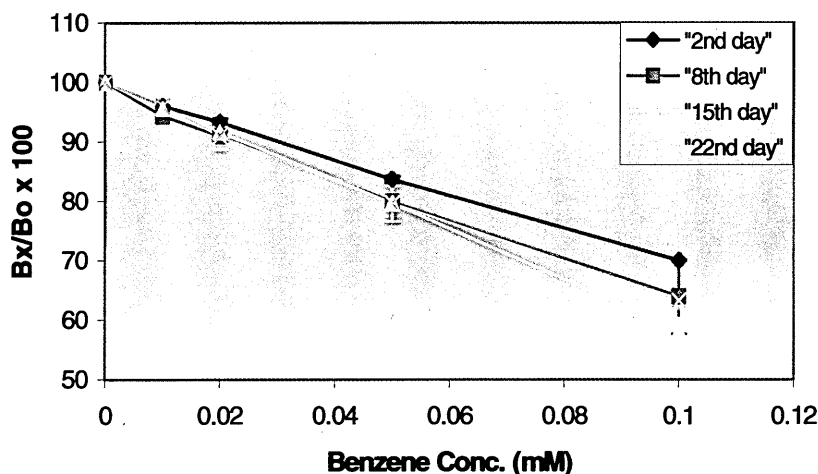


Figure 4.10. Biosensor response over 22 days of continuous use in batch mode. When not in use, the membrane was stored at 4°C in a 1 mM benzene-buffer solution. B_x/B_o (%): the percentage decrease from the original baseline where, B_x = sample/standard baseline, and B_o = original baseline. Error bars are the standard deviation from the mean, based on triplicate calibrations curves.

The use of stored culture aliquots (1 ml volumes taken from the same 18-hour bacterial culture and stored at 4°C until required) to allow new immobilisations without repeated growth of fresh bacterial cultures was also investigated over a 7-day period. A variation coefficient of 32% in sensor response was obtained among 4 different aliquots used on days 1, 3, 5 and 7, with no decreasing trend in sensitivity over storage time (results not shown). This method is beneficial in that it eliminates the need for repeated growth of 18-hour bacterial cultures, hence saving the time and costs associated with bacterial culturing. It also allows the bacteria to be transported to *in situ* locations. Schmidt *et al.* (1996) also reported up to 10 days of use with negligible loss in sensor response utilising the same method, stating the aforementioned advantages.

4.5. Effect of Interfering Compounds

Bacterial biosensors can have the disadvantage of non-specificity to a particular target analyte with respect to enzyme-based sensors (Mulchandani and Rogers, 1998), which is a considerable problem for samples such as wastewater containing large amounts of organic substances. Similarly workplace air samples will contain various volatile organic compounds, depending on the type of processes or activities undertaken there, which could be potentially interfering compounds. In the initial stages of the project, the biosensor response to toluene, catechol, and glucose was investigated in batch mode. Toluene is a benzene derivative and another commonly found compound in industrial air, and catechol is an intermediary compound in the benzene-degradation chain. The sensor response to glucose was also investigated at this stage as a means of comparison of the oxygen consumed for the degradation of this simple sugar, with respect to the benzene derivatives.

Table 4.2 shows the response of the bacterial biosensor to 0.1 mM concentrations of benzene, toluene, catechol, and glucose. The highest response was obtained with benzene (a 36% decrease from the original baseline), with a negligible response to toluene resulting in a 4% decrease from the original baseline. A slight response to toluene was also obtained by Tan *et al.* (1994), using *P. putida* ML2; and a much greater response was obtained by Campanella *et al.* (1996). However, a different *Pseudomonas* strain was used in the latter case. Table 4.2 also shows that similar responses were obtained for catechol and glucose, resulting in a decrease of around 15% from the original baseline, thus indicating less oxygen consumption for the degradation of the less complex molecular structure of these compounds with respect to benzene. The testing of further benzene derivatives is detailed in Chapters 5 and 6.

Table 4.2.
Biosensor response to 0.1 mM concentrations of benzene, toluene, catechol, and glucose in batch mode. Results presented as a percentage decrease from the original sensor baseline.

Compound	Decrease from the baseline (%)
Benzene	36
Toluene	4
Catechol	13
Glucose	16

4.6. *Escherichia Coli* JM109

As described in Section 3.4, *Escherichia coli* (*E. coli*) JM109, genetically modified with the genes encoding the benzene deoxygenase enzymatic system from *P. putida* ML2, was tested on the biosensor. The sensitivity and stability of the bacterial cells on the immobilisation membrane was compared with that of *P. putida* ML2. As previously shown in Figure 3.5, *E. coli* grown in a nutrient rich LB medium caused the growth of a significantly higher number of cells after 18 hours. Thus cell loadings from 2 and 1 ml culture volumes resulted in no oxygen passage through the membranes, in contrast to relatively unrestricted oxygen passage using similar culture volumes of *P. putida*. An *E. coli* culture volume of 0.25 ml (corresponding to around 0.7 mg dry weight) was found to have a similar sensitivity to benzene to that of *P. putida* ML2 immobilised using a culture volume of 2 ml (corresponding to a cell loading of 1.2 mg dry weight, results not shown).

Despite a similarity in sensitivity between the two strains of bacteria, the stability of the *E. coli* cells on the immobilisation membrane was not comparable. A 50% decrease from the original sensor response to 0.05 mM benzene was found with *E. coli* over one day of use, (Figure 4.11), compared to no decrease in sensitivity as displayed in Figure 4.6 for *P. putida* for the same concentration of benzene. This could be due to the natural ability of the *P. putida* ML2 cells to survive in minimal competitive conditions as they

would do in their natural soil habitat, acquired by adaptation over generations, thus accounting for their robustness and survival between injections on the immobilisation membrane. Since in this case the GM *E. coli* have not occurred naturally in such an environment, they may lack the necessary physiological components, hereditarily acquired during binary fission in bacterial cell division, for their survival during biosensor measurements.

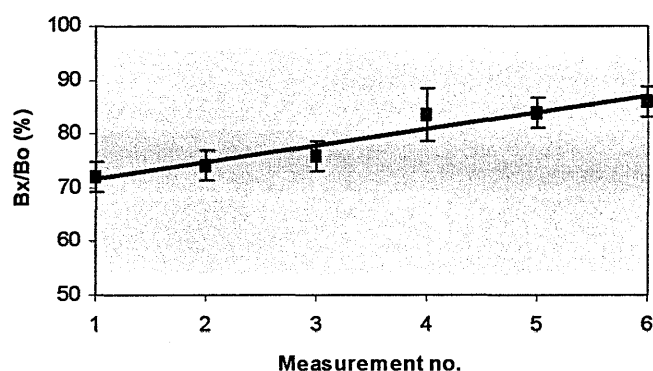


Figure 4.11. The stability of the sensor response to 0.05 mM benzene using genetically modified *E. coli* JM109 cells. B_x/B_o (%): the percentage decrease from the original baseline where, B_x = sample/standard baseline, and B_o = original baseline. Error bars are the standard deviation from the mean, based on triplicate repetitions.

4.7. Discussions

Chapter 4 has described the set-up and optimisation of the biosensor in batch mode, in a stirred solution of potassium phosphate buffer. Measurements of bacterial cell numbers and mass from liquid cultures described in Chapter 3 were used as a basis for the construction of the biosensor. Viable cell counts revealed an average of around $50 - 60 \times 10^8$ viable cells ml^{-1} after 18 hours of growth. This was also established by Axcell & Geary (1975) when *P. putida* ML2 cells were grown for 18 hours under the same conditions. However, the viable cell counts were found to be subject to error, and hence not an accurate means of investigating the effects of cell loading on the biosensor

conditions. However, the viable cell counts were found to be subject to error, and hence not an accurate means of investigating the effects of cell loading on the biosensor response. Dry and wet weight measurements of the bacterial cells after centrifugation have been commonly utilised in the literature as a means of investigating cell loading effects (Katrlik *et al.* 1996, Mulchani *et al.* 2002, Reshetilov *et al.* 1997a, Taranova *et al.* 2002). The effect of cell loading using bacterial dry weights on the sensor response in the present work is described in Chapter 5.

The effects of the immediate external environment on the bacterial response to benzene were investigated, showing an increased response and shorter response time with increasing temperatures up to 35°C. A temperature of 30 – 35°C is closer to the optimum temperature for the growth of *P. putida*, and similar increases in sensor response were also reported by Tan *et al.* (1994), using the same bacteria over this temperature range in batch mode. However, the sensor operated almost equally as well at a room temperature of 25°C (with a slightly longer response time), reflecting indoor workplace measurement conditions, hence eliminating the need for a water bath (provided the room temperature is controlled). This was also reported by Mulchandi *et al.* 2002, in which an increase in *Moraxella* sp. response to *p*-nitrophenol up to 37°C was thought to be due to an increase of both enzyme reaction and mass transport rates. Decreases in sensor response above this temperature were reported to be due to enzyme denaturation. Despite the optimum performance of the sensor at 37°C, Mulchandi and co-workers (2002) continued to operate the Clark oxygen electrode-based sensor at a temperature of 20°C to overcome the problem of evaporative losses during the course of the experiment and for ease of operation. They also concluded that because the solubility of oxygen is an inverse function of the temperature, operating at higher temperatures would reduce the linear and dynamic ranges of the biosensor (Mulchandi *et al.* 2002).

No significant differences in sensitivity were found over a pH range between 5 and 9, perhaps due to the bacterial ability to naturally buffer the intracellular enzymes by the active transport of ions inside and outside of the cell, thus maintaining a neutral pH within the cell itself. This is contrary to the results obtained by Tan *et al.* (1994), in

which the highest sensor responses were obtained between a pH of 7 and 8, which is the optimum pH for the growth of *Pseudomonas putida* ML2. Katrlík *et al.* (1996) also reported independence of the *Aspergillus niger* response to glucose over a pH range from 5 to 8, despite reports of the glucose oxidase enzyme dependence on pH. The pH independence of the biosensor in this case was reported to be due to the location of the glucose oxidase enzyme in the peroxisomes inside the cells, meaning that changes of the external pH had no influence on the value of the intracellular pH (Katrlík *et al.*, 1996).

The lifetime of the bacterial cells on the immobilisation membrane was investigated by utilising two commonly used membrane storage methods in the literature, that is at room temperature and at 4°C. The cells are usually in contact with a substrate solution in both cases. This allows to the sensor to be used for prolonged periods of time, hence reducing time and costs in the preparation of fresh bacterial cultures and new membranes. For the room temperature storage method in the present work, a significant decrease in sensor response was found over three days, despite the presence of benzene. Reshetilov *et al.* (1997a) and Liu *et al.* (2000) reported similar decreases in response utilising this storage method. However, Tan *et al.* (1994) found stable and reproducible responses for 3 months with storage at room temperature in a 1.35 mM benzene-buffer solution, also using *P. putida* ML2.

The decrease in response in the present work could have been due to the faster growth of the cells at room temperature, hence faster utilisation of the benzene source (1 mM benzene in potassium phosphate buffer), thus leaving the cells without a carbon source for a prolonged period during the night. Storage of the membrane at 4°C with benzene proved to be much more effective for pro-longing the lifetime of the membrane, where a sensitive response to benzene was maintained over 22 days. Similar findings were also reported by Mulchandi *et al.* (2002), where membrane storage at 4°C maintained 90% of the original response for a two-week period in which the sensor was used 20 times. This storage method was also utilised by Schmidt *et al.* (1996), where the membrane retained 50% of its initial activity from 7 to 25 days over the assay period. This is in contrast to Katrlík *et al.* (1996) where a greater membrane lifetime was reported at room

temperature than at 4°C, reported to be due to better regeneration conditions of the cells at room temperature, however the sensor still lost 25% of its initial sensitivity after 20 days of use.

The influence of interfering compounds on the *Pseudomonas putida* ML2 response to benzene was investigated, in order to determine the specificity of the biosensor. The sensor proved to be highly specific for benzene, with a slight response to toluene. A similar response to toluene was also obtained by Tan *et al.* (1994), using *P. putida* ML2; and a much greater response was obtained by Campanella *et al.* (1996). A comparison of the sensor response to glucose and catechol revealed more oxygen consumption for the degradation of benzene with respect to the less complex molecular structure of the former two compounds.

With the biosensor operating in batch mode under optimised conditions, a linear range between 0.025 – 0.1 mM benzene, with a lower detection limit of 0.01 mM was obtainable. This was based on the immobilisation of 1 ml of an 18-hour bacterial culture, of 1.2 mg dry weight (after centrifugation). These results are comparable with those obtained by Tan *et al.* (1994), and Campanella *et al.* (1996), in which a lower detection limit of 0.07 mM and 0.01 mM benzene was obtained, respectively, for a benzene biosensor based on *P. putida*. Similar response and baseline recovery times between 10 – 12 minutes, and between 30 – 40 minutes respectively, were also obtained, depending on the concentration of benzene injected.

Chapter 4 has presented the results obtained from the biosensor in batch mode, in a stirred solution of potassium phosphate buffer. Although the results presented in this chapter are comparable with those obtained in the literature for Clark oxygen electrode-based biosensors in batch mode, a faster analysis time and more automated system is desirable for the analysis of large numbers of samples, and for the potential testing of workplace air samples. The next Chapter therefore describes the adaptation of the biosensor in a Flow Injection Analysis (FIA) system.

CHAPTER 5

RESULTS

BIOSENSOR FUNCTION IN THE KINETIC MODE (FLOW INJECTION ANALYSIS)

CHAPTER 5: BIOSENSOR FUNCTION IN THE KINETIC MODE USING FLOW INJECTION ANALYSIS

The use of flow injection analysis (FIA) has become increasingly popular in recent years, particularly for on-line continual monitoring (Karl and Pacey, 1989). It allows a more systematic analysis, offering precise injection volumes and often allowing faster analysis times. It also permits the development of an automated system, hence without requiring the constant presence of trained personnel (Karl and Pacey, 1989). In this Chapter, the response of the bacterial biosensor in the kinetic mode is investigated. The dissolved oxygen probe, with the immobilised bacterial membrane, was inserted into a water-jacketed custom-made flow cell as part of a flow injection analysis (FIA) system as described in Chapter 2. The effects of various parameters on the sensor response, including pH, temperature, cell loading, time, and bacterial membrane storage conditions were investigated. Within the FIA system, the effects of different flow rates and injection volumes were also investigated. This part of the project represents the development of the biosensor system to be used for air sample analyses.

In the kinetic mode, although the dissolved oxygen electrode is the same as that used in the batch mode (Chapter 4), and the principles of measurement are the same, the flow system means that the sensor output signal is represented differently. In batch mode, the original baseline is seen to decrease to a secondary steady baseline after injection of benzene, which represents the oxygen consumption of the bacteria as the benzene is degraded. The secondary baseline in this case, representing the maximum respiratory activity necessary for the concentration of benzene injected, which remains as a baseline until all of the benzene has been consumed and the respiratory activity decreases. With flow injection analysis, a continual flow of buffer through the flow cell (containing the dissolved oxygen probe and immobilised bacteria), into which samples are injected, is represented as a “peak” from the sensor output signal. After sample injection, benzene passes into the flow cell and diffuses through the cellulose acetate membrane where it is assimilated by the immobilised bacteria. This causes an increase in bacterial respiration rate and oxygen consumption, which results in a decrease in the concentration of dissolved oxygen near the electrode surface. This is accompanied by a corresponding decrease in the output sensor signal until the injected sample starts to leave the flow cell

and at which point the sensor signal starts to recover back to the original baseline. Thus the sensor response is determined by the height of the peak, which corresponds to the amount of dissolved oxygen consumed by the bacteria, and hence to the concentration of benzene injected. Figure 5.1 shows the typical biosensor response to increasing concentrations of benzene over time, using the FIA system. The “peaks” of the sensor output signal here represent the concentrations of benzene injected, and typically occur between 5 and 6 minutes after injection. The red line on the graph indicates the original baseline to which the peaks recover, taking between 10 to 15 minutes, depending on the concentration of benzene injected.

Furthermore, in contrast to the batch mode, in which standard solutions of benzene can be injected into the 10 ml of buffer in the reaction vessel to obtain the concentration desired, for the FIA system, the desired concentration of standard must be made prior to injection. The carrier stream is not used for diluting in this case, but for carrying the injected sample to the flow cell.

5.1. The Effect of Flow rates and Injection Volumes

For the detection of benzene using the flow injection analysis system, a range of flow rates and injection volumes were tested, in order to achieve the greatest sensitivity with a relatively fast baseline recovery time. At the onset of the development of the FIA system, a sample injection loop volume of 200 μl was selected, as this was thought to be closest to the dead volume of the flow cell. However, poor sensitivity was achieved under those conditions due to the dispersion and dilution of this small injection volume in the FIA carrier stream. The bacterial cells need an increased exposure time to their substrate with respect to an enzyme-based sensor, as benzene has to pass through their cell walls before its degradation through an enzymatic chain, as detailed in Chapter 1.

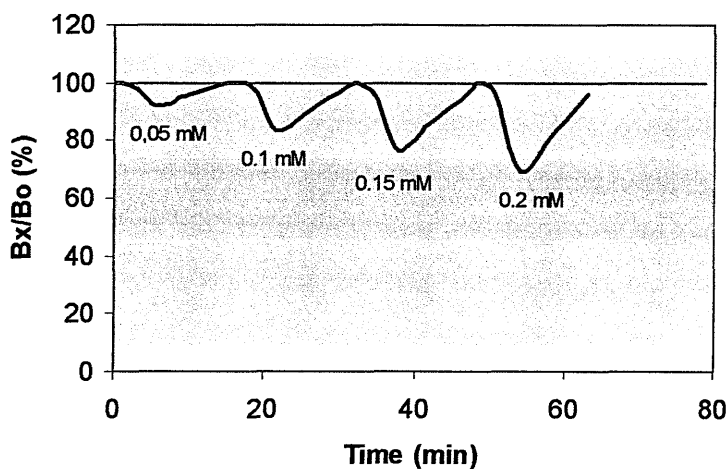


Figure 5.1. Typical response over time of the biosensor operating in kinetic mode during the conductance of calibration curves for benzene. The red line represents the original baseline of the sensor output signal. FIA conditions: flow rate: 0.5 ml min^{-1} ; injection loop: 1 ml. B_x/B_o (%): the percentage decrease from the original baseline where, B_x = sample/standard baseline, and B_o = original baseline.

Higher injection volumes between 1 and 3 ml were therefore investigated. The effect of increasing injection loop volumes on the sensor response to 0.1 mM benzene were investigated at a flow rate of 0.5 ml min^{-1} , following conditions reported in the literature for a BOD bacterial biosensor based on flow injection analysis (Liu *et al.*, 2000). Figure 5.2 shows an increasing sensor response from the 200 μl to 3 ml loop (reflected by a 4 – 24% decrease from the original baseline), however these responses were also coupled with respective increases in response and baseline recovery times. This increasing sensitivity could be attributed to the larger amount of sample passing through the flow cell, hence an increased presence of the same concentration of benzene in the flow cell over time. This would allow a greater amount of benzene to be assimilated by the bacteria. Smaller injection volumes are more prone to dispersion and dilution in the FIA carrier stream, which is further influenced by the carrier flow rate and the distance between the injection valve and the flow cell. A 1 ml loop was selected as the optimum injection volume allowing a relatively fast response and baseline recovery time, despite the less sensitive response.

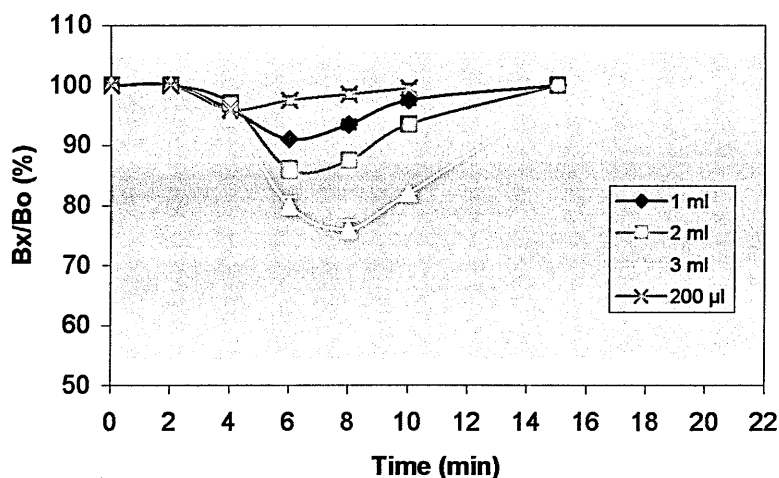


Figure 5.2. The effect of different injection loop volumes on the sensor response to 0.1 mM benzene at a flow rate of 0.5 ml min^{-1} . B_x/B_o (%): the percentage decrease from the original baseline where, B_x = sample/standard baseline, and B_o = original baseline.

Based on the 1 ml injection loop, Figure 5.3 shows the effect of different flow rates on the sensor response to 0.1 mM benzene. An increase in sensor response can be seen as the flow rate is decreased from 1 ml min^{-1} to 0.25 ml min^{-1} , this is reflected by a respective 14 - 44% decrease from the original baseline. This can be attributed to an increase in the occupancy time of benzene in the flow cell at the lowest flow rate. This allows a greater amount of benzene to diffuse through the dialysis membranes, hence a higher amount for the bacteria to catabolise, with respect to the higher flow rates investigated. However, a flow rate of 0.25 ml min^{-1} was also coupled with the longest response and baseline recovery times. A flow rate of 0.5 ml min^{-1} was therefore selected as the optimum condition, as this showed a relatively high sensitivity with a faster response and baseline recovery time (6 minutes and 10 minutes, respectively).

Similar results were also reported by Han *et al.* (2002), in which a compromise was made between sensitivity and baseline recovery time. A total analysis time of 20 minutes was achieved with a flow rate of 0.09 ml min^{-1} , and an injection volume of 1 ml, giving the best sensor response to 0.5 mg l^{-1} trichloroethylene (Han *et al.*, 2002).

In the present work, with the biosensor operating in batch mode, a response time of 10 minutes and a baseline recovery time of 30 minutes was obtained at a 0.1 mM concentration of benzene. The batch mode operation was also coupled with an increased sensitivity of a 37% decrease from the original baseline with respect to a 22% decrease obtained with the FIA system at the same benzene concentration.

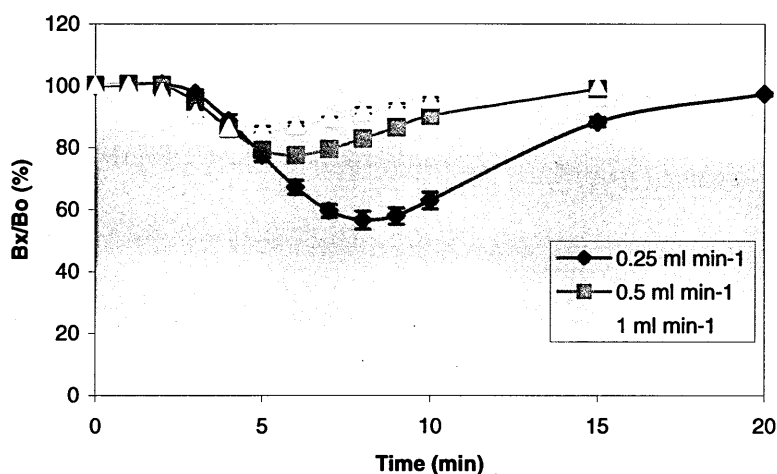


Figure 5.3. The effect of different flow rates on the sensor response to 0.1 mM benzene, using a 1 ml sample injection loop. B_x/B_o (%): the percentage decrease from the original baseline where, B_x = sample/standard baseline, and B_o = original baseline.

5.2. The effect of cell loading

As aforementioned in Chapters 3 and 4, the biosensor response is dependent on the number of viable cells immobilised on the membrane. Viable cell counts revealed an average number of 57×10^8 viable cells ml^{-1} after 18 hours of growth (stationary phase cells) under the conditions described in Chapter 3. Previous experiments in batch mode relied on the immobilisations of cultures containing different numbers of viable cells, but this was concluded to be an inaccurate means of investigating cell loading (Section 3.2). In this investigation, immobilisations of increasing volumes of an 18-hour bacterial culture were conducted. In each case, the dry weight of the corresponding bacterial

bacterial pellet of such volumes (after centrifugation) was used as a means of comparison of cell loading on the immobilisation membrane (Figure 3.4 displays the dry weight of such pellets with the corresponding volume of bacterial culture). Figure 5.4 shows the effect of increasing cell loadings on the sensor response to 0.5 mM benzene. An increase in sensor response up to 1.7 mg dry weight was obtained, however cell loadings over this amount resulted in a restriction of oxygen passage through the membranes. These findings are also in accordance with Im Lee & Karube (1996) in which a cell loading above 110 mg (wet weight) caused a decline of the sensor response. This was reported to have been due to the thickness of the immobilised membrane, resulting in a diffusion limitation of oxygen.

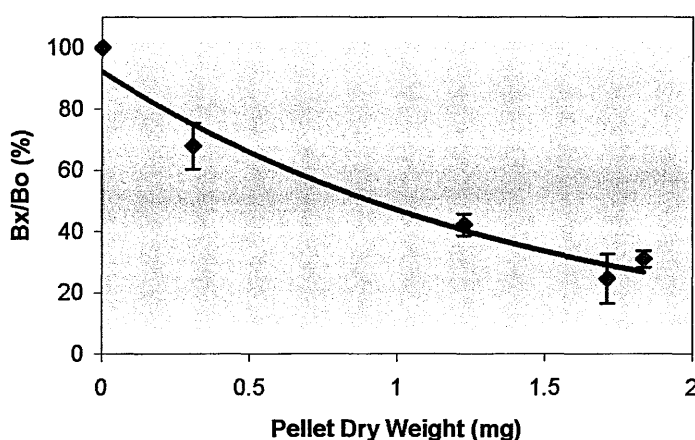


Figure 5.4. The effect of bacterial pellet dry weight on the sensor response to 0.5 mM benzene. FIA conditions: 0.5 ml min⁻¹, 1 ml injection loop. B_x/B_o (%): the percentage decrease from the original baseline where, B_x = sample/standard baseline, and B_o = original baseline. Error bars are the standard deviation from the mean, based on 5 repetitions of the assay.

Following all immobilisations, or re-fitting of stored membranes, it was necessary to wait up to one hour for the baseline of the dissolved oxygen meter to rise and stabilise before use. This is because the bacteria are still actively respiring and/or undergoing cell division during this time (resulting from their storage in the substrate solution, or from their transfer from the culture medium in the case of a new immobilisation), resulting in

a low dissolved oxygen concentration near the electrode surface. For the cell loading investigation, the time taken for the baseline to rise and stabilise was seen to increase with increasing cell loading. Figure 5.5. shows that from a cell loading of 0.9 – 1.5 mg, the baseline stabilisation time rose greatly from around 60 to 200 minutes, respectively.

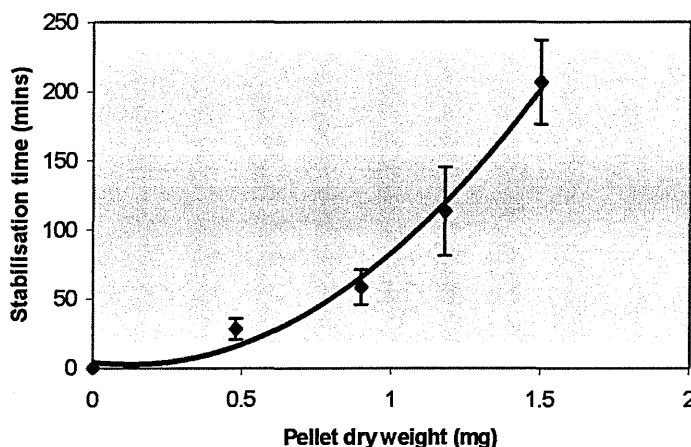


Figure 5.5. The effect of cell loading on the stabilisation time of the bacterial membrane following immobilisation. Error bars are the standard deviation from the mean, based on triplicate immobilisations.

A 2 ml culture volume (between 0.9 - 1.2 mg dry weight) was found to be the optimal cell loading for the biosensor in the present work, allowing the greatest sensitivity with minimal restriction of the oxygen passage through the membrane. This allowed the construction and use of the biosensor within in a reasonable period of time.

5.3. The Influence of Culture Growth Phase and Age of Bacterial Cells

As previously mentioned in Chapter 1, most microbial biosensors have been constructed using cells taken from the late exponential or stationary phases of growth (D'Souza, 2001). In this investigation, bacterial cells were taken from the exponential phase and from the stationary phase (by spectrophotometrical measurements of a sub-culture as

described in Section 3.1) and compared for their response to benzene. Figure 5.6 shows that there was no difference in biosensor sensitivity to 0.1 mM benzene between the two growth phases. Stationary phase cells were therefore continued to be used throughout the project, due to the easier obtainment of this growth phase (an 18-hour overnight culture) with respect to the exponential, which for the latter, involved growth of an overnight culture followed by a 6-hour sub-culture the following day (as described in Section 3.1).

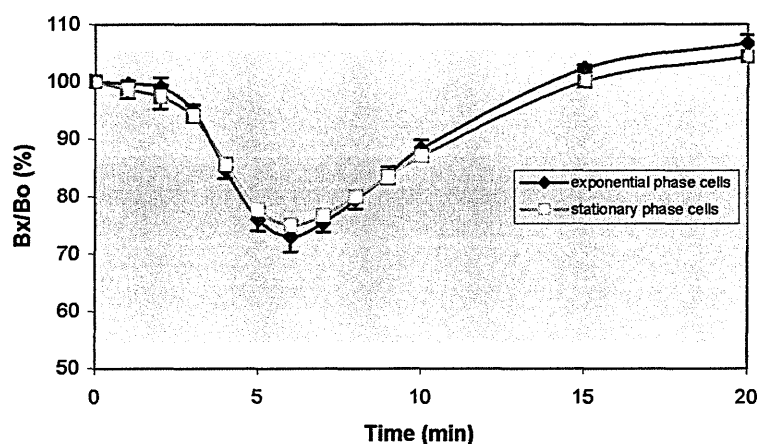


Figure 5.6. A comparison of the biosensor response to 0.1 mM benzene using cells taken from the exponential and stationary phases of growth. FIA conditions: 0.5 ml min^{-1} , 1 ml injection loop. B_x/B_o (%): the percentage decrease from the original baseline where, B_x = sample/standard baseline, and B_o = original baseline. Error bars are the standard deviation from the mean, based on triplicate measurements.

Over the course of the project, *P. putida* ML2 cells were maintained on agar plates by the monthly transfer of cells to fresh agar, and stored at 4°C . Stock solutions of the original cell culture grown at the start of the project were also stored at -80°C in 8% glycerol, as described in Chapter 2. Throughout the project, bacterial cultures for the biosensor were grown using cells taken from the agar plates. Since it is possible that natural mutations can occur over time with the continual growth of bacteria (Prescott *et*

al., 1993), the biosensor response using cells taken from original culture stored at -80°C were compared with the cells that were maintained monthly over the last 2 years.

Figure 5.7 shows that there was no difference in the biosensor sensitivity to benzene between the original stock solution cells, and the monthly-maintained agar plate cells. This suggests that the *P. putida* ML2 cells have remained a pure and stable culture throughout the 2 years of repeated growth, without showing any changes in their ability to degrade benzene. However, upon studying the culture under the light microscope, it was noted that the cells had a more circular shape with respect to the rod-shaped structure typical of pseudomonads (this image has not been presented due to the poor resolution of the photograph). One explanation for this could be that over the course of 30 years, in which the bacteria have been grown as a laboratory-based culture, the cells have naturally mutated to a more circular shape. This could be due to the fact that in their natural environment the rod shape of the cells, coupled with their flagella, provides the cells with motility which is essential for their survival in a competitive environment.

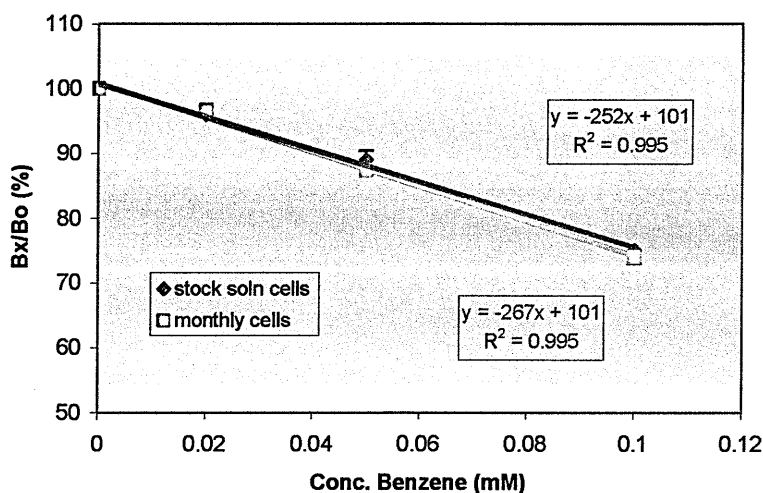


Figure 5.7. A comparison of calibration curves for benzene using cells taken from an original stock solution, and cells maintained monthly on nutrient agar. FIA conditions: 0.5 ml min^{-1} , 1 ml injection loop. B_x/B_o (%): the percentage decrease from the original baseline where, B_x = sample/standard baseline, and B_o = original baseline. Error bars are the standard deviation from the mean, based on triplicate measurements.

In the laboratory environment however, isolated cultures are typically grown in specific media where all the essential growth nutrients for that strain are provided, hence there is no competition between strains of bacteria. However, a more detailed study of wild-type and laboratory based strains using a scanning electron microscope would be necessary to confirm such a theory.

5.4. The Effect of pH and Temperature

As aforementioned for a microbial biosensor based on respiratory activity, the control of temperature is important not only for the optimal functioning of the bacteria, but also for the consistency of the dissolved oxygen concentration. Previous experiments on the effect of temperature in batch mode, showed an increase in sensitivity from 18 – 35°C. For the FIA system, the temperature of the water bath was increased to the desired temperature, and the bottle containing the carrier buffer was immersed prior to the experimentation. Additionally, the flow cell containing the dissolved oxygen probe and immobilised bacteria was also connected to the water bath so that a continual flow of heated water surrounded the oxygen probe. In contrast to the batch mode however, the biosensor showed no significant differences in response over a temperature range of 21.5 – 35°C (results not shown). The difference found in this case could be attributed to the cooling of the buffer through the FIA system before reaching the sensor, despite the heated flow cell. In batch mode, the bacteria are in direct contact with a measurable constant temperature, thus the biosensor is perhaps more sensitive to changes in temperature in this mode.

For the pH investigation, 100 ml volumes of 50 mM potassium phosphate buffer were adjusted to the desired pH and connected to the flow cell as the FIA carrier stream. Conversely to the batch mode, the biosensor in the kinetic mode showed a variation in response within a pH range from 5 to 9. Figure 5.8 shows the highest sensor response to 0.5 mM benzene at pH 6 (a 65% decrease from the original baseline), with a slight decrease in response at pH 7 and 5, and the lowest responses at pH 8 and 9 (a 35 and 30% decrease from the original baseline, respectively). A pH of 7 was tested before and

after this part of the investigation, yielding the same response, therefore the testing of higher and lower pH values did not cause any permanent damage to the cells. It is unclear why the biosensor failed to show any variation in response during pH testing in batch mode, but perhaps because the benzene is left for a longer time within the reaction vessel after injection, the bacteria are able to assimilate it.

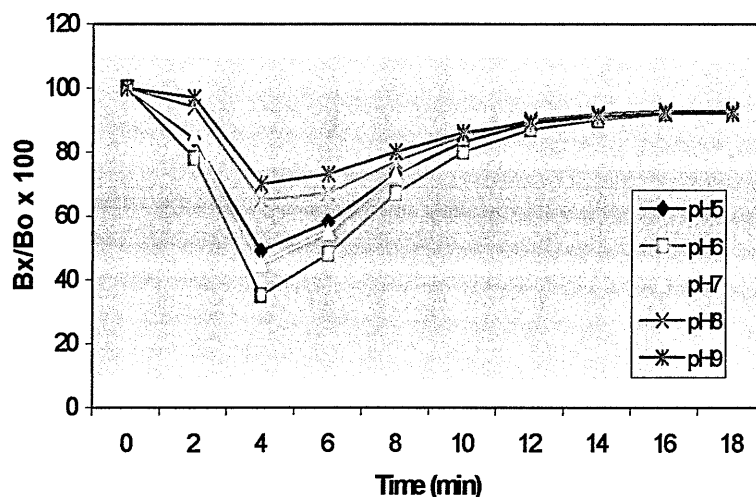


Figure 5.8. The effect of pH on the sensor response in the kinetic mode to 0.5 mM benzene. FIA conditions: flow rate 0.5ml/min, 1 ml loop. B_x/B_o (%): the percentage decrease from the original baseline where, B_x = sample/standard baseline, and B_o = original baseline.

5.5. Reproducibility and Lifetime of the Bacterial Membrane

Investigations conducted in batch mode revealed that the biosensor demonstrated good intra- and inter-assay reproducibility, in the latter case based on the storage of the membrane at 4°C in a 1 mM benzene-buffer solution when not in use (Section 4.4). For the FIA system, Table 5.1 shows a stable sensor response to 0.1 mM benzene over 6 measurements taken at random times throughout a day of continual use. This stability has been further demonstrated in Chapter 6 for air sample analysis. As discussed in Chapter 4, due to the time taken and costs incurred for the repeated growth of bacterial

cultures, it is more beneficial to use one bacterial membrane for as long as the sensitivity of the sensor response persists. This requires the use of an effective storage method when the biosensor is not in use. In batch mode, the biosensor displayed a stable response over a 22-day period, without a decrease in sensitivity. When the biosensor was not in use, the cap of the oxygen electrode (containing the bacterial membranes) was stored at 4°C in a solution of 50 mM potassium phosphate buffer containing 1 mM benzene. This storage method was also utilised for the investigation of the bacterial membrane lifetime with the FIA system. For this investigation, one bacterial membrane was used continually over a 28-day period, and the response to 0.5 mM benzene plotted at random intervals as shown on Figure 5.9.

Table 5.1

Intra-assay reproducibility of the bacterial biosensor response to 0.1 mM benzene in the kinetic mode based on 6 measurements over one day. Measurements 1-3 were conducted at the beginning of the day, and measurements 4-6 were conducted at the end of the day, during a day of calibration curves.

Measurement	Sensor Response to 0.1 mM benzene. Decrease from baseline (%)
1	15
2	14
3	14
4	16
5	14
6	14

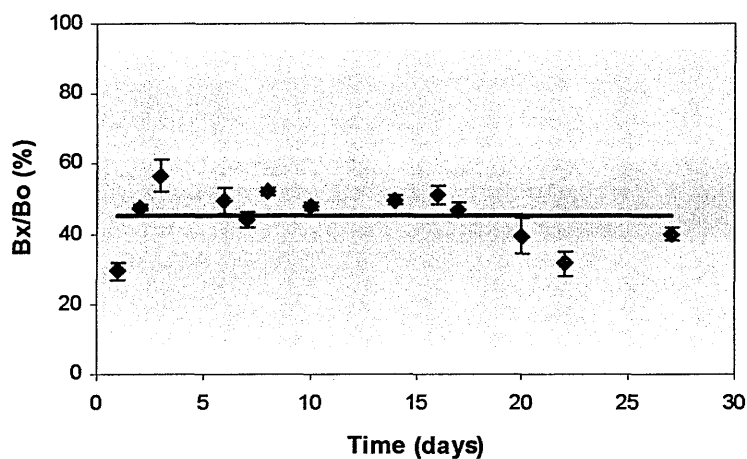


Figure 5.9. Biosensor response over time to 0.5 mM benzene, based on the same bacterial membrane (stored at 4°C in potassium phosphate buffer with 1 mM benzene when not in use). B_x/B_o (%): the percentage decrease from the original baseline where, B_x = sample/standard baseline, and B_o = original baseline. Membrane immobilised with 1.3 mg bacterial dry weight. (mean response: 45.1, SD 8.0, CV 18%).

No decrease in sensitivity was found over this assay period. Another bacterial membrane was analysed over a 47-day period based on non-continuous use, that is the membrane was stored at 4°C in a 1 mM benzene-buffer solution, and analysed once every week for its response to 0.5 mM benzene. The results from this investigation are shown in Figure 5.10 and it can be seen that no decrease in sensitivity occurred over the assay period.

The results from both the continuous and non-continuous use assays highlight the stability of the bacterial membrane under the storage conditions used, and thus eliminate the need for the continual growth of fresh bacterial cultures. This stability also proved to be beneficial for *in situ* analysis, which is discussed in Chapter 6.

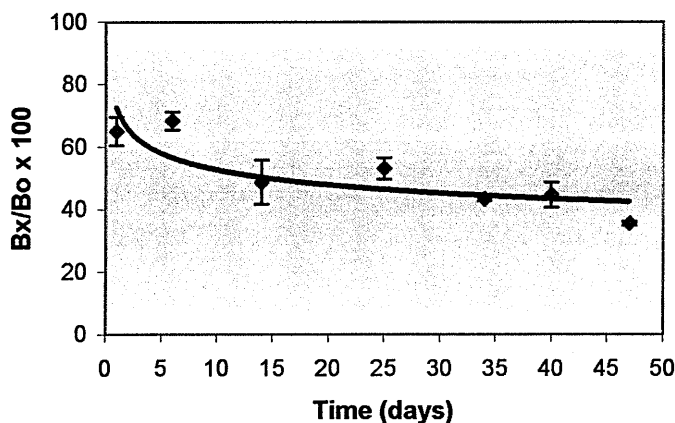


Figure 5.10. Sensor response to 0.5 mM benzene based on non-continuous use over 47 days. Used only on days shown, and stored at 4°C in a 1 mM benzene-buffer solution. Membrane immobilised with 1 mg bacterial dry weight. (mean response: 51.2, SD 11.9, CV 23%). B_x/B_o (%): the percentage decrease from the original baseline where, B_x = sample/standard baseline, and B_o = original baseline.

5.6. Sensitivity and Linear Range

With the biosensor optimised in the FIA system, typically a linear range between 0.05 and 0.2 mM benzene was obtained. A lower detection limit of 0.01 mM benzene was also obtainable for bacterial membranes showing high sensitivity, but this was generally excluded from calibration curves, instead using the more profound responses for the linear trend line equations, as explained in Chapter 6 for the analysis of air samples. When the sensitivity of the sensor response with the FIA system is compared with that in the batch mode, it can be seen that a higher sensitivity is obtained in the latter case (Figure 5.11). The lesser sensitivity obtained in the FIA system with respect to the batch could be attributed to the dilution and dispersion of benzene in the FIA carrier stream, and the flow rate of the benzene through the flow cell, thus limiting its diffusion through the bacterial membrane.

Concentrations of benzene creating sensor responses greater than a 60% decrease from the baseline were generally avoided due to the irreproducibility of responses exceeding this amount, and also due to the longer baseline recovery required. It has been well reported in the literature that higher concentrations are coupled with longer baseline recovery times, in both batch and kinetic mode (Mulchandani and Rogers, 1998).

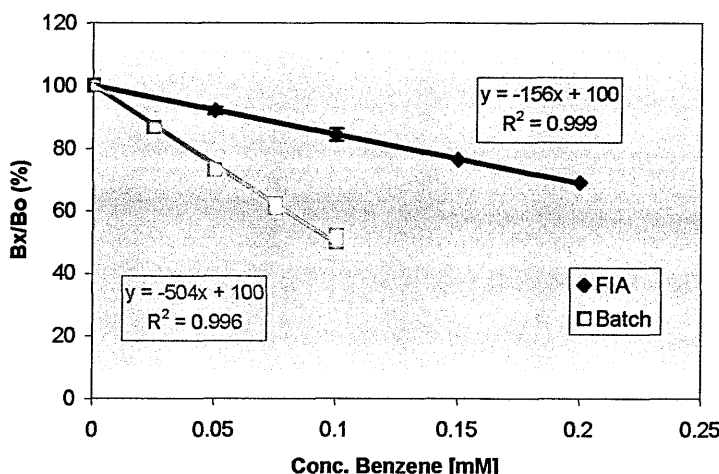


Figure 5.11. Comparison of calibration curves for benzene in batch and kinetic mode. FIA conditions: flow rate 0.5 ml min^{-1} , 1 ml loop, bacterial cell loading of 1.2 mg dry weight. B_x/B_o (%): the percentage decrease from the original baseline where, B_x = sample/standard baseline, and B_o = original baseline. The error bars are the standard deviation from the mean, based on 3 repetitions of the assay.

5.7. Interfering Compounds

As previously mentioned, bacterial biosensors can have the disadvantage of non-specificity to a particular target analyte with respect to enzyme biosensors (Mulchandani and Rogers, 1998), and in particular for samples containing large amounts of organic compounds. In batch mode, the biosensor response to toluene, catechol, and glucose was investigated (Section 4.5), however for the analysis of workplace air, a range of volatile organic compounds (VOCs) will usually be present depending on the type of

processes and activities undertaken there. Therefore, the sensor response to other benzene-related hydrocarbons was investigated with the FIA system.

Figure 5.12 shows the sensor response to 0.5 mM concentrations of benzene, toluene, p-xylene, and n-hexane with the FIA system, with negligible sensor responses to the latter three. These findings are in agreement with those obtained by Tan *et al.* (1994), where a negligible response was found for the same compounds using *P. putida* ML2, and also for ethylbenzene and MTB ether. The effect of interfering compounds has also been included in the following chapter, which describes the optimisation of the biosensor for air sample analysis. The selectivity of the *P. putida* ML2 cells for benzene, can be attributed to the specificity of the benzene deoxygenase enzymatic pathway for the cleaving of the benzene ring (Mason, 1994). This is in contrast to *P. putida* F1, which contains the genes encoding the toluene deoxygenase enzymatic pathway, enabling the bacteria to utilise toluene, benzene and ethylbenzene as sources of carbon (Woo *et al.*, 2000), thus limiting its use as a recognition element for a substrate specific biosensor.

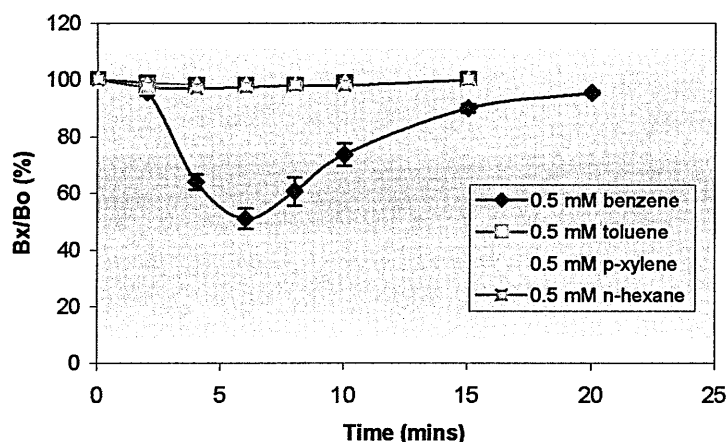


Figure 5.12. Biosensor response to benzene-related hydrocarbons. FIA flow rate 0.5 ml min⁻¹, 1 ml injection loop. B_x/B_o (%): the percentage decrease from the original baseline where, B_x = sample/standard baseline, and B_o = original baseline. Error bars are the standard deviation from the mean, based on triplicate measurements.

5.8. The Development of an Automated system coupled with Autolab® Measurements

The Clark dissolved oxygen electrode is an amperometric device, that is dissolved oxygen is reduced at a fixed potential, hence changes in the dissolved oxygen concentration near the electrode surface are represented by changes in the sensor output current over time. However, as described previously, the units of measurement on the oxygen meter used for this project are arbitrary values. Therefore, investigations into the response of the oxygen probe in units of current were conducted by connection of the oxygen electrode to a potentiostat (Autolab®). Figure 5.13 shows the graph obtained from the amperometric software displaying the biosensor response to 0.5 mM benzene. It can be seen that a decrease in current of around 5 nA from the original baseline current was obtained in response to 0.5 mM benzene, hence allowing calibration of the arbitrary oxygen meter units into current readings (Figure 5.14).

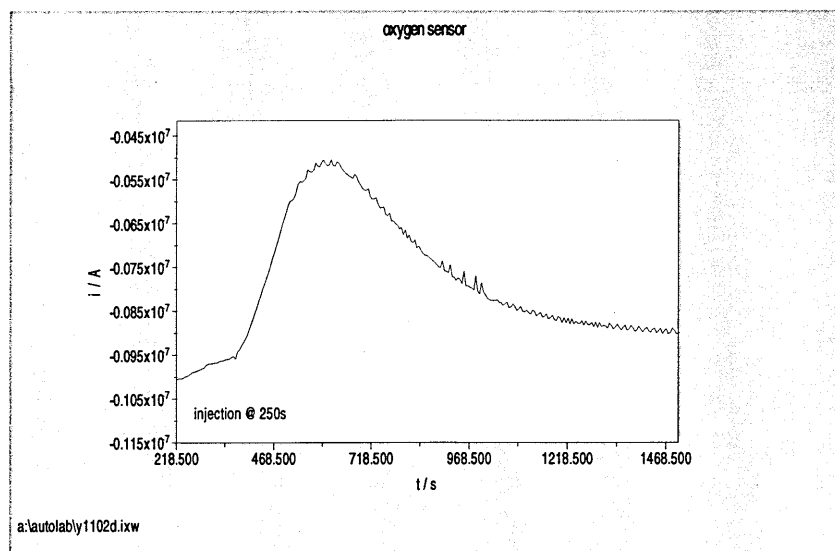


Figure 5.13. The biosensor response to 0.5 mM benzene with the oxygen electrode connected to a potentiostat (Autolab®).

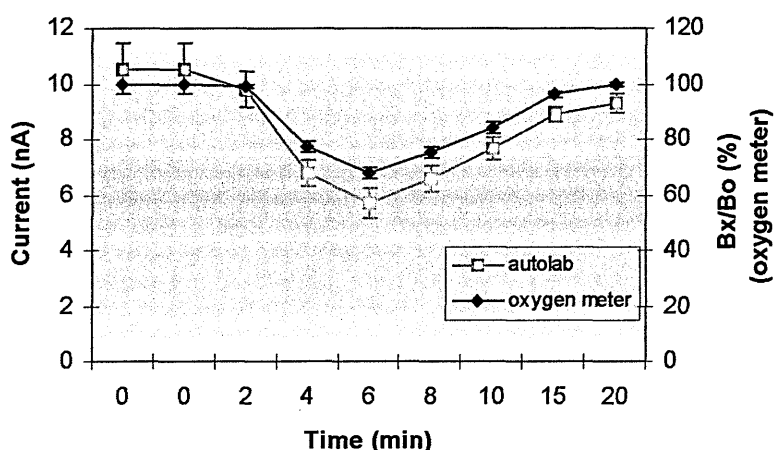


Figure 5.14. A comparison of the results obtained from the biosensor response to 0.5 mM benzene using the oxygen meter, Bx/Bo (%), and the Autolab, current. Error bars are the standard deviation from the mean, based on triplicate measurements.

The calibration curve conducted for benzene using the Autolab®, revealed a reasonable linearity of response between 0.1 – 0.5 mM benzene (Figure 5.15). However, due to the low current range of the oxygen electrode, a large amount of background noise (external power-line noise, or the use of other electrical equipment in the laboratory) was often apparent when connected with the Autolab®. The oxygen electrode could also be attached to the PalmSens® portable potentiostat (pictured in Figure 2.2, Chapter 2) to allow *in situ* measurements, but this device was also subject to a large amount of background noise, particularly when re-charging the battery. The PalmSens® is described in more detail in Chapter 7, for investigations with bacterially-modified screen-printed electrodes.

Investigations of the biosensor connected with an automated FIA system were also conducted (*Ismatec* Automated Sample Injection Analyser). This system was fully programmable and consisted of 2 pumps, each with 4 channels. One of the pumps was used to continually pump buffer through the flow cell (the carrier stream), while the other was used to introduce standards or samples to the carrier stream through automatic injection. Such a system could theoretically be programmed to run a series of analyses automatically, without requiring the presence of personnel.

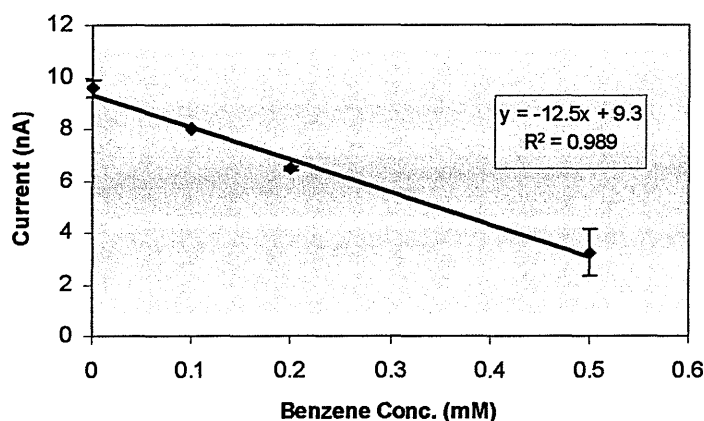


Figure 5.15. Calibration curve for benzene using the Autolab[®]. FIA conditions: 0.5 ml min⁻¹, 1 ml injection loop. Error bars are the standard deviation from the mean, based on triplicate measurements.

However, for a bacterial biosensor for benzene analysis, such a system was seen to have some limitations. Firstly, the calibration of the biosensor requires pre-dilutions of a benzene stock solution in potassium phosphate buffer, and needs to be conducted periodically throughout the day to account for any unpredictable changes in sensitivity (as described in Chapter 6 for air sample analysis). Moreover, standard solutions need be freshly prepared prior to injection due to the highly volatile nature of benzene, which favours the head space of the vial more than the aqueous buffer phase (Johansen and Wendleboe, 1981), hence pre-prepared standard solutions cannot be left for long periods of time. Secondly, the characteristics of a bacterial biosensor are such that any changes in the baseline recovery time following unknown sample injections of high concentration cannot be predicted, hence a pre-programmed injection time would introduce the next sample to the sensor before recovery of the baseline from the last sample. Thirdly, the automated FIA system was laboratory-based, hence could not be transported to *in situ* locations.

5.9. Discussions

Chapter 5 has described the optimisation of the biosensor within a Flow Injection Analysis system. The two main influencing factors on the sensor response of such a kinetic system, aside from the parameters associated with the bio-part of the sensor outlined in Chapter 4, have been the sample injection volume and the flow rate of the carrier stream. An increased response was found with an increase in the volume of the injected sample, but this was also coupled with an increase in response time. Similarly, a decrease in the carrier stream flow rate caused an increase in the sensor sensitivity, but this was also coupled with an increase in response time. This can be attributed to amount of time that benzene is present in the flow cell, in contact with the bacterial membrane. Therefore, at higher flow rates and with smaller injection volumes, there is less benzene in contact with the bacterial membrane over time, hence less time for diffusion through the membrane. The injected sample is also subject to dilution and dispersion in the carrier stream, again influenced by the injection volume and carrier stream flow rate, and also the distance between the sample injection valve and the flow cell. Similar results were also reported by Han *et al.* (2002), in which a compromise was made between sensitivity and baseline recovery time. A total analysis time of 20 minutes was achieved with a flow rate of 0.09 ml min^{-1} , and an injection volume of 1 ml, giving the best sensor response to 0.5 mg l^{-1} trichloroethylene (Han *et al.*, 2002).

For cell loading investigations, Chapter 5 has described the results obtained from dry weight measurements after culture centrifugation, in contrast to Chapter 4 based on less accurate viable cell counts. In the present work, an increase in sensor response to benzene up to 1.7 mg dry weight was obtained, however cell loadings over this amount resulted in a restriction of oxygen passage through the membranes. These findings are also in accordance with Im Lee & Karube (1996) in which a cell loading above 110 mg (wet weight) caused a decline of the *Pseudomonas fluorescens* response to gaseous cyanide. This was reported to have been due to the thickness of the immobilised membrane, resulting in a diffusion limitation of oxygen. Mulchandi *et al.* (2002), also reported a similar trend and concluded that an initial increase of the catalytic activity

was due to increasing cell amount, however this was also coupled with increased mass transfer resistance due to the increased thickness of the biomass layer.

As part of the cell loading investigations, cells taken from the exponential and stationary growth phases of the liquid culture were investigated revealing no differences in response characteristics between the two. Similarly, there were no differences between original stock solution cells stored at -80°C in 8% glycerol, and cells maintained monthly on nutrient agar at 4°C .

Investigations of temperature and pH effects were also conducted with the FIA system. In contrast to the batch mode however, the biosensor showed no significant differences in response over a temperature range of $21.5 - 35^{\circ}\text{C}$. The difference found in this case could be attributed to the cooling of the buffer through the FIA system before reaching the sensor, despite the heated flow cell. For a pH range between 5 and 9, higher sensitivity was found at pH 6, with the least sensitivity at values of 8 and 9. This is in contrast to the batch mode, where the sensor response was found to be independent of the pH. Nevertheless, a buffer pH of 7 was continued to be used for all further experimentation due to the relatively high sensitivity of the sensor response and for ease of buffer preparation. Further literature on these parameters for bacterial biosensors has been discussed in Chapter 4.

The lifetime of the sensor operating in kinetic mode was found to be similar to that of the batch mode, based on the same storage method when the sensor was not in use (4°C , in a 1 mM benzene-buffer solution). In the present work, investigations of continuous and non-continuous use revealed a maintained sensitivity for 28 and 47 days respectively, with the assay being terminated due to time limitations, rather than failure of response after this period. Further literature on these parameters for bacterial biosensors has been discussed in Chapter 4.

The influence of benzene-related compounds on the selectivity of the sensor to benzene revealed negligible sensor responses to toluene, p-xylene, and n-hexane with the FIA system. These findings are in agreement with those obtained by Tan *et al.* (1994), where

a negligible response was found for the same compounds using *P. putida* ML2, and also for ethylbenzene and MTB ether. The selectivity of the *P. putida* ML2 cells for benzene, can be attributed to the specificity of the benzene deoxygenase enzymatic pathway for the cleaving of the benzene ring (Mason, 1994).

Connection of the oxygen electrode to a potentiostat allowed calibration of the arbitrary units of the oxygen meter with amperometric current readings. Further integration of the biosensor into an automated FIA system was found to be unsuitable in the present work, due to the instability of benzene in the aqueous phase, and the response characteristics of the bacteria, which can change depending on the concentration of benzene injected (as previously discussed in Chapter 4). This prevents the development of a fully automated system, which in turn, would render the biosensor suitable for laboratory-based analyses only.

With the biosensor optimised in the portable FIA system, a linear detection range between 0.05 and 0.2 mM benzene was obtained. A lower detection limit of 0.01 mM benzene was also obtainable for bacterial membranes showing high sensitivity, but this was generally excluded from calibration curves, instead using the more profound responses for the linear trend line equations. The response time in the kinetic mode was between 5 and 6 minutes, with a baseline recovery between 10 – 15 minutes, depending on the concentration of benzene. It has been well reported in the literature that higher concentrations are coupled with longer baseline recovery times, in both batch and kinetic mode (Mulchandani and Rogers, 1998), related to the respiratory activity of the bacterial cells. Batch mode investigations revealed a similar linear range, with an enhanced sensitivity, but twice the response and baseline recovery time. The difference in sensitivity between the batch and FIA systems can be attributed to the flow characteristics in the latter case, resulting in less contact time of the benzene with the bacterial membrane.

Chapter 5 has presented the results obtained from the biosensor in the kinetic mode, using a custom-made flow cell containing the dissolved oxygen probe and immobilised bacteria. Flow Injection Analysis has allowed the development of a more automated

system with respect to the batch experiments detailed in Chapter 4, and in addition providing faster response and baseline recovery times. In the next chapter, the FIA system has been used for the development and optimisation of the biosensor for air sample analysis.

CHAPTER 6

RESULTS

BIOSENSOR APPLICATION TO AIR SAMPLE ANALYSIS

CHAPTER 6: BIOSENSOR APPLICABILITY TO AIR SAMPLE ANALYSIS

Chapter 5 described the optimisation of the biosensor in the kinetic mode using a Flow Injection Analysis (FIA) system. Such a system allowed more automated and precise analyses with a faster response and baseline recovery time, with respect to the batch system, described in Chapter 4. The continual flow of fresh buffer further allowed a more straightforward instrumental operation. Chapter 6 focuses on the final part of this project, which is the development of the biosensor for the analysis of benzene in workplace air. This will include air collection and benzene extraction methods which are compatible with the bacterial biosensor, and the applicability of the compatible methods for the analysis of benzene in workplace air. All biosensor analyses in this chapter have been based on FIA conditions incorporating a 0.5 ml min^{-1} flow rate of 50 mM potassium phosphate buffer (pH 7), and a 1 ml injection loop.

6.1. Examining Air Sampling Methods

The preliminary air sampling method investigated in this project was undertaken in a benzene exposure room of controlled concentration at the FSM Scientific Institute of Pavia, Italy (as described in Section 2.7.2). This method incorporated the use of an air pump and glass air bubbling device to draw benzene contaminated air at 15 ppm and effervesce it through a collection buffer of potassium phosphate (50 mM, pH 7). However GC analyses revealed that this method resulted in a benzene concentration in the collection buffer, which was lower than the 0.01 mM detection limit of the biosensor (Table 6.1). This can be attributed to the high volatility of benzene and its poor solubility in water without the presence of other solvents, meaning that the benzene passes through the collection buffer to the air on the other side. This air sampling method highlighted that for volatile compounds such as benzene, a type of sorbent is required to collect and pre-concentrate the airborne vapours. As discussed in Chapter 1, the use of sorbents for air sampling, also requires a method of desorption of the compound of interest, in order to analyse the sample. This is done most commonly

section therefore presents the findings from a range of solvents tested and their effects on the bacterial biosensor.

Table 6.1.
The concentration of benzene obtained in solution following air sampling using a gas bubbling device at a fixed exposure room benzene concentration.

Air sample volume (L)	Exposure room benzene conc. (ppm)	Benzene conc. (mM) obtained in solution. GC results (analytical error $\pm$ 5%)
15	14.6	0.0035 ($\pm$ 0.00018)
30	14.6	0.0037 ($\pm$ 0.00019)
45	14.8	0.0086 ($\pm$ 0.00043)
150	16.8	0.0083 ($\pm$ 0.00042)

6.2. The Compatibility of Desorption Solvents with the Biosensor

Following the adsorption of benzene vapour on a charcoal air sampling tube, it is important to find a suitable method of desorption from the solid sorbent that is compatible with analysis by the biosensor. The biosensor operates in the liquid phase, hence the first point of consideration is the effective desorption of benzene using a solvent that is soluble in the water-based sample carrier stream (potassium phosphate buffer) of the FIA system. It is also necessary to dilute the sample eluent (containing the extracted benzene) in this buffer prior to injection into the FIA system, in order to create a benzene concentration within the detection range of the biosensor. Moreover, it is necessary to dilute the desorption solvent to a concentration which does not interfere with either the biosensor response, or viability of the bacterial cells. A range of desorption solvents were tested with the biosensor in order to find the most compatible combination. Table 6.3 summarises the solvents tested and their associated advantages and disadvantages.

Initial investigations with carbon disulphide (CS₂) proved that this solvent was not compatible for use with the biosensor. Firstly, CS₂ has only a 0.3% solubility in water, thus rendering sample or standard dilutions in a water-based buffer unfeasible.

Furthermore, a 0.2% CS₂ concentration was found not to be completely miscible in the phosphate buffer even after 15 minutes, and was seen to affect the bacterial cells in such a way that no biosensor response to benzene was obtained after exposure to the partially diluted solvent. CS₂ was also seen to cause a diminution of the sensor response even without the presence of the bacterial cells, hence this would create difficulties in distinguishing sensor response readings to benzene and those to CS₂. This was also found to be the case with dimethylsulfoxide and acetonitrile, which are commonly used solvents for solid-phase extraction (Thurman and Mills, 1998). These two solvents are both soluble in water, hence allow sample dilutions and are easily miscible with the FIA carrier stream following injection. Figure 6.1 shows the biosensor response to acetonitrile at a 10% concentration, and it can be seen that the response to this solvent alone, is 30% greater than that of benzene. The sensitivity to both 10% acetonitrile and benzene together was the same as that to benzene alone with a faster response time in the presence of the solvent (6 minutes and 4 minutes, respectively).

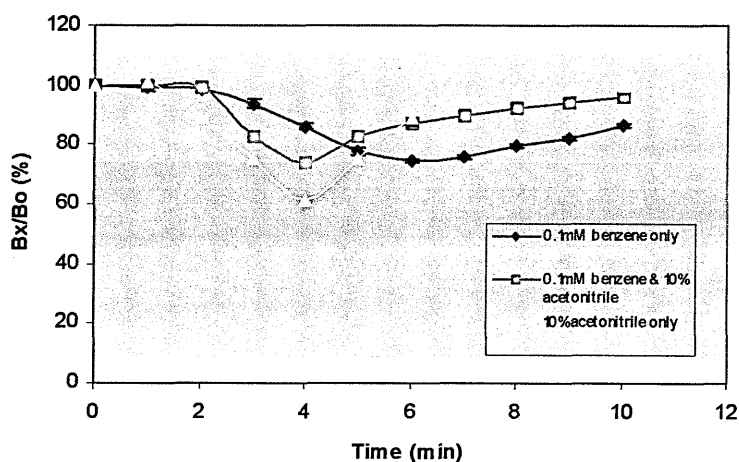


Figure 6.1. The biosensor response to 10% acetonitrile, with and without benzene. B_x/B_o (%), where B_x = sample/standard maximum sensor output signal, and B_o = original baseline.

These results demonstrate that acetonitrile could not be used as a desorption solvent due to its large interference in the sensor response to benzene. A similar response was also obtained with dimethylsulfoxide (DMSO), however as well as the interference in sensor response, both DMSO and acetonitrile are usually mixed with CS_2 for effective recovery of benzene from charcoal (Harper, 2000), thus emphasising further the unsuitability of these two desorption solvents.

Methanol was also tested as an alternative desorption solvent, which is soluble in water, but required a silica solid phase due to methanol's reportedly poor desorption of non-polar compounds, such as benzene, from charcoal (Harper, 2000). Figure 6.2 shows that a 44% decrease in the biosensor sensitivity to benzene was found in the presence of 10% MeOH. A further decrease in the biosensor response to benzene was found with higher concentrations of methanol (results not shown).

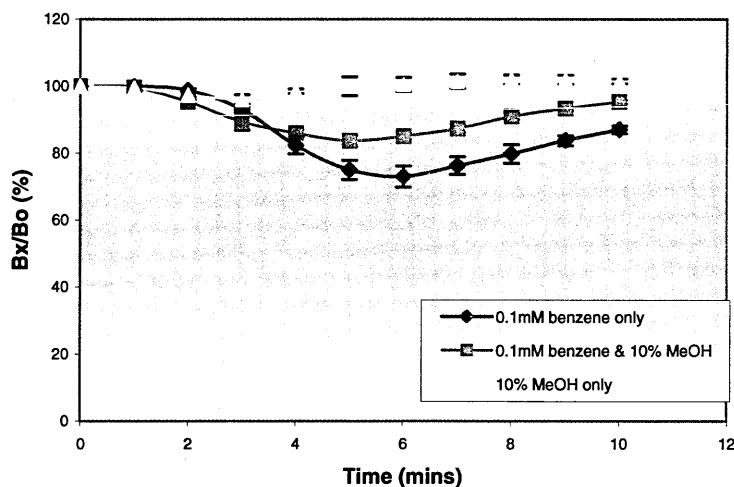


Figure 6.2. Biosensor response to methanol. B_x/B_o (%), where B_x = sample/standard maximum sensor output signal, and B_o = original baseline. Error bars are the standard deviation from the mean, based on triplicate assays.

For a further investigation of methanol, air samples were collected from the benzene exposure room at a 10 ppm concentration using silica air sampling tubes and desorbed with methanol. However, the biosensor results from these samples revealed that under

the sampling conditions used, no greater than 5% benzene was recovered from the silica (Table 6.2), thus it was concluded that silica was ineffective for the collection of benzene vapours, reportedly having a higher affinity for water vapour (Harper, 2000).

Table 6.2.
Biosensor results showing the eluent benzene concentration obtained from air sampling using silica sorbent tubes with MeOH desorption, following a 1:100 dilution in the biosensor cell.

Air sample volume (L)	Exposure room benzene conc. (ppm)	Theoretical benzene conc. in biosensor cell (mM)	Benzene conc. obtained from biosensor response (mM)	Benzene recovery from silica (%)
45	10.0	0.20	0.01	5
105	10.0	0.48	0.02	4
180	10.0	0.82	0.02	2

Theoretical conc. based on 100% benzene recovery, under the sampling conditions used, and a 1:100 eluent dilution in the biosensor cell.

Final investigations were conducted with *N,N*-Dimethylformamide (DMF), which has previously been tested in the literature as an alternative desorption solvent for the extraction of benzene from charcoal (Johansen and Wendelboe, 1981). It has not been extensively used alone as a desorption solvent in the literature, but its desorption efficiency has been compared to that of CS₂, and it has been reported to be a good solvent for polar compounds, and a reasonable solvent for non-polar compounds (usually 80% recovery or more) (Harper 2000). In the present work, DMF was found to be the most compatible solvent for use with the biosensor, being completely soluble in water, having negligible interference with the sensor response up to a 2% concentration, and having no toxic effects on the bacterial cells (Section 6.3). Furthermore, DMF was found to have no effect on the sensor response without the presence of the bacterial cells, indicating that DMF itself did not cause any chemical change in the dissolved oxygen concentration. It also allowed the use of a charcoal sorbent which is the most effective for benzene collection (Harper, 2000). Table 6.3 summarises the desorption solvents tested in this section, and their associated advantages and disadvantages. The next section presents the results from the exposure effects of DMF on the bacterial biosensor.

Table 6.3.

Summary of desorption solvents tested with the biosensor and associated advantages and disadvantages.

Solvent	Properties ^[1]	Advantages	Disadvantages
Carbon Disulphide	CS ₂ 0.294% solubility in water	Best solvent for benzene extraction from a charcoal sorbent ^[2]	Found to be toxic to bacterial cells Found to be insoluble in water-based buffer at 0.2% conc., hence sample dilution unfeasible
Dimethyl-sulfoxide (DMSO)	(CH ₃) ₂ SO Soluble in water	Alternative solvent for solid-phase extraction ^[3] Allows water-based sample dilution Not found to be toxic to bacterial cells over conc. range tested (0 – 20%)	Biosensor response to DMSO alone similar to that of benzene
Acetonitrile	CH ₃ CN Miscible with water	Alternative solvent for solid-phase extraction ^[3] Allows water-based sample dilution Not found to be toxic to bacterial cells over conc. range tested (0 – 20%)	Biosensor response to acetonitrile alone similar to that of benzene
Methanol (MeOH)	CH ₃ OH Miscible with water	Alternative desorption solvent from a silica sorbent ^[2] Allows water-based sample dilution Not found to be toxic to bacterial cells over conc. range tested (0 – 20%)	Decrease in biosensor response to benzene with increasing MeOH conc. Poor solvent for desorption of benzene from charcoal. Required use of a silica sorbent, which has a higher affinity for water than benzene ^[2]
N,N-Dimethyl-Formamide (DMF)	HCON(CH ₃) ₂ Miscible with water	Alternative desorption solvent from a charcoal sorbent ^[4] Allows water-based sample dilution Not found to be toxic to bacterial cells over conc. range tested (0 – 20%) Negligible effect on biosensor response up to 2% conc.	Reported to be less effective for the desorption of benzene from charcoal (80% recovery with respect to 100% for CS ₂) ^[4]

References

^[1] Budavari *et al.* (1996), ^[2] Harper (2000), ^[3] Thurman and Mills (1998), ^[4] Johansen and Wendelboe (1981).

6.3. The Influence of DMF on the Biosensor Response

Prior to testing the efficiency of DMF for the desorption of benzene from charcoal, the effect of differing concentrations of this solvent on the biosensor response was investigated. Figure 6.3 shows the effect of increasing DMF concentration on the sensor response to 0.1 mM benzene, and also includes the sensor response to DMF alone. It can be seen that 5 and 10% concentrations of DMF alone caused a decrease from the sensor baseline of 14 and 32% respectively, compared to a 1 and 2% DMF concentration, which caused a maximum decrease of 6% from the baseline. A maximum of 2% DMF was therefore selected as the highest concentration that could be used with the biosensor. This allowed a maximum of 100 μ l of sample eluent (extracted benzene in pure DMF) to be diluted in 5 ml of phosphate buffer (which was the volume used to flush and entirely fill the 1 ml sample injection loop of the FIA system). Thus, during air sampling, it is important to collect a sufficient amount of benzene on the charcoal, to allow an eluent dilution of the above proportions (this is further influenced by the volume of DMF used during desorption, which is discussed in the following section). A larger analyte collection quantity also necessitates the use of larger air sampling tubes, hence a total of 600 mg of charcoal (400/200 mg for the primary and back-up sections, respectively) was used in the present work.

Figure 6.4 shows the influence of a 1% concentration of DMF on the biosensor response to 0.1 mM benzene, in which twice the sensitivity was obtained with respect to benzene alone. This could be due to the increased solubility of benzene in the buffer in the presence to DMF. The lower vapour pressure of DMF with respect to benzene may also prevent some benzene evaporation into the headspace of the vial during standard solution preparations. Johansen and Wendelboe (1981) also reported a greater sample stability during storage over time in DMF, with respect to CS_2 . The increase in sensitivity of the biosensor response in the presence of DMF, required all calibration curves to be conducted from a benzene stock solution (10 mM) in DMF, which was diluted in phosphate buffer to the desired benzene (and DMF) concentration, to correspond with the air sample eluent benzene and DMF concentrations.

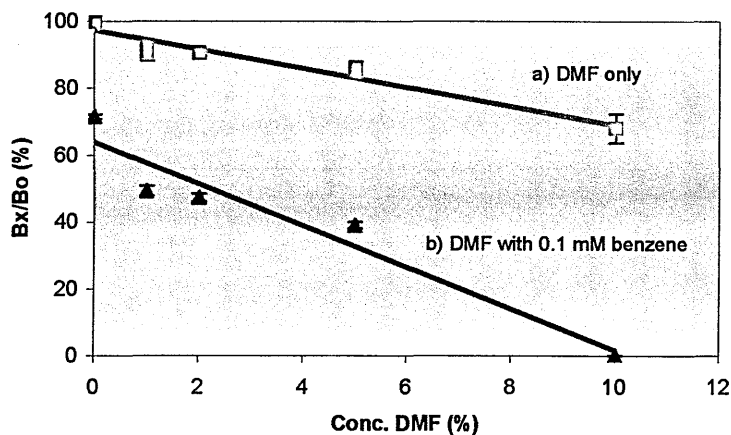


Figure 6.3. The effect of increasing DMF concentration on the sensor response: a) the effect of DMF only on the sensor response, b) the effect of increasing DMF concentration on the sensor response to 0.1 mM benzene. B_x/B_o (%), where B_x = sample/standard maximum sensor output signal, and B_o = original baseline. Error bars are the standard deviation from the mean, based on triplicate assays.

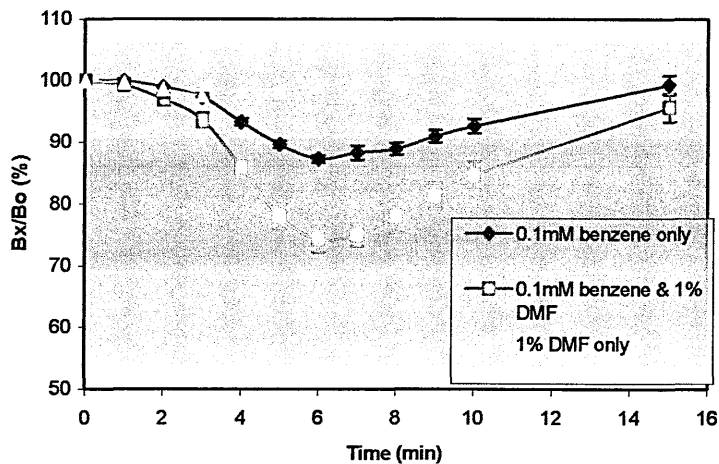


Figure 6.4. The effect of 1% DMF on the sensor response to 0.1 mM benzene. B_x/B_o (%), where B_x = sample/standard maximum sensor output signal, and B_o = original baseline. Error bars are the standard deviation from the mean, based on triplicate assays.

A detection range between 0.025 – 0.15 mM benzene with a maximum DMF concentration of 2% gave a trend line equation of $y = -313x$ (y intercept 101) with an r^2 value 0.998 and a variation coefficient of 3% ($n = 17$). A lower detection limit of 0.01 mM was obtainable, though this was generally excluded from calibration curves due to the higher benzene eluent concentrations analysed after air sampling (Section 6.6). The biosensor response time was between 5 and 6 minutes, with a baseline recovery time between 10–15 minutes, depending on the concentration of benzene, as previously described in Chapter 5.

Without the presence of the bacterial cells, no sensor response to DMF was obtained, indicating that the solvent alone does not effect the dissolved oxygen concentration. It is unclear if DMF can be degraded by the bacterial cells, but nevertheless it caused a decrease in the sensor signal with the presence of the bacteria, with a response time of between 3 and 4 minutes, with respect to 6 minutes for benzene (either alone or with DMF). The sensor response to DMF alone was more evident above 2% concentrations, but was undetectable when mixed with benzene, expressed as always in one peak between 5 and 6 minutes.

The lifetime of the same bacterial membrane over 8 days of exposure to a maximum of 2% DMF was investigated. This was based on daily calibration curves for benzene, based on dilutions of a 10 mM benzene stock solution in pure DMF. The sensor response was seen to maintain its sensitivity throughout the assay period, with a maximum variation coefficient of 3% ($n = 17$), based on previously optimised membrane storage conditions when the biosensor was not in use, as described in Chapters 4 and 5.

6.4. DMF Desorption Efficiency

As aforementioned, DMF was reported to be less efficient for the desorption of non-polar compounds such as benzene from charcoal, with respect to CS_2 (Johansen and Wendelboe, 1981). In the present work, the efficiency of DMF as a solvent for benzene

desorption from charcoal was investigated in the laboratory prior to the testing of air samples containing benzene. This involved injections of liquid benzene directly onto the primary sections of charcoal air sampling tubes, followed by the transfer of the charcoal into gas sampling vials for solvent desorption (following NIOSH air sample desorption procedures, Kennedy *et al.* 1995). For each experiment, 4.5 μ l of benzene was injected into the primary charcoal section, and DMF volumes between 2 and 10 ml were used to investigate desorption efficiency (benzene recovery). Each eluent was diluted in potassium phosphate buffer (between 1:250 - 1:50), prior to injection into the FIA system in order to obtain a theoretical 0.1 mM benzene concentration for each eluent (in the case of 100% desorption). Corresponding reference solutions containing 4.5 μ l of benzene over the same DMF volume range (without charcoal) were also made to represent 100% benzene recovery solutions (diluted in the same way prior to injection into the FIA system). The reference results were then compared with the results obtained from the charcoal eluents after desorption (calculated from the biosensor standard calibration curves).

The results from these preliminary investigations showed a benzene recovery between 70 - 80%, over a DMF volume range between 2 and 10 ml (a 1 ml volume was not sufficient to immerse all of the charcoal), with a slightly improved recovery with increasing volume (Figure 6.5) (an 80% recovery is in agreement with that obtained for benzene by Johansen and Wendelboe, 1981). However, in contrast to the slightly improved benzene recovery with increasing amount of DMF, a 10 ml desorption volume showed a slight decrease in benzene recovery. This could have been due to the larger vials used for the 10 ml volumes, which had a larger amount of headspace. Johansen and Wendelboe (1981) suggested that a distribution ratio of an analyte exists between the solvent and headspace of a vial, however if the analyte is more in favour of the headspace (such as benzene) this will result in a more negative calculation of the desorption efficiency.

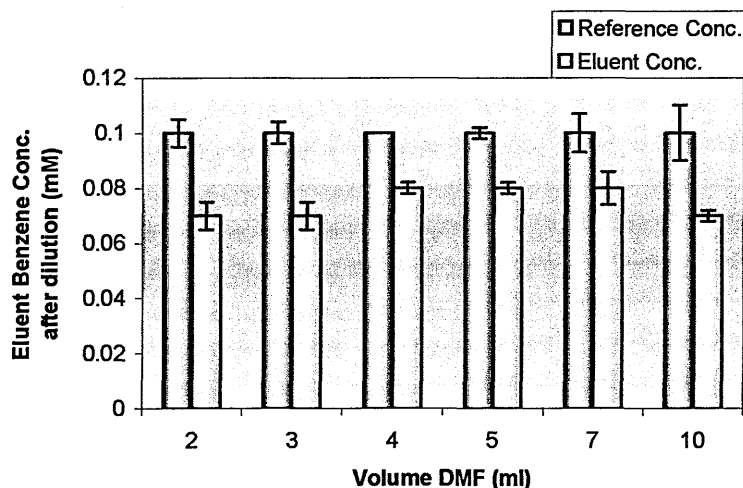


Figure 6.5. The effect of increasing volume of DMF on the recovery of benzene from the charcoal sorbent. Reference concentrations based on benzene injected directly in the corresponding volume of DMF, representing solutions of 100% benzene recovery. Eluent concentrations based on the benzene obtained after charcoal desorption over the DMF volume range tested. All solutions diluted between 1:250 – 1:50 in potassium phosphate buffer prior to injection into the FIA system. Concentrations calculated using the biosensor standard calibration curves. Error bars based on triplicate desorptions.

The results shown in Figure 6.5 were based on a total desorption time of 30 minutes, with 10 seconds of manual shaking every 5 minutes. Increased desorption time was seen not to improve benzene recovery beyond 80%, even after 20 hours as previously tested by Johansen and Wendelboe (1981). Furthermore, the effect of vigorous agitation using a magnetic stirrer bar did not improve the recovery with respect to regular manual agitation. Moreover, the presence of the back-up section with respect to only the primary section of charcoal (a total of 600 mg or 400 mg of charcoal, respectively) was not seen to influence the desorption efficiency of DMF.

6.5. Interfering Compounds

As previously mentioned in Chapters 4 and 5, typically a mixture of volatile compounds including benzene, toluene, ethylbenzene and xylene (BTEX compounds) will be present in the air of many industries or factories, depending on the type of processes undertaken there. Investigations in Chapter 5 involved the testing of some benzene related compounds diluted in buffer prior to injection in the FIA system. Since in this Chapter DMF is being used as a desorption solvent for benzene extraction, for industrial air samples other VOCs may also be extracted along with benzene. Therefore in this section the effect of all the BTEX compounds on the biosensor response was investigated at a concentration of 0.1 mM in 2% DMF. Negligible sensor responses were obtained for each individual compound (toluene, ethylbenzene and xylene, Figure 6.6) and furthermore, the presence of all compounds together was seen not to affect the sensor response to benzene, thus emphasising the biosensor selectivity for benzene as described in Chapter 5.

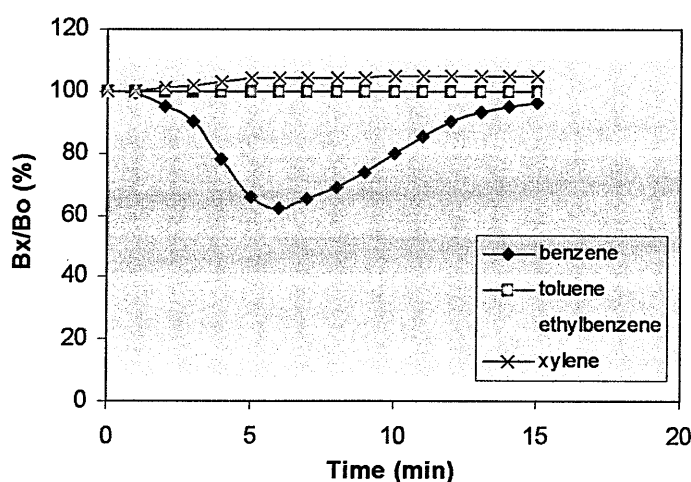


Figure 6.6. Biosensor response to BTEX compounds at a concentration of 0.1 mM in 2% DMF. FIA conditions: flow rate: 0.5 ml min⁻¹; injection loop: 1 ml. B_x/B_o (%), where B_x = sample/standard maximum sensor output signal, and B_o = original baseline.

6.6. Applicability of the biosensor for the analysis of benzene in air samples

6.6.1. Active Air Sampling

As described in Chapter 1, the analysis of particular compounds in air usually involves a pre-concentration step due to their low concentrations in air, in order to obtain a measurable quantity within the detection limits of the analytical instrumentation used (Czaplicka and Klejnowski, 2002). For benzene, the most commonly used pre-concentration step involves the pumping of air through a charcoal sorbent, referred to as active sampling (Harper, 2000). For personal exposure measurements, this is usually undertaken over an 8-hour working period, with the employee wearing the sampling apparatus. Pre-concentration can also be achieved using a passive sampling method, in which the employee wears a charcoal sorbent device into which benzene diffuses naturally over the 8-hour working period. The latter method is further described in Section 6.6.2.

The applicability of the biosensor for the analysis of benzene in air samples using the active sampling method was investigated using the benzene exposure room previously used in this Chapter. Air samples were taken using charcoal adsorption tubes, which were attached to pre-calibrated air pumps (1 l min^{-1}) inside the exposure room of 7-8 ppm benzene concentration. Several samples of 50, 75, and 100 l total volume of air were taken, and after the sampling period, the charcoal was transferred to a small vial and desorbed with 5 ml DMF, following the procedures used previously in the laboratory (Section 6.4). For each sample, the theoretical benzene concentration of the eluent was calculated based on the known exposure room concentration, the total air sample volume, and the amount of DMF used for desorption. The eluents were then diluted accordingly to a concentration within the detection range of the biosensor (typically 1:50 in potassium phosphate buffer). For GC cross-analyses of the air samples, 5 μl of the eluent was injected into a gas-tight vial and allowed to evaporate for 30 minutes at 37°C prior to injection of the gas phase into the gas chromatographic instrumentation.

Sample concentrations were calculated based on the trend line equation of the calibration curves conducted each day for the biosensor. The original concentration of the exposure room could then be calculated based on the eluent dilution, the DMF desorption volume, and the total volume of air sampled. Table 6.4 shows the data obtained from all of the samples taken using the active sampling method. This data has been used to create comparisons between the known exposure room concentration (analysed by GC), and the exposure room concentration calculated from the biosensor results (Table 6.5). The known room concentration was analysed by taking periodical samples of air directly from the room without pre-concentration (known as “grab sampling” (SEPAC, 1997), used in this case due to the relatively high concentration of benzene) and injecting it into the gas chromatographic system.

Table 6.5 shows the comparison between the known exposure room benzene concentration and that calculated from the biosensor results. The means from each of the 3 different air volumes sampled have been displayed, and it can be seen that in each case, a higher exposure room benzene concentration was calculated from the biosensor results with respect to the known concentration analysed by the GC.

Table 6.5.
Exposure room benzene concentrations calculated from the biosensor results, compared with known exposure room concentrations analysed by gas chromatography. Biosensor analytical error based on that obtained from the standard calibration curves. Standard GC error for exposure room concentrations = 5%. Biosensor SD based on variation in response between samples, GC SD based on fluctuations in control room conc. on day of sampling.

	Exposure Room Benzene Conc. (ppm)	SD	CV (%)
Biosensor 100 l samples	9.1 (± 0.6)	0.8	9 (n = 10)
Known room conc. (GC)	7.6 (± 0.4)	0.4	5 (n = 12)
Biosensor 75 l samples	11.0 (± 0.8)	0.6	5 (n = 8)
Known room conc. (GC)	8.1 (± 0.4)	0.1	1 (n = 8)
Biosensor 50 l samples	9.8 (± 1.2)	0.9	9 (n = 7)
Known room conc. (GC)	8.2 (± 0.4)	0.2	2 (n = 8)
Mean room conc. calculated from biosensor results (all samples)	10.0 (± 0.9)	1	10 (n = 25)
Mean known room conc. (GC)	8.0 (± 0.4)	0.3	4 (n = 28)

Table 6.4.
Data from active sampling in benzene exposure room using charcoal adsorption tubes with DMF desorption.

Sample identification	Sample conditions (actual exposure room benzene conc. (ppm) and number of air litres sampled (l))		Exposure room benzene conc. calculated from biosensor results (ppm) ¹	Exposure room benzene conc. calculated from GC results (ppm) (±5%)
A 23/02	7.0	100	8.7	11.3
B 23/02	7.0	100	8.7	10.3
A 24/02	7.7	100	9.4	13.3
B 24/02	7.7	100	9.4	12.7
C 24/02	7.7	100	8.1	11.0
D 24/02	7.7	100	9.4	12.5
E 24/02	7.3	100	7.5	11.2
F 24/02*	7.3	100	4.4	6.6
A 25/02	8.0	100	9.8	10.9
B 25/02	8.0	100	9.8	10.6
C 25/02	8.0	100	10.2	11.6
D 25/02*	8.0	100	6.2	7.8
E 25/02	8.0	50	8.7	7.3
F 25/02*	8.0	50	2.5	1.9
A 26/02	8.5	50	10.0	7.8
B 26/02	8.5	50	10.0	8.4
C 26/02	8.2	50	10.0	8.3
D 26/02	8.2	50	8.7	7.9
E 26/02	8.2	50	11.2	7.8
F 26/02	8.2	50	10.0	7.8
G 26/02	8.2	75	11.6	11.8
H 26/02	8.2	75	10.8	11.4
I 26/02	8.2	75	10.8	10.2
J 26/02	8.2	75	10.0	10.4
A 27/02	8.0	75	11.6	11.6
B 27/02	8.0	75	10.8	11.7
C 27/02	8.0	75	11.6	11.4
D 27/02	8.0	75	10.8	11.3

¹Error for biosensor analyses is displayed in Table 6.5.
*Samples in which the sample air pump stopped working at an unknown time due to the batteries. With the exception of sample F (25/02 50 l), in which the charcoal was spilt during transfer for desorption. These samples have not been included in the statistics of Table 6.5, or Figure 6.7.

Overall, the biosensor displayed a 20% increase in exposure room benzene concentration with respect to the known concentration. The reproducibility of the sampling method for the exposure room calculations is displayed by a 10% variation coefficient, based on all of the 25 samples, compared to 4% obtained from the GC ($n = 28$) (based on fluctuations of exposure room concentration throughout the days of sampling).

Figure 6.7 shows the results obtained from the biosensor and GC cross-analyses of the air sample eluents from the charcoal tubes, that is the 5 ml of DMF containing the extracted benzene following desorption of the charcoal. This figure also shows the theoretical eluents that should have been obtained based on the air sampling conditions used, and 100% benzene recovery. It can be seen that for all the air samples taken, the recovery of benzene from the charcoal after desorption was no less than 100%, despite a lower recovery obtained for the laboratory desorption experiments as aforementioned (Section 6.4). There has been no data published in the literature on the use of DMF alone for the desorption of actual air samples, only data relating to laboratory-based desorption efficiency experiments as previously described (Johansen and Wendelboe, 1981). Hence it is unknown if the improved benzene recovery in the present work was due to the difference in adsorption nature of benzene vapour from the air phase as opposed to liquid benzene following standard injection. Perhaps the more equal distribution of benzene molecules within the charcoal following active sampling facilitated an easier displacement of the molecules by DMF during desorption.

Figure 6.7 also shows that similar results were obtained for both the biosensor and GC analysis of the 75 l air sample eluents, but both were higher than the calculated theoretical eluent concentration (based on the known exposure room concentration and sampling conditions used). Similar concentrations were obtained between the biosensor, GC and calculated theoretical eluent concentration for the 50 l air samples, corresponding to the lowest amount of pre-concentrated benzene. The highest variation among the GC, biosensor, and theoretical eluent concentrations was found for the 100 l air samples, corresponding to the highest amounts of pre-concentrated benzene. A theoretical 1.3 and 2.4 mg amount of benzene would be adsorbed onto the charcoal for

the 50 and 100 l samples, respectively, based on the exposure room concentration and air pump flow rate used.

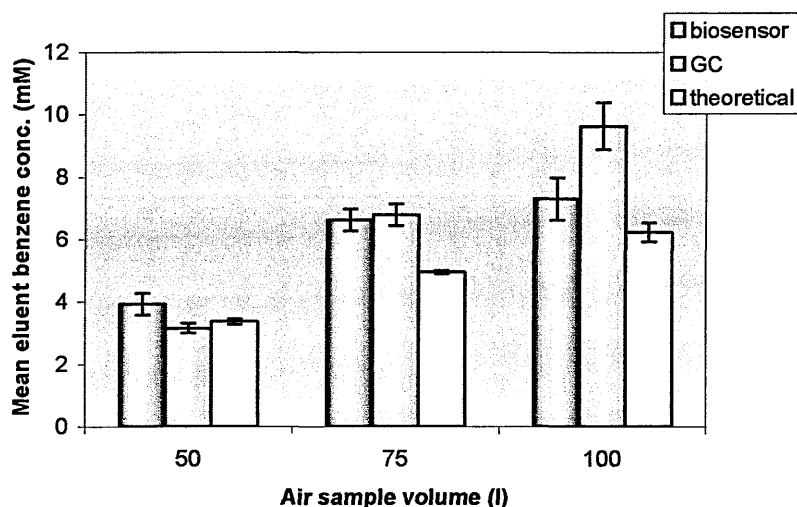


Figure 6.7. The mean air sample eluent benzene concentrations calculated from the biosensor and GC results. Theoretical eluent concentrations based on known exposure room concentration (errors bars based on fluctuations), total volume of air sampled and DMF desorption volume. Biosensor and GC error bars based on 7, 8, and 10 samples for the 50, 75, and 100 litre air volumes, respectively.

Overall, the main difference in the results from the active sampling has been the higher benzene concentration obtained for the air samples taken using charcoal adsorption tubes, with respect to the known benzene concentration of the exposure room, which was analysed by grab sampling (using air taken directly with a syringe and injected into the gas chromatographic system). This difference is more evident for the 75 and 100 l air samples. Nevertheless, the biosensor linear detection range of 0.025 – 0.15 mM benzene in a maximum concentration of 2% DMF would allow the analysis of air containing between 3 – 16 ppm benzene, based on a 60-minute sampling period (at an air pump flow rate of 1 l min⁻¹). However, since European legislation has set the exposure limit for benzene at 1 ppm (TWA) (EC Directive 97/42/EC) this would mean a longer sampling time for biosensor analyses to allow the collection of a greater

amount of benzene on the charcoal cartridge. However, the short-term exposure limit (STEL) of 5 ppm would be detectable under the present sampling conditions.

For the active sampling investigations, the biosensor apparatus was easily transported to the institute's benzene exposure room, thus demonstrating the portability of the sensor for *in situ* measurements. The same bacterial membrane was used throughout the 5 days in which the investigations took place without a decrease in sensitivity (2% coefficient of variation, $n = 11$), based on previously optimised membrane storage conditions (Section 5.5). In addition, no interference in response was obtained from blank samples created by the desorption of blank charcoal (results not shown).

6.6.2. Passive Air Sampling

As aforementioned, due to the low levels of benzene present in workplace air, pre-concentration to a quantity within the detection range of the analytical instrument is usually required. The preceding section described the use of active sampling for this pre-concentration step, based on air pumps to draw benzene through a charcoal sorbent. In this section, the use of passive air sampling is described, which is based on the natural diffusion of benzene through a charcoal sorbent, without using an air pump.

For this investigation, twenty air samples were taken from employees wearing a charcoal diffusion tube for a 7-hour working period in a Prague factory, Czech Republic. Since DMF has been reported in the literature to have a less efficient benzene recovery from charcoal with respect to the standard carbon disulphide (CS_2) solvent (Harper, 2000), 10 of the air samples were desorbed using CS_2 , and 10 were desorbed using DMF. In the preceding section, a DMF volume of 5 ml was used for the desorption of a total of 600 mg of charcoal, upon its transfer into gas-tight vials, where it was regularly agitated for a total desorption period of 30 minutes prior to dilution and analysis. For the passive diffusion tubes, the charcoal sorbent was retained within a tight wire mesh as shown in Figure 2.5. The standard desorption procedure for these diffusion tubes, according to the manufacturer guidelines (Radiello® 2004) is to add 2

ml of the desorption solvent into the glass vial containing the wire mesh-enclosed charcoal, and leave for a desorption period of 2 hours with occasional agitation.

All of the 20 air samples were analysed by gas chromatography, prior to the biosensor analysis of the DMF-desorbed samples. Table 6.6 shows all of the GC results obtained for both the CS₂- and DMF-desorbed samples. It can be seen that the concentration of benzene in the factory was low, for both the CS₂- and DMF-desorbed samples, thus indicating similar desorption efficiencies between the two solvents in this case, though the differences in the concentrations obtained will also be influenced by the particular part of the factory in which the employee was working at the time of sampling.

For the investigation of the air samples with the biosensor, 100 µl of the sample eluents after desorption were diluted in 5 mL of potassium phosphate buffer and injected into the FIA system. Figure 6.8 shows the biosensor response to one of the samples containing the highest amount of benzene (0.676 ppm), and the response to a blank sample (2 ml of DMF used for the desorption of a blank charcoal sorbent, diluted in buffer to a 2% concentration). It can be seen that there was a 6% decrease from the baseline for both the sample and the blank, which is the typical biosensor response to a 2% concentration of DMF only, indicating that the concentration of benzene in the sample was lower than the detection limit of the biosensor. These results indicate that for the concentrations of benzene found in the Prague factory, the passive method of air sampling was unsuitable for biosensor analyses. The natural diffusion of benzene through a charcoal sorbent is a slow process (80 ml min⁻¹) with respect to the active sampling method (Skov *et al.*, 2001). For a typical 0.004 ppm benzene concentration in the case of the Prague Factory, the amount of benzene adsorbed on the charcoal would have been 0.44 µg after the 7-hour sampling period. This is in contrast to the active sampling measurements, in which 1.3 mg of benzene was obtained on the charcoal cartridge following a 60-minute sampling period at an air pump flow rate of 1 l min⁻¹ at a 7 ppm exposure room concentration.

Table 6.6.

Factory air concentrations of benzene, calculated from the GC analysis of samples desorbed using CS₂ and DMF.

Sample ID & Desorption solvent		Air Conc. in Factory (ppm)	Sample ID & Desorption solvent		Air Conc. in Factory (ppm)
669E	CS ₂	0.003	680E	DMF	0.004
668E	CS ₂	0.003	664E	DMF	0.002
665E	CS ₂	0.003	661E	DMF	0.009
667E	CS ₂	0.013	662E	DMF	0.676
666E	CS ₂	0.010	671E	DMF	0.009
676E	CS ₂	0.004	672E	DMF	0.003
677E	CS ₂	0.002	673E	DMF	0.002
678E	CS ₂	0.004	674E	DMF	0.012
679E	CS ₂	0.003	675E	DMF	0.024
663E	CS ₂	0.002	670E	DMF	0.004

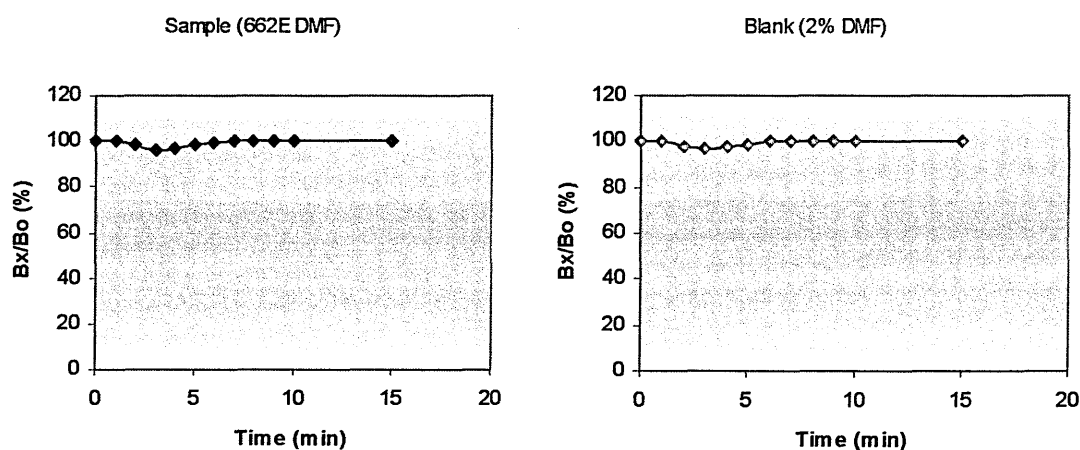


Figure 6.8. A comparison of the biosensor response to an air sample taken by passive diffusion and that to a blank sample. FIA conditions: flow rate: 0.5 ml min⁻¹; injection loop: 1 ml. B_x/B_o (%), where B_x = sample/standard maximum sensor output signal, and B_o = original baseline.

This highlights the much higher sample pre-concentration quantities required for biosensor analyses, hence the passive sampling method would necessitate a much longer sample period, which may not be appropriate for personal exposure measurements.

6.7. Discussions

In this chapter, the FIA-based bacterial biosensor has been developed for the analysis of benzene in workplace air samples. Initial investigations incorporated the testing of air sampling methods and desorption solvents, to find the most compatible combination for use with the bacterial biosensor. Due to the detection limits of the biosensor, which are higher than those for gas chromatography, a benzene pre-concentration method was required. This was achieved using charcoal adsorption tubes with solvent desorption of the entrapped benzene. Initial desorption solvents including carbon disulphide, acetonitrile, dimethylsulfoxide and methanol were all found to be unsuitable due to either their toxicity to the bacterial cells, interference in the sensor output signal, or poor

recovery of benzene from charcoal. Dimethylformamide (DMF) was found to be the most compatible solvent, being the second sole solvent (after carbon disulphide) that can be used for effective benzene recovery from charcoal with around 80% recovery (Harper 2000, Johansen and Wendelboe, 1981). This solvent caused negligible effects on the sensor response up to a 2% concentration.

For the active sampling investigations conducted, comparisons between the exposure room benzene concentration calculated from the biosensor results and the known room concentration analysed by GC, revealed a 20% higher concentration calculated from all of the biosensor results. Moreover, both the biosensor and GC analyses of the eluents collected after the air sampling, and in particular the higher air volumes sampled, revealed a higher concentration than the theoretical calculated from the known room concentration. This could be due to the difference in the two sampling methods utilised for this investigation where the known room concentration was analysed by GC without a pre-concentration step due to the high amount of benzene present. Thus the pre-concentration of benzene on the charcoal may have resulted in the collection of more benzene than calculated. This is particularly evident for the higher volumes of air sampled, and is perhaps enhanced due to the high concentration of benzene present in the exposure room. Real workplace environments have exposure limits of 1 ppm, hence pre-concentration effects would be much less at this concentration.

A passive air sampling method based on the diffusion of benzene onto a charcoal cartridge, used for the analysis of benzene concentrations in a factory in Prague, Czech Republic was also investigated. However, GC results revealed concentrations much lower than the detection limits of the biosensor. The passive diffusion of benzene is known to be a much slower process than the active pumping method (Skov *et al.*, 2001), hence unsuitable for the collection of samples to be analysed by the biosensor.

In summary, from the active sampling investigations using charcoal adsorption tubes with DMF desorption, a linear detection range between 0.025 – 0.15 mM benzene in a maximum concentration of 2% DMF was obtained, allowing the analysis of air containing between 3 – 16 ppm benzene, based on a 60-minute sampling period at an air

pump flow rate of 1 l min^{-1} . However, increased sampling times would be required for the analysis of samples within the legislative limits of benzene concentration in the workplace, up to 1 ppm.

The biosensor response characteristics were unchanged with the presence of DMF up to 2%, as described in Chapter 5. Furthermore, the biosensor response to benzene was not influenced by the presence of other benzene-related compounds in the same solution. In addition, the same bacterial membrane maintained a sensitive response over 5 days of continual use in an *in situ* location for the exposure room investigations (2% coefficient of variation, $n = 11$).

Overall, Chapter 6 has described the development of the FIA-based bacterial biosensor for the analysis of benzene in workplace air. The results from the air samples taken by active sampling have shown that the biosensor could be used as an early warning device for benzene concentrations in the workplace requiring further monitoring, with the added advantages over the conventional GC methods of low operation costs, ease of use, and portability for *in situ* measurements.

CHAPTER 7

RESULTS

BIOSENSOR FUNCTION USING SCREEN-PRINTED ELECTRODES

CHAPTER 7: BIOSENSOR FUNCTION USING SCREEN-PRINTED ELECTRODES

The use of biosensors based on screen-printed electrodes (SPEs) allows the development of much smaller and portable technologies for environmental analyses, which are low cost and can be mass-produced. Commonly used biological recognition elements for such sensors include enzymes, antibodies, and DNA. The use of bacteria in combination with SPEs has not been widely publicised in the literature, perhaps due to the limitation of electrochemical exchanges between bacteria and the electrode surface. Moreover, the planar nature of the electrode structure means that a conventional gas-permeable membrane cannot be fitted for measurements of dissolved oxygen consumption, which is important for keeping interfering substances, which may be reduced at the same potential as oxygen, away from the working electrode. However, the transformation of particular compounds by specific enzyme systems within microbial cells generate products or co-substrates which can be electrochemically active, and hence be detected by standard electrochemical techniques such as amperometry or potentiometry. This generally involves the use of mediators which replace the electron transfer performed by oxygen, thus preventing the problem of having a low oxygen concentration. However, the use of mediators has not been successful for some strains of bacteria (Skládal *et al.*, 2002).

Schmidt *et al.* (1996) and Timur *et al.* (2003), have demonstrated the use of bacterially-modified SPEs for the detection of free fatty acids and phenol, respectively, based on the dissolved oxygen consumption of the cells, where results were obtained without the use of a gas-permeable membrane. The papers describe the respective immobilisations of *Arthrobacter nicotianae* using Ca-alginate, and a *Pseudomonas putida* strain of bacteria in a gelatin membrane cross-linked with glutaraldehyde directly on the working electrode.

This chapter describes the use of gold screen-printed electrodes, modified with *Pseudomonas putida* ML2, for the analysis of benzene based on the respiratory activity of the bacterial cells. This part of the project focuses on an alternative type of transducer for the measurement of dissolved oxygen consumption with respect to the conventional

Clark dissolved oxygen probe. A novel immobilisation method and investigation of the sensitivity and stability of the bacterially-modified SPE is described. All measurements for dissolved oxygen detection were conducted in 0.1 M KCl in 50 mM potassium phosphate (pH 7) to provide the necessary chloride ions for the silver pseudo-reference electrode to make Ag/AgCl. The gold working electrode was held at a fixed potential of -0.7 V versus the Ag/AgCl for the reduction of dissolved oxygen, using a PalmSens[®] portable potentiostat. All measurements were conducted in batch mode, in 10 ml of the KCl-potassium phosphate buffer solution. After immersion of the bacterially-modified electrode, the reaction cell was sealed with Parafilm[®] to prevent benzene evaporation. Benzene was introduced into the biosensor cell by injection with a Hamilton syringe, as for the measurements conducted for batch mode using the Clark electrode (Chapter 4).

7.1. Dissolved Oxygen Detection using Screen-Printed Electrodes

The Clark dissolved oxygen utilises a gas-permeable membrane for the selective reduction of oxygen at the platinum working electrode, which minimises interference from any other compounds in a sample matrix that may be reduced at the same potential. In this section, the use of bare gold screen-printed electrodes without any form of gas-permeable membrane were tested for their ability to detect dissolved oxygen. Sodium sulphite was used to chemically remove oxygen from the potassium phosphate buffer solution in the reaction vessel, resulting in almost complete removal of the oxygen with 2 injections of 0.8 M concentration, which was clearly detected by the SPE (Figure 7.1). Nitrogen bubbling was also tested as a displacement means of removing oxygen from the buffer, which was also clearly detected (Figure 7.2). The bubbling however, was found to wet the electrode connector and hence interfere with the conductance of the electrodes causing a large interference in the sensor signal.

Sodium sulphite and nitrogen were also tested with the Clark electrode for the removal of dissolved oxygen, and yielded the same response as the screen-printed electrode. These results demonstrated the effective detection of dissolved oxygen without the use of the gas-permeable membrane, however the absence of this membrane could create interference in samples containing complex organic matrices such as wastewater.

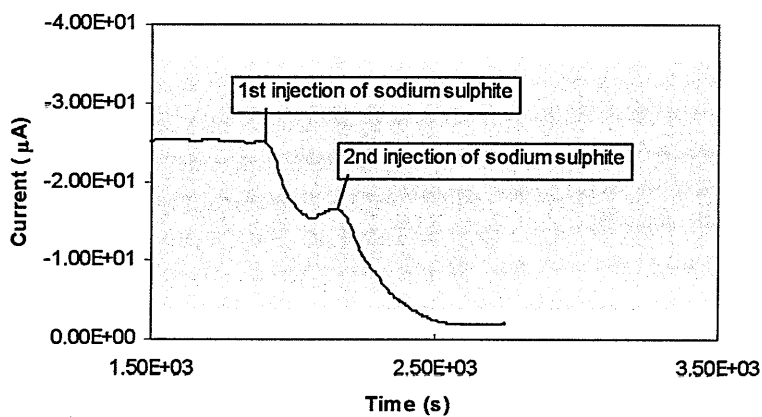


Figure 7.1. The testing of dissolved oxygen detection using a bare gold SPE at -0.7 V. Dissolved oxygen removed from the potassium phosphate buffer solution by two additions of 0.8 M sodium sulphite.

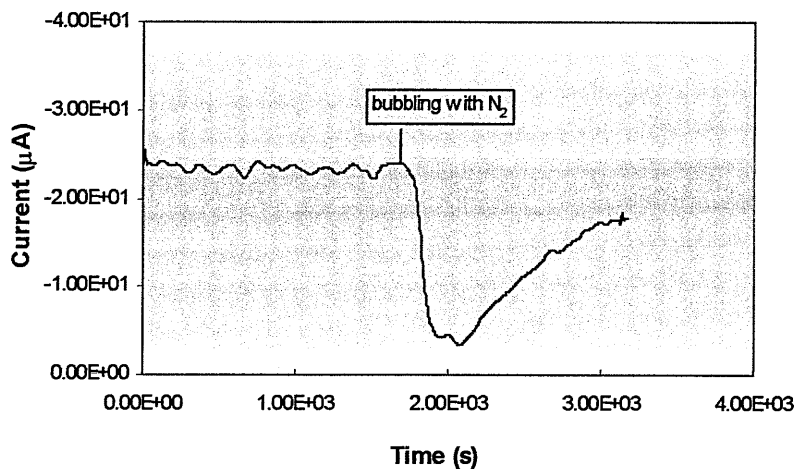


Figure 7.2. Dissolved oxygen detection using a bare gold SPE at -0.7 V, by bubbling with nitrogen.

7.2. Microbial Immobilisation

Initial experiments were conducted following an immobilisation method used by Timur *et al.* (2003) in which bacterial cells were immobilised directly onto the working electrode surface using bloom gelatin cross-linked with glutaraldehyde. In the present work however, this method yielded no response to either benzene or glucose, a substrate which is easily aerobically degraded by most bacterial strains. Furthermore, the use of this technique for bacterial immobilisation onto the gas permeable membrane of the Clark electrode (to replace the normally used cellulose acetate) also yielded no biosensor response to either benzene or glucose. D'Souza (2001) stated that the chemical cross-linking reagents used for such kinds of immobilisations can often affect the cell viability, thus the cross-linking technique would be useful for the immobilisation of non-viable cell preparations containing active intracellular enzymes.

An alternative immobilisation technique was therefore investigated, which involved the immobilisation of the bacterial cells onto a pre-cut circular piece of cellulose acetate (CA) membrane, as described in Chapter 2. The cells were obtained from the centrifugation of 1 ml of an 18-h bacterial culture, with re-suspension of the bacterial pellet in 30 μ l of potassium phosphate buffer (50 mM, pH 7). The cell paste was pipetted onto the CA membrane, allowing to dry for about 5 minutes, then the membrane was then stuck onto an adhesive ring around the working electrode with the bacterial cells in direct contact with the sensor surface. This created a kind of microenvironment, therefore allowing the free movement of ions, gases and solutes around the cells, and in which, bacterially induced changes in dissolved oxygen concentration could be measured.

Blank membranes were tested with the screen-printed electrodes prior to the investigation with the bacteria to investigate any interfering effects from the adhesive ring on the detection of oxygen. The response to dissolved oxygen removal using sodium sulphite and nitrogen bubbling was the same as for the bare electrodes outlined in Section 7.1.

7.3. Biosensor Response to Benzene

Prior to testing the bacterially modified SPEs with benzene, blank screen-printed electrodes (attached cellulose acetate membrane without bacteria) were tested with benzene to investigate any influence on the sensor response. Figure 7.3 shows that the natural drift of the baseline was unaffected by the injection of benzene into the reaction cell. Moreover, the injection of buffer also caused no changes in the current baseline.

The bacterial response to benzene is shown in Figure 7.4, where increasing concentrations of benzene were injected into the same 10 ml of buffer in the reaction vessel. In this graph, the dissolved oxygen consumption by the bacteria and corresponding baselines for each benzene concentration can be easily detected. The results from this figure have been used to determine the linearity of the sensor response, shown in Figure 7.5.

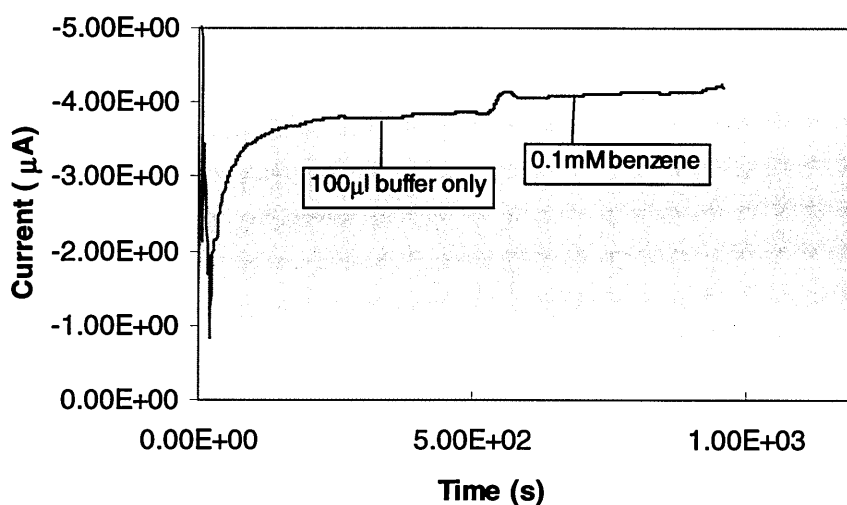


Figure 7.3. Screen printed electrode with blank cellulose acetate membrane (no bacteria). Sensor response to benzene without the presence of the bacteria.

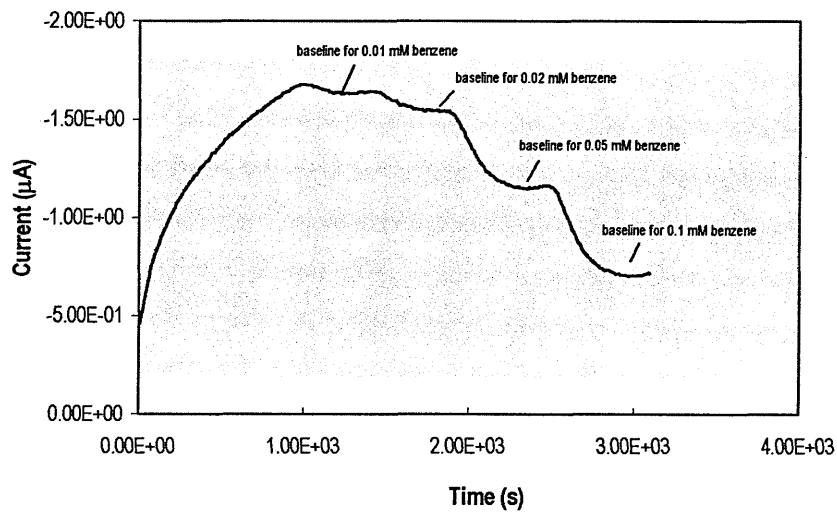


Figure 7.4. SPE-biosensor response to increasing concentrations of benzene over time.

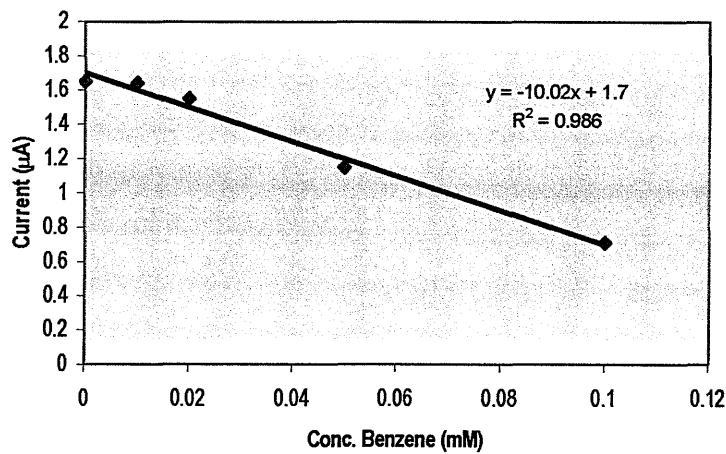


Figure 7.5. Linearity of the SPE-biosensor response to benzene, based on data from Figure 7.4.

The response times of the SPE experiments for the detection of benzene were the same as for the Clark electrode, that is the time taken after benzene injection to reach a secondary stable baseline following a decrease in current due to the increased oxygen consumption of the bacteria. For both the Clark electrode and SPE in batch mode, the biosensor response time was between 10 to 12 minutes, depending on the concentration of benzene.

Figure 7.6 shows the sensitivity of the biosensor to benzene using the SPE and using the Clark electrode. A similar sensitivity has been achieved for both types of transducer in batch mode. However, the absence of error bars in Figure 7.5 is a result of the poor reproducibility of the sensor response when using the screen-printed electrodes, meaning that the responses described were obtained only on one occasion. The succeeding section will therefore focus on the reproducibility and stability of the bacterially-modified SPE.

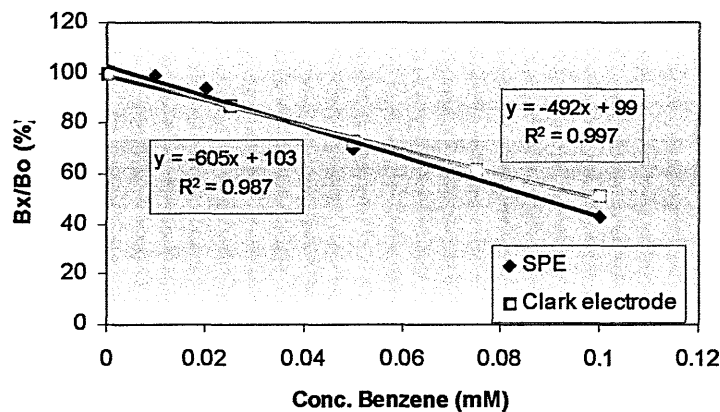


Figure 7.6. Comparison of the biosensor sensitivity to benzene using the SPE and the Clark electrode, both in batch mode. Bx/Bo (%), where B_x = sample/standard maximum sensor output signal, and B_o = original baseline.

7.4. Reproducibility and Stability

Previous investigations with the Clark oxygen electrode revealed excellent reproducibility and stability of the bacterial membrane coupled with this type of transducer (as described in Chapters 4, 5 and 6). However, the results described in Section 7.3 were obtained only on one occasion, and were marked by a large decrease in sensitivity upon immediate repetition of the same experiment, or unaccountable rising and falling of the current, not corresponding to standard injections. Repeated immobilisations under the same conditions more often resulted in no sensor response, or failure of the baseline to stabilise. This could have been due to a limitation of oxygen passage through the bacterial membrane related to mass transfer resistance due to the thickness of the biomass layer (Mulchandi *et al.*, 2002). Variations in the distribution of the bacterial cell paste over the membrane may have emphasised this effect in some immobilisations more than others. Timur *et al.* (2003), also reported a maximum response based on 50 μ l of culture containing exponential phase cells (OD_{560} 0.450), after which a decrease in the signal was obtained due to diffusional problems.

Figure 7.7 shows the results obtained from 4 repeated injections of 0.1 mM benzene, where a variation coefficient of 9% ($n = 4$) was obtained in this case. This figure demonstrates the time response of the biosensor showing the decrease in current with the oxygen consumption by the bacteria, and after the secondary baseline, the changing of the buffer and the recorded increase in current as the baseline recovers. These results have been plotted with the order of measurement against the sensor response with a further 2 measurements made afterwards, which demonstrates a decreasing trend of the sensor response (Figure 7.8).

Schmidt *et al.* (1996) used each bacterially-modified SPE for a 1-hour period and could perform a total of four assays per day using this method, though the sensor response after the hour or how many measurements were made during the hour, were not reported. Timur *et al.* (2003) in contrast immobilised a new electrode daily, and reported a stable sensor response over 25 measurements in one day (over 6 hours).

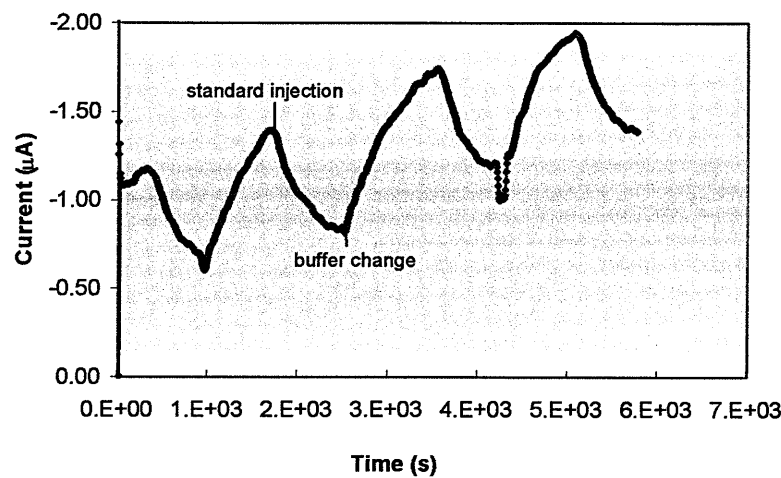


Figure 7.7. The reproducibility of the sensor response based on 4 injections of 0.1 mM benzene, showing the response and baseline recovery over time.

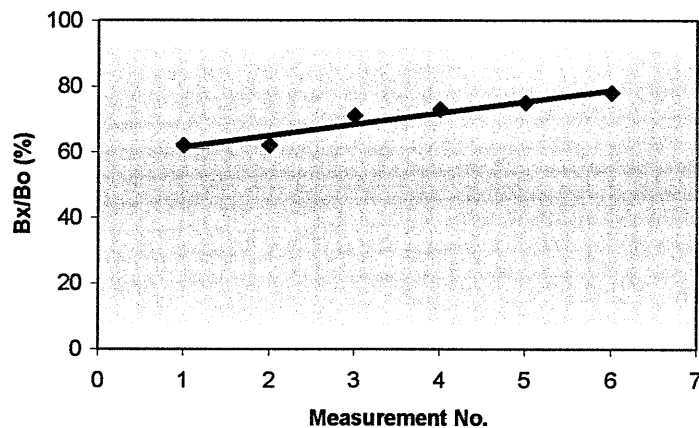


Figure 7.8. The stability of the biosensor response to repeated injections of 0.1 mM benzene. B_x/B_o (%), where B_x = sample/standard maximum sensor output signal, and B_o = original baseline.

7.5. Discussions

This chapter has described the use of an alternative transducer to the Clark electrode using low-cost and mass-producible gold screen-printed electrodes for the biosensing of benzene. Some preliminary results have been obtained using a novel immobilisation method based on the adhesion of a cellulose acetate membrane. Although the SPEs were able to detect dissolved oxygen in the potassium phosphate buffer without the use of a gas-permeable membrane, the immobilisation of the bacteria was found to be irreproducible. The sensitivity and response kinetics of the bacterially-modified SPEs were comparable with the Clark electrode, but their lifetime was seen to be short and unstable. This may suggest potential application of the modified SPEs as single-use devices.

Noise levels were sometimes noted during experimentation with the bacterially-modified electrodes, and in particular when the battery of the potentiostat was charging, or if other electrical appliances were in operation at that time. Experiments with the bare electrodes generated more noise, and also a higher baseline current of around 25 μA with respect to around 3 or 4 μA with the presence of a blank membrane, related to the diffusion limitation of the dissolved oxygen to the electrode surface. The immobilised bacteria caused a further slight decrease in the baseline current, of around 2 μA , also representing their endogenous respiration rate.

Overall, the Clark electrode has demonstrated more stability in terms of immobilisation, reproducibility and lifetime, with respect to the SPEs. The major limitation of the SPEs at this stage in their development is the immobilisation procedure, which is further limited by the non-viability of *Pseudomonas putida* ML2 on exposure to the cross-linking chemicals tested.

CHAPTER 8

DISCUSSIONS

CHAPTER 8. DISCUSSIONS

The development of an amperometric bacterial biosensor for the detection of airborne benzene has been described in this thesis. The biosensor incorporated a naturally occurring benzene degrader, *Pseudomonas putida* ML2, which was immobilised between two cellulose acetate membranes and fixed onto a Clark dissolved oxygen electrode. The bacteria consume oxygen upon degradation of benzene, and comparisons were made between sensor responses in batch (stirred solution) and kinetic (flow injection analysis) mode. The biosensor sensitivity and reproducibility was tested over a variety of parameters, including pH, temperature, time, cell loading, and the lifetime of the bacterial membrane under differing storage conditions. Additionally, for the flow injection analysis (FIA) system the effects of different flow rates and injection loop volumes were investigated. The biosensor was then further developed for the analysis of benzene in workplace air samples, involving investigation of different air sampling techniques and their associated compatibilities with the biosensor. Additional work in this thesis also incorporated investigations of a change of transducer, using screen-printed electrodes. This chapter will summarise the main points of discussion from all of the results chapters, on the development of the biosensor for the detection of benzene in workplace air samples.

8.1. Biosensor Operation in Batch and Kinetic Mode (Flow Injection analysis)

Similar linear detection ranges between 0.025 – 0.1 mM benzene, with a lower detection limit of 0.01 mM were obtainable in both batch and kinetic mode, though a more sensitive response was obtained in batch mode. This was based on the immobilisation of 1 ml of an 18-hour bacterial culture, of 1.2 mg dry weight (after centrifugation). These results are comparable with those obtained by Tan *et al.* (1994), and Campanella *et al.* (1996), in which a lower detection limit of 0.07 mM and 0.01 mM benzene was obtained, respectively, for a benzene biosensor based on *P. putida* operating in batch mode. Similar response and baseline recovery times to these publications of between 10

– 12 minutes, and between 30 – 40 minutes respectively, were also obtained in batch mode and found to be influenced by the concentration of benzene injected. In the present work, a faster response time was obtained using the flow injection analysis system, where the maximum sensor output signal was reached between 5 and 6 minutes, with respect to 10 - 12 minutes in batch mode. A faster baseline recovery time of around 10-15 minutes, depending on the substrate concentration, was also obtained in the kinetic mode with respect to 30 – 40 minutes obtained in batch mode.

The differences in sensitivity between the kinetic and batch mode can be attributed to both the flow and injection characteristics of the FIA system, which limit the presence of benzene in the flow cell and hence the contact time with the bacterial membrane through which the benzene diffuses. Decreased sensitivity could also be due to the dispersion and dilution of the injected sample in the carrier stream with respect to the stirred solution, which is further influenced by the injection volume and flow rate of the carrier stream and also the distance between the injection valve and the flow cell. In contrast, Liu *et al.* (2000) found a good correlation between results obtained in the kinetic and batch modes for BOD measurements of various types of wastewater, using a dissolved oxygen electrode as the transducer.

The sensitivity of the biosensor was found to be influenced by the bacterial cell mass on the immobilisation membrane. Higher cell loadings in both batch and kinetic mode were found in most cases, to give a higher response. However, cell loadings that were too high caused a limitation of the diffusion of dissolved oxygen through the membranes, and in some cases, no passage at all. Mulchandani and Rogers (1998) also state that a high membrane loading causes a high diffusion resistance, which results in a low sensor signal.

The sensitivity of the biosensor was also tested over a pH range between 5 and 9, and a temperature range between 16 – 35°C, and 21 – 35°C in batch and kinetic mode, respectively. An increasing sensor response was found from 16 – 35°C in batch mode, however in the kinetic mode, no significant differences were observed. The differences found here could be attributed to the cooling of the buffer through the FIA system

before reaching the sensor, despite the heated flow cell. A temperature of 30 – 35°C is closer to the optimum temperature for the growth of *P. putida*, and similar increases in sensor response were also reported by Tan *et al.* (1994), using the same bacteria over this temperature range in batch mode. However, operation of the biosensor in kinetic mode without the use of the water bath further enhances the portability of the instrumentation.

A variation in sensor response for a pH range between 5 and 9 was found only in the kinetic mode, with the greatest response at pH 6, and the lowest at pH 8 and 9. There were no differences in sensor response found over the same pH range in batch mode, despite significant differences obtained by Tan *et al.* (1994). However, Katrlík *et al.* (1996) reported independence of the *Aspergillus niger* response to glucose over a pH range from 5 to 8, despite reports of the glucose oxidase enzyme dependence on pH. The pH independence of the biosensor in this case was reported to be due to the location of the glucose oxidase enzyme in the peroxisomes inside the cells, meaning that changes of the external pH had no influence on the value of the intracellular pH (Katrlík *et al.*, 1996).

In this work, the biosensor has shown good intra- and inter-assay reproducibility in both batch and kinetic modes, based on optimised membrane storage conditions. In batch mode, the biosensor maintained a sensitive response to benzene over 22 days of continual use. Similarly with the FIA system, the same bacterial membrane was used for 28 days, and another for 47 days based on continuous and non-continuous use, respectively. This was achieved by storage of the bacterial membranes at 4°C in a 1 mM benzene-buffer solution when not in use. Such storage methods have also been utilised in the literature for the prolonged use of bacterial membranes (Mulchandani *et al.*, 2002; Schmidt *et al.*, 1996), and have thus been found to reduce the time and costs associated with repeated bacterial culturing.

In summary, between the two modes of operation, the use of the FIA system has allowed a more rapid sensor response and baseline recovery with respect to the batch system, hence allowing a greater number of samples to be analysed over a given period

of time. However this has been slightly compromised by a decrease in sensitivity. The FIA system permits an increased simplicity of operation, enabling the development of a more automated system without requiring the constant presence of trained personnel. Though a fully automated system was found to be unsuitable in the present work due to the volatility of benzene and the unpredictable nature of the bacterial response over several sample analyses. In addition, the constant flow rate and precise injection volume of the FIA system give rise to a more accurate sample analysis. Furthermore, the enclosed nature of the FIA tubing and flow cell also prevents the evaporation of volatile compounds such as benzene into the more favourable gas phase, which is present in the reaction cell of the batch mode.

8.2. Optimisation of the Biosensor for Air Sample Analysis

Initial investigations incorporated the testing of air sampling methods and desorption solvents to find the most compatible combination for use with the bacterial biosensor. Due to the detection limits of the biosensor, which are higher than those for gas chromatography, a benzene pre-concentration method was required. This was achieved using charcoal adsorption tubes with solvent desorption of the entrapped benzene. Initial desorption solvents including carbon disulphide, acetonitrile, dimethylsulfoxide and methanol were all found to be unsuitable due to either their toxicity to the bacterial cells, interference in the sensor output signal, or poor recovery of benzene from charcoal. Dimethylformamide (DMF) was found to be the most compatible solvent, being the second sole solvent (after carbon disulphide) that can be used for effective benzene recovery from charcoal with around 80% recovery (Harper 2000, Johansen and Wendelboe, 1981). This solvent caused negligible effects on the sensor response up to a 2% concentration.

One of the air sampling methods investigated was based on the pumping of air through charcoal adsorption tubes using pre-calibrated air pumps, known as “active sampling”, inside a benzene exposure room of controlled concentration. Comparisons between the exposure room benzene concentration calculated from the biosensor results and the

known room concentration analysed by GC, revealed a 20% higher concentration calculated from all of the biosensor results. Similarly the biosensor and GC analyses of the eluents collected after the air sampling, and in particular the higher air volumes sampled, revealed a higher concentration than that calculated based on the known room concentration. This could be due to the difference in the two sampling methods utilised for this investigation where the known room concentration was analysed by GC without a pre-concentration step due to the high amount of benzene present (known as “grab sampling”). Thus the pre-concentration of benzene on the charcoal may have resulted in the collection of slightly more benzene than calculated. This was particularly evident for the higher volumes of air sampled, and was perhaps enhanced due to the high concentration of benzene present in the exposure room. Real workplace environments should have concentrations no greater than 1 ppm, hence pre-concentration effects would be much less at this level.

A passive air sampling method based on the diffusion of benzene onto a charcoal cartridge, used for the analysis of benzene concentrations in a factory in Prague, Czech Republic was also investigated. However, GC results revealed concentrations much lower than the detection limits of the biosensor. The passive diffusion of benzene is known to be a much slower process than the active pumping method (Skov *et al.*, 2001), hence in this case, unsuitable for the collection of samples to be analysed by the biosensor.

In summary, from the active sampling investigations using charcoal adsorption tubes with DMF desorption, a linear detection range between 0.025 – 0.15 mM benzene in a maximum concentration of 2% DMF was obtained, allowing the analysis of air containing between 3 – 16 ppm benzene, based on a 60-minute sampling period at an air pump flow rate of 1 l min⁻¹. However, increased sampling times would be required for the analysis of samples within the legislative limits of benzene concentration in the workplace, up to 1 ppm.

The biosensor response characteristics were not influenced by the presence of DMF up to a 2% concentration. Furthermore, the biosensor response to benzene was not

influenced by the presence of other benzene-related compounds in the same solution. In addition, the same bacterial membrane maintained a sensitive response over 5 days of continual use in an *in situ* location for the exposure room investigations (2% coefficient of variation, $n = 11$).

Overall, this thesis has described the development of an FIA-based bacterial biosensor for the analysis of benzene in workplace air. The results from the air samples taken by active sampling have shown that the biosensor could be used as an early warning device for benzene concentrations in the workplace requiring further monitoring, with the added advantages over the conventional GC methods of low operation costs, ease of use, and portability for *in situ* measurements.

8.3. Screen-Printed Electrodes

Additional work in this thesis also focused on the use of an alternative transducer to the Clark electrode using low-cost and mass-producible gold screen-printed electrodes (SPEs) for the biosensing of benzene. Some preliminary results were obtained using a novel immobilisation method based on the adhesion of a cellulose acetate membrane, entrapping the bacterial cells next to the sensor surface. Although the SPEs were able to detect dissolved oxygen in the potassium phosphate buffer without the use of a gas-permeable membrane, the immobilisation of the bacteria was found to be irreproducible. The sensitivity and response kinetics of the bacterially-modified SPEs were comparable with the Clark electrode, but their lifetime was seen to be short and unstable. This may suggest their potential application as single-use devices.

Overall, the Clark electrode has demonstrated more stability in terms of immobilisation, reproducibility and lifetime, with respect to the SPEs. The major limitation of the SPEs at this stage in their development is the immobilisation procedure, which is further limited by the non-viability of *Pseudomonas putida* ML2 on exposure to the cross-linking chemicals tested.

Chapter 8 has summarised the main points of discussion from the results chapters of this thesis, highlighting the main features of the bacterial biosensor through its development for the analysis of benzene in workplace air. Chapter 9 will summarise the main conclusions that can be drawn from this project.

CHAPTER 9

CONCLUSIONS

CHAPTER 9. CONCLUSIONS

The presence of toxic volatile organic compounds (VOCs) such as benzene in workplace air has accounted for the death of many occupationally exposed workers over the last century (DEFRA, 2003). Benzene is included in the list of carcinogenic substances in Annex I of European Directive 67/548/EEC. The current permissible exposure level to benzene in the workplace in Europe is 1 ppm for an eight-hour time weighted average, with a 5 ppm short-term exposure limit (Council Directive 90/394/EEC amended by Directive 97/42/EC). Conventional chromatographical methods for the monitoring of airborne benzene are often complex, costly, require skilled personnel and are in most cases, laboratory-based. Thus, the development of low cost, easy to use and portable methods for the rapid detection of benzene in the workplace is desirable for occupational risk assessment.

In this work, *Pseudomonas putida* ML2, originally isolated from refinery soil as a benzene degrader, was immobilised between two cellulose acetate membranes, and fixed onto an amperometric Clark-type dissolved oxygen electrode. The bacterial biosensor was developed in batch mode, and then adapted into a Flow Injection Analysis (FIA) system with which the applicability of the biosensor for the analysis of air samples containing benzene was investigated. Air samples were collected by active sampling from a controlled-concentration benzene exposure room using charcoal adsorption tubes, and benzene extracted with solvent desorption using dimethylformamide (DMF). DMF proved to be compatible for use with the biosensor, causing minimal interference with the sensor response and causing no toxic effects on the bacterial cells. The biosensor displayed a linear detection range between 0.025 – 0.15 mM benzene based on standard solutions containing a maximum of 2% DMF, with a response time between 5 - 6 minutes and a baseline recovery time of 10 – 15 minutes. This linear detection range allowed the analysis of air containing between 3 – 16 ppm benzene, based on a 60-minute sampling period (at an air pump flow rate of 1 l min⁻¹). A longer sampling period would be required for biosensor analyses of benzene concentrations within the 1 ppm regulatory exposure limits.

A passive air sampling method also investigated was found to be unsuitable for biosensor analyses due to the slow diffusion rate of benzene through the charcoal sorbent without the use of an air pump, which resulted in a collection of benzene that was below the detection limits of the biosensor.

The FIA system was easily transported to an *in situ* location, and a correlation was obtained between the biosensor and gas chromatography (GC) results for the exposure room air samples investigated. Moreover, the biosensor displayed no interference to other benzene related compounds in the BTEX range. The results from this thesis have shown that the biosensor has potential applications for the analysis of benzene in workplace air samples, with the added advantages over the conventional GC methods of low operation costs, ease of use, and portability for *in situ* measurements.

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APPENDICES

APPENDIX 1

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Flow Injection Analysis of Benzene Using an Amperometric Bacterial Biosensor

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ABSTRACT

A bacterial biosensor integrated within a flow injection analysis (FIA) system has been developed for the detection of benzene, based on its aerobic catabolism by *Pseudomonas putida* ML2. *P. putida* ML2 cells were immobilised between two cellulose acetate membranes and fixed onto a Clark dissolved oxygen electrode. Biosensor responses were investigated with the FIA system, resulting in a linear detection range between 0.01–0.1 mM benzene. A response and baseline recovery time of 6 and 10–15 min, respectively, was obtained. A stable and reproducible sensor response has been found up to 28 days of use based on the same bacterial membrane, and the sensor has shown a high specificity to benzene, with a

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negligible response to other benzene-related compounds. Its ease of operation, rapid and sensitive response, and cost-effective production, demonstrate that the *P. putida* ML2 biosensor has potential applications for the analysis of samples containing benzene. The optimisation of the biosensor-flow injection system is described.

Key Words: Bacterial biosensor; Flow injection analysis; Benzene; Dissolved oxygen electrode.

INTRODUCTION

The presence of toxic volatile organic compounds (VOCs) such as benzene in workplace air has accounted for the death of many occupationally exposed workers over the last century.^[1] The main route of human exposure to benzene is mainly through inhalation; therefore, individuals employed in industries that make or use benzene may be exposed to the highest levels of this hydrocarbon. These industries include benzene production (petrochemicals, petroleum refining, and coke and coal chemical manufacturing), rubber tire manufacturing, and storage or transport of benzene and petroleum products containing benzene.

Many epidemiological studies have been undertaken, which have established benzene as a cause of various human haematologic cancers and diseases.^[2] The United States Environment Protection Agency have reported increased incidence of leukaemia in humans occupationally exposed to benzene, and have therefore classified it as a Group A, known human carcinogen of medium carcinogenic hazard.^[2] In Europe, the current permissible exposure level to benzene in the workplace is 1 ppm in air for an 8-hr time weighted average (Council Directive 90/394/EEC amended by Directive 97/42/EC).

The conventional method for monitoring employee exposure to benzene involves the collection of benzene vapour on charcoal adsorption tubes, which are desorbed with a solvent such as carbon disulphide, and subsequently analysed by gas chromatography.^[3] Despite the high accuracy and precision associated with chromatographical methods, they are often time-consuming, costly, and complex to use. Moreover, they often have slow analysis times and do not permit in-situ monitoring.

Microbial biosensors are devices incorporating a biological sensing element (a micro-organism) that can specifically recognise species of interest, either intimately connected to or integrated within a suitable transducing system. The transducer functions to quantitatively convert the biochemical signal into an electronic signal that can be suitably processed and outputted. In this way, analytes of interest may be measured by using the assimilation

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capacity of the micro-organism as an index of the respiration activity or of the metabolic activity.^[4] Microbial biosensors based on respiratory activity have had several applications in environmental monitoring in recent years.^[5,6]

Pseudomonas putida ML2 is a naturally occurring micro-organism, originally isolated from refinery soil, which can utilise benzene as its sole carbon and energy source.^[7] The biochemical pathway utilised by *P. putida* ML2 in the aerobic catabolism of benzene is shown in Fig. 1. In the microbial cell, benzene is first oxidised by the multicomponent benzene dioxygenase enzyme system to *cis*-1,2-dihydroxycyclohexa-3,5-diene or *cis*-benzene glycol in the presence of NADH and ferrous ions. The *cis*-benzene is subsequently dehydrogenated to catechol and finally undergoes ring cleavage to *cis*-*cis*-muconic acid. Degradation of this acid then occurs via the 3-oxoadipate pathway to give tricarboxylic acid (TCA) cycle intermediates.^[8] Therefore, the increase in respiration rate (and corresponding oxygen consumption) of *P. putida* ML2 during the aerobic assimilation of benzene can be used as the basis of a biosensor for the detection of this hydrocarbon in solution.

In this work, *P. putida* ML2 cells were immobilised between two cellulose acetate membranes, and fixed onto an amperometric Clark-type dissolved oxygen electrode. This paper describes the optimisation of the biosensor within a flow injection system for the detection of benzene. The aim of this work was to develop a biosensor that could have applications for the measurement of airborne benzene levels in the workplace, and prove a reliable, fast, and inexpensive method for compliance with health and safety regulations.

EXPERIMENTAL

Reagents

Bacterial culture constituents: KH_2PO_4 and K_2HPO_4 were purchased from Acros Organics (Fisher Scientific UK Ltd, Loughborough); tryptone was purchased from Oxoid S.p.A. (Milan, Italy); $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ and $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ were purchased from Fluka (Milan, Italy). $(\text{NH}_4)_2\text{SO}_4$ and benzene were purchased from Sigma (Milan, Italy).

Buffer components (KH_2PO_4 and $\text{K}_2\text{HPO}_4 \cdot 3\text{H}_2\text{O}$) and KCl (used for the electrolyte solution) were purchased from Merck (Milan, Italy).

Bacterial Cultivation

P. putida ML2 cells (kindly provided by Prof. F. Briganti, Florence University, Italy) were grown in a minimal liquid medium, containing the

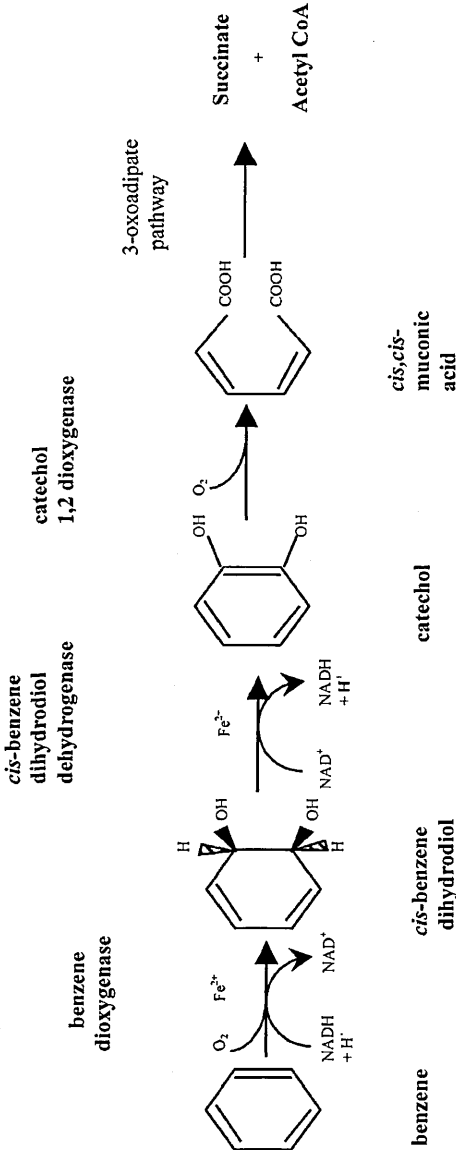


Figure 1. The catabolism of benzene by *P. putida* ML2.^[8]

following constituents per litre: KH_2PO_4 (1.02 g), K_2HPO_4 (3.06 g), $(\text{NH}_4)_2\text{SO}_4$ (1 g), Tryptone (0.2 g), $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ (0.4 g), and $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ (0.04 g) (dissolved in deionised water and adjusted to pH 7.2). Benzene was added to a final concentration of 10 mM. A 50-mL culture was grown for 18 hr, at 30°C and shaken at 150 rpm (Gerhardt Thermoshake, Königswinter, Germany) to obtain cells in the stationary phase of growth. Bacterial growth was measured using a Cary 3 UV-VIS spectrophotometer (Varian Instruments, Italy); an average absorbance (660 nm) of 1.2 after 18 hr of growth contained an average of 57×10^8 viable cells mL^{-1} .

P. putida ML2 colonies were sub-cultured monthly, and maintained on minimal medium agar plates, with the same constituents as the liquid medium, with the addition of 15 g of agar per litre. Viable cell counts were conducted by serial dilutions of the liquid medium culture, spread onto Luria Bertani medium agar plates.

Instrumentation

An Idronaut Oxyliquid model 23 dissolved oxygen probe and meter (Idronaut s.r.l., Italy) was used as the transducer and measuring device, respectively. A carrier stream of potassium phosphate buffer (50 mM, pH 7) (saturated with atmospheric air) was pumped using a peristaltic pump (Gilson Minipuls 3, Gilson Medical Electronics, France) through a custom-made water-jacketed flow cell containing the dissolved oxygen probe (Fig. 2).

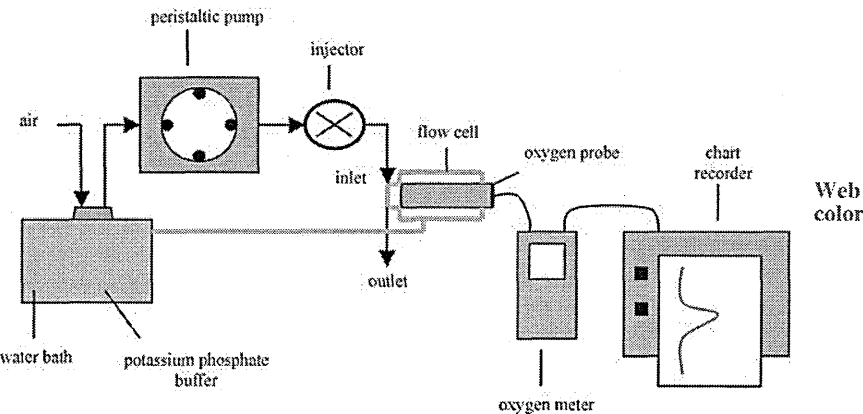


Figure 2. Scheme of the bacterial biosensor within the FIA system. (View this art in color at www.dekker.com.)

211 A Rheodyne model 7125 syringe loading sample injector (Rheodyne, CA) was
212 used to introduce standard solutions into the carrier stream. The sensor
213 response was continuously monitored on a chart recorder (Linseis Inc., Italy).

214 All experiments were conducted at 30°C, as the optimum working
215 temperature for the bacteria, and to minimise variations in dissolved oxygen
216 concentration.

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Immobilisation

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Measurement Scheme

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The steady state baseline of the oxygen meter prior to the injection of standard solutions represents the endogenous respiration rate of the immobilised bacteria. Dissolved oxygen diffuses continuously through the dialysis membranes from the potassium phosphate buffer to the surface of the platinum working electrode of the oxygen probe, where it is reduced at a fixed potential of -600 mV vs. a Ag/AgCl reference electrode. After injection, benzene diffuses through the cellulose acetate membrane and is assimilated by the immobilised bacteria, resulting in an increase of bacterial respiration rate and oxygen consumption. This causes a decrease in the concentration of dissolved oxygen near the electrode surface, resulting in a decrease in the output sensor

signal until a new steady state or “peak” (in the case of FIA) is obtained. This decrease in dissolved oxygen, and corresponding decrease in the sensor signal is proportional to the concentration of benzene injected. All results are presented as a percentage decrease from the original baseline, B_x/B_o (%), where B_x = sample/standard baseline and B_o = original baseline.

RESULTS AND DISCUSSION

The sensitivity and reproducibility of the sensor response to varying concentrations of benzene were investigated to establish the detection limits of the biosensor. The effects of flow rates and injection volumes were also investigated for the optimisation of the flow injection system. Further variables including pH, temperature, membrane loading, interfering compounds, and life-time of the bacterial membrane were also investigated. A comparison was made between the results obtained in batch mode and those obtained with the FIA system.

Effect of Flow Rates and Injection Volumes

For the detection of benzene using the FIA system, a range of flow rates and injection volumes were tested, in order to achieve the greatest sensitivity with a relatively fast response and baseline recovery time. Figure 3 shows the

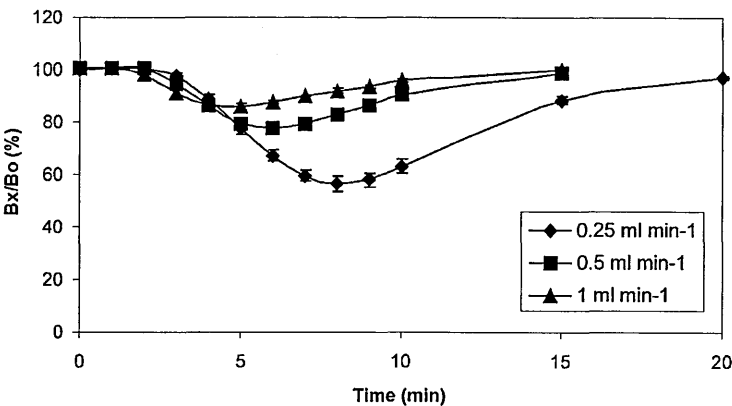


Figure 3. The effect of different flow rates on the sensor response to 0.1 mM benzene, using a 1-mL sample injection loop. B_x/B_o (%): the percentage decrease from the original baseline, where B_x = sample/standard baseline and B_o = original baseline.

effect of different flow rates on the sensor response to 0.1 mM benzene. An increase in sensor response can be seen as the flow rate is decreased from 1 to 0.25 mL min⁻¹, this is reflected by a respective 14–44% decrease from the original baseline.

This can be attributed to an increase in the occupancy time of benzene in the flow cell at the lowest flow rate. This allows a greater amount of benzene to diffuse through the dialysis membranes, hence a higher concentration for the bacteria to catabolise, with respect to the higher flow rates investigated. However, a flow rate of 0.25 mL min⁻¹ was also coupled with the longest response and baseline recovery times. A flow rate of 0.5 mL min⁻¹ was therefore selected as the optimum condition, as this showed a relatively high sensitivity with a faster response and baseline recovery time (6 and 10 min, respectively). These results are in agreement with those in the literature, where similar findings were obtained for a trichloroethylene microbial sensor.^[9]

The effect of increasing injection loop volumes on the sensor response to 0.1 mM benzene was investigated at a flow rate of 0.5 mL min⁻¹. Figure 4 shows an increasing sensor response from the 200 µL to 3 mL loop (reflected by a 4–24% decrease from the original baseline), however, these responses were also coupled with respective increases in response and baseline recovery times. This increasing sensitivity could be attributed to the larger amount of sample passing through the flow cell, hence an increased presence of the same concentration of benzene in the flow cell over time. This would allow a greater amount of benzene to be assimilated by the bacteria. Smaller

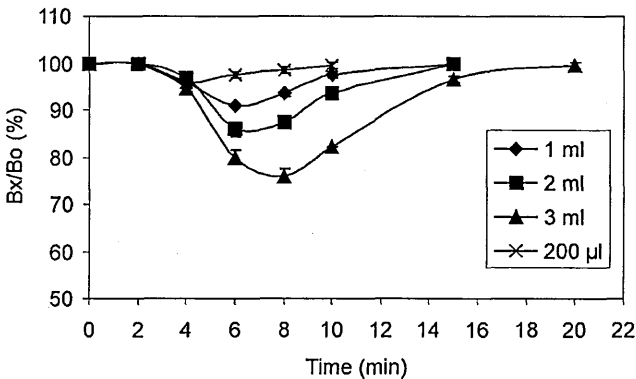


Figure 4. The effect of different injection loop volumes on the sensor response to 0.1 mM benzene at a flow rate of 0.5 mL min⁻¹. B_x/B_o (%): the percentage decrease from the original baseline, where B_x = sample/standard baseline and B_o = original baseline.

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injection volumes are more prone to dispersion and dilution in the FIA carrier stream, which is further influenced by the carrier flow rate and the distance between the injection valve and the flow cell. A 1-mL loop was selected as the optimum injection volume allowing a relatively fast response and baseline recovery time.

With the biosensor operating in batch mode, a response time of 10 min and a baseline recovery time up to 20 min was obtained at a 0.1 mM concentration of benzene. This was also coupled with an increased sensitivity of a 37% decrease from the original baseline with respect to a 22% decrease obtained with the FIA system at that benzene concentration. The batch results are in agreement with those reported by Tan et al.^[10]

Effect of Cell Loading

The biosensor response is dependent on the number of viable cells immobilised on the membrane. Viable cell counts revealed an average number of 57×10^8 viable cells mL⁻¹ after 18 hr of growth (stationary phase cells) under the conditions described in the Experimental section. In this investigation, immobilisations of increasing volumes of an 18-hr bacterial culture were conducted. In each case, the dry weight of the corresponding bacterial pellet of such volumes was used as a means of comparison of cell loading on the immobilisation membrane. Figure 5 shows the effect of increasing cell loadings on the sensor response to 0.5 mM benzene. An increase in sensor response up to 1.7 mg dry weight was obtained, however, cell loadings over

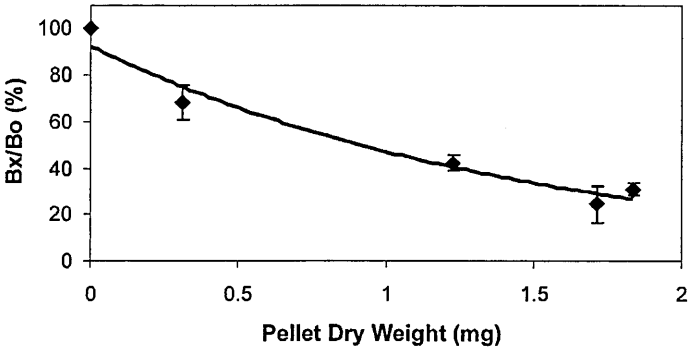


Figure 5. The effect of bacterial pellet dry weight on the sensor response to 0.5 mM benzene. FIA conditions: 0.5 mL min⁻¹, 1-mL injection loop. B_x/B_o (%): the percentage decrease from the original baseline, where B_x = sample/standard baseline and B_o = original baseline.

this amount resulted in a restriction of oxygen passage through the membranes. These findings are also in accordance with Im Lee and Karube^[11] in which a cell loading above 110 mg (wet weight) caused a decline of the sensor response. This was reported to have been due to the thickness of the immobilised membrane, resulting in a diffusion limitation of oxygen.

A 2-mL culture volume (1.2 mg dry weight) was found to be the optimal cell loading for the biosensor in the present work, allowing the greatest sensitivity without restricting the oxygen passage through the membrane.

Effect of pH and Temperature

For a microbial biosensor based on respiratory activity, the control of temperature is important not only for the optimal functioning of the bacteria, but also for the consistency of the dissolved oxygen concentration.

The biosensor showed no significant differences in response over a temperature range of 21.5–35°C with the FIA system. This is in contrast to previous experiments conducted in batch mode, which displayed an increasing response over the same temperature range. The differences found in this case could be attributed to the cooling of the buffer through the FIA system before reaching the sensor, despite the heated flow cell.

Conversely, for a pH range between 5 and 8 (in 50-mM potassium phosphate buffer), no significant differences in sensor response were found in batch mode. However, with the FIA system, a higher sensor response to 1 mM benzene was found at pH 6 (a 65% decrease from the original baseline), with a slight decrease in response at pH 7 and 5, and the lowest responses at pH 8 and 9 (a 35% and 30% decrease from the original baseline, respectively) (results not shown).

Lifetime of the Bacterial Membrane

Due to the time taken and consumption of materials used for growth of an 18-hr bacterial culture, it is more beneficial to prepare one bacterial membrane and use it for as long as the sensitivity and reproducibility of the sensor response persists. In this investigation, the lifetime of one bacterial membrane was investigated over a 28-day period with storage at 4°C in a 1-mM benzene solution (dissolved in 50-mM potassium phosphate buffer) when not in use. Figure 6 shows the sensor response to 0.5 mM benzene over this assay period with the FIA system, without a decrease in sensitivity. This storage method was also utilised by Schmidt et al.^[12] at 4°C in a 1-mM substrate solution, where the membrane retained 50% of its initial activity from 7 to 25 days over the assay

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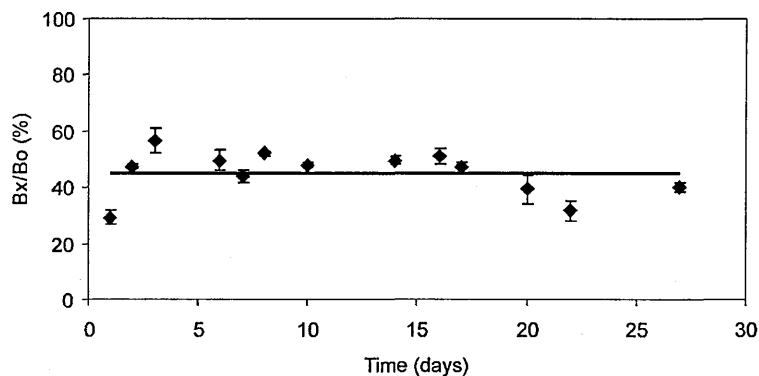


Figure 6. Biosensor response over time to 0.5 mM benzene, based on the same bacterial membrane (stored at 4°C in potassium phosphate buffer with 1 mM benzene when not in use). B_x/B_o (%): the percentage decrease from the original baseline, where B_x = sample/standard baseline and B_o = original baseline.

period. Tan et al.^[10] found stable and reproducible responses for 3 months with bacterial membrane storage at room temperature in a 1.35-mM substrate solution. However, this method caused a significant decrease in sensor response over a 3-day assay period in the present work. Similar decreases in response utilising storage methods at room temperature have also been reported in the literature.^[13,14]

The use of culture aliquots (2 mL volumes taken from the same 18-hr bacterial culture and stored at 4°C until required) to allow a new immobilisation each day was also investigated over a 2-week period. The sensitivity of each membrane remained the same over the investigation, thus this method also eliminates the need for repeated growth of 18-hr bacterial cultures.

Following all immobilisations, or re-fitting of stored membranes, it was necessary to wait up to 1 hr for the baseline of the dissolved oxygen meter to rise and stabilise. This is because the bacteria are still actively respiring and/or undergoing cell division during this time (resulting from their storage in the substrate solution, or from their transfer from the culture medium in the case of a new immobilisation), resulting in a low-dissolved oxygen concentration near the electrode surface.

Effect of Interfering Compounds

Bacterial biosensors can have the disadvantage of non-specificity to a particular target analyte with respect to enzyme biosensors.^[15] Therefore,

the sensor response to other benzene-related hydrocarbons was investigated. Figure 7 shows the effect of 0.5 mM concentrations of benzene, toluene, *p*-xylene, and *n*-hexane in the FIA system, with negligible sensor responses to the latter three. These findings are in agreement with those obtained by Tan et al.,^[10] where a negligible response was found for the same compounds, and also for ethylbenzene and MTB ether.

Benzene Linear Detection Range

With the biosensor optimised in the FIA system, a linear detection range between 0.01–0.1 mM benzene has been obtained (slope $y = -267x$, $R^2 = 0.995$, greatest CV = 1.4% ($n = 3$) for 0.1 mM benzene). These results are based on a flow rate of 0.5 mL min⁻¹, a 1-mL injection loop, and a bacterial membrane loading of 1.2 mg. The same linear range was also obtained in batch mode but with an increased sensitivity (slope $y = -363x$, $R^2 = 0.996$, greatest CV = 2.4% ($n = 3$) for 0.1 mM benzene) (results not shown). These results are comparable with those obtained by Tan et al.,^[10] and Campanella et al.,^[16] in which a lower detection limit of 0.07 and 0.01 mM benzene was obtained, respectively, for a benzene biosensor in batch mode.

The biosensor displayed good intra-assay reproducibility without a decrease in sensor response over 1 day of use in the present work. The lesser sensitivity obtained in the FIA system with respect to the batch over the same concentration range can be attributed to the dilution and dispersion of benzene

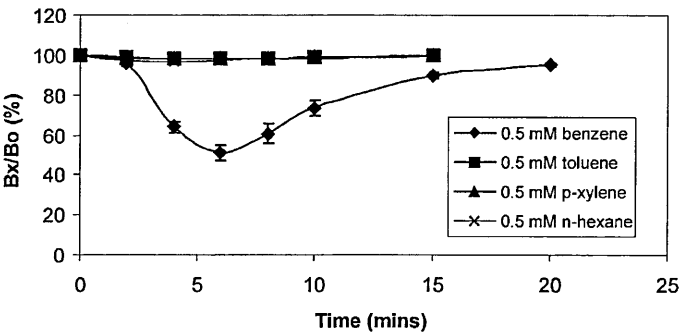


Figure 7. Biosensor response to benzene-related hydrocarbons. FIA flow rate: 0.5 mL min⁻¹, 1-mL injection loop. B_x/B_o (%): the percentage decrease from the original baseline, where B_x = sample/standard baseline and B_o = original baseline.

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in the FIA carrier stream, and the flow rate of benzene through the flow cell, thus limiting its diffusion through the bacterial membrane.

CONCLUSIONS

An FIA-based bacterial biosensor has been developed in this work with a linear detection range between 0.01–0.1 mM benzene. The optimised flow injection conditions allow a response time of 6 min and a baseline recovery time between 10–15 min, depending on the concentration of benzene. The biosensor has shown a good intra-assay reproducibility based on continuous use over 1 day and has also displayed good inter-assay reproducibility without a decrease in the sensor response over 28 days of use.

The use of the FIA system allows are more rapid sensor response and baseline recovery with respect to the batch system, hence allowing a greater number of samples to be analysed over a given period. It also permits an increased simplicity of operation, and could enable the development of an automated system without requiring the constant presence of trained personnel. Moreover, the constant flow rate and precise injection volume, give rise to a more accurate sample analysis.

Over all, its ease of operation, rapid and sensitive response, and cost-effective production, demonstrate that the *P. putida* ML2 biosensor has potential applications for the testing of workplace air samples containing benzene.

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APPENDIX 2

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Benzene analysis in workplace air using an FIA-based bacterial biosensor

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Abstract

A bacterial biosensor based on flow injection analysis (FIA) has been developed for the determination of benzene in workplace air samples. Benzene can be used by the bacteria *Pseudomonas putida* ML2 as a sole carbon source, and its aerobic degradation can be measured using a dissolved oxygen electrode. The bacterial cells were immobilised between two cellulose acetate membranes and fixed onto a Clark dissolved oxygen probe, which was inserted into a custom-made flow cell. The applicability of the biosensor for the analysis of air samples containing benzene was investigated. Air samples were collected from a controlled exposure room using charcoal adsorption tubes, and benzene extracted with solvent desorption using dimethylformamide (DMF). The biosensor displayed a linear detection range between 0.025 and 0.15 mM benzene based on standard solutions containing a maximum of 2% DMF, with a response time of 6 min. This linear detection range allows the analysis of air containing between 3 and 16 ppm benzene based on a 60-min sampling period.

DMF proved to be compatible for use with the biosensor, causing minimal interference with the sensor response and causing no toxic effects on the bacterial cells. The FIA system was easily transported to an in situ location, and a correlation was obtained between the biosensor and gas chromatography (GC) results for the preliminary air samples investigated. Moreover, the biosensor displayed no interference to other benzene related compounds in the BTEX range. The results from this work have shown that the biosensor has potential applications for the analysis of benzene in workplace air samples, with the added advantages over the conventional GC methods of low operation costs, ease of use, and portability for in situ measurements.

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Keywords: Bacterial biosensor; Flow injection analysis; Benzene; Dissolved oxygen electrode

1. Introduction

The presence of toxic volatile organic compounds (VOCs) such as benzene in workplace air has accounted for the death of many occupationally exposed workers over the last century (DEFRA, 2003). Benzene has been classified by the Environmental Protection Agency as a Group A, known human carcinogen of medium carcinogenic hazard (EPA, 2003). In Europe, the current permissible exposure level to benzene in the workplace is 1 ppm for an 8-h time weighted

average, with a 5 ppm short-term exposure limit (Council Directive 90/394/EEC amended by Directive 97/42/EC).

The conventional method for monitoring employee exposure to benzene incorporates the collection of benzene vapour on charcoal adsorption tubes, which are desorbed using carbon disulphide (CS₂), and subsequently analysed by gas chromatography (GC) (OSHA, 2003). CS₂ is the most common solvent used for the desorption of benzene from a charcoal sorbent (Harper, 2000). It has a high heat of adsorption on activated carbon, displacing other molecules, and is a good solvent for non-polar compounds (Harper, 2000). However it is also highly toxic with a high vapour pressure (275 mmHg at 20 °C), hence it is very difficult to

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avoid human contact with the vapours. Moreover, possible evaporation of CS₂ during and after addition of precise amounts to the charcoal constitutes a possible source of error (Johansen and Wendelboe, 1981).

N,N-Dimethylformamide (DMF) has been tested and compared to CS₂ as an alternative solvent for the desorption of organic vapours from charcoal (Johansen and Wendelboe, 1981). Although DMF is in the same toxicity range as CS₂, its low vapour pressure (2.65 mmHg at 20 °C) makes it easier to handle both in safety and analytical senses (though it can be absorbed through the skin). DMF was found to be less efficient than CS₂ for the desorption of hydrocarbons from charcoal, resulting in an 80% recovery for benzene (Johansen and Wendelboe, 1981). However, it was reported that a desorption efficiency close to 100% does not necessarily result in a better quantification than a less efficient desorption such as 80%, apart from a gain in sensitivity. NIOSH air sampling guidelines state that analyte recovery should be 75% or greater (Kennedy et al., 1995). Johansen and Wendelboe (1981) also reported that DMF provided greater sample stability after desorption than CS₂, which is important if samples are left for several days prior to analysis.

Despite the high accuracy and precision associated with chromatographical methods of analysis, they are often time-consuming, costly, and complex to use. Moreover, they often have slow analysis times and do not permit in-situ monitoring. Microbial biosensors are devices incorporating a biological sensing element (a microorganism) that can specifically recognise species of interest, either intimately connected to or integrated within a suitable transducing system. The transducer functions to quantitatively convert the biochemical signal into an electronic signal that can be suitably processed and outputted. In this way, analytes of interest may be measured by using the assimilation capacity of the microorganism as an index of the respiration activity or of the metabolic activity (Mulchandani and Rogers, 1998). Microbial biosensors based on respiratory activity have had several applications in environmental monitoring in recent years (Tothill and Turner, 1996; D'Souza, 2001).

Pseudomonas putida ML2 is a naturally occurring microorganism, originally isolated from refinery soil, which can utilise benzene as its sole carbon and energy source (Axcell and Geary, 1975; Mason, 1994). The increase in respiration rate (and corresponding oxygen consumption) of *P. putida* ML2 during the aerobic assimilation of benzene can be used as the basis of a biosensor for the detection of this hydrocarbon in solution (Lanyon et al., 2004). In this work, *P. putida* ML2 cells were immobilised between two cellulose acetate membranes, and fixed onto an amperometric Clark-type dissolved oxygen electrode. This paper describes the development of an FIA-based bacterial biosensor for the analysis of benzene in workplace air samples, including the optimisation of air sample collection and extraction techniques that are compatible for use with the biosensor.

2. Experimental

2.1. Reagents

Bacterial culture constituents: KH₂PO₄ and K₂HPO₄ were purchased from Acros Organics (Fisher Scientific UK Ltd., Loughborough); tryptone was purchased from Oxoid S.p.A. (Milan, Italy); MgSO₄·7H₂O and FeSO₄·7H₂O were purchased from Fluka (Milan, Italy). (NH₄)₂SO₄ and benzene were purchased from Sigma (Milan, Italy). Buffer components (KH₂PO₄ and K₂HPO₄·3H₂O), KCl (used for the electrolyte solution), and *N,N*-dimethylformamide were purchased from Merck (Milan, Italy).

2.2. Bacterial cultivation

Pseudomonas putida ML2 cells (kindly provided by Prof. F. Briganti, Florence University, Italy) were grown in a minimal liquid medium, containing the following constituents per litre: KH₂PO₄ (1.02 g), K₂HPO₄ (3.06 g), (NH₄)₂SO₄ (1 g), tryptone (0.2 g), MgSO₄·7H₂O (0.4 g), and FeSO₄·7H₂O (0.04 g) (dissolved in deionised water and adjusted to pH 7.2). Benzene was added to a final concentration of 10 mM. A 50 ml culture was grown for 18 h, at 30 °C and shaken at 150 rpm (Gerhardt Thermoshake, Königswinter, Germany) to obtain cells in the stationary phase of growth. Bacterial growth was measured using a Cary 3 UV–vis Spectrophotometer (Varian Instruments, Italy); an average absorbance (660 nm) of 1.2 after 18 h of growth contained an average of 57×10^8 viable cells ml⁻¹. *P. putida* ML2 colonies were sub-cultured monthly, and maintained on minimal medium agar plates, with the same constituents as the liquid medium, with the addition of 15 g of agar per litre. Viable cell counts were conducted by serial dilutions of the liquid medium culture, spread onto Luria Bertani medium agar plates.

2.3. Instrumentation

An Idronaut Oxyliquid model 23 dissolved oxygen probe and meter (Idronaut s.r.l., Italy) was used as the transducer and measuring device, respectively. A carrier stream of potassium phosphate buffer (50 mM, pH 7) (saturated with atmospheric air) was pumped using a peristaltic pump (Gilson Minipuls 3, Gilson Medical Electronics, France) through a custom-made water-jacketed flow cell containing the dissolved oxygen probe. A Rheodyne model 7125 syringe loading sample injector (Rheodyne, CA, USA) was used to introduce standard or sample solutions into the carrier stream. FIA operating conditions incorporated a carrier stream flow rate of 0.5 ml min⁻¹, and a 1-ml sample injection loop as previously optimised (Lanyon et al., 2004). The sensor response was continuously monitored on a chart recorder (Linseis Inc., Italy). All experiments were conducted at room temperature (23–25 °C).

2.4. Immobilisation

Bacterial cells were harvested by centrifugation of 1 ml of an 18-h culture (A_{660} 1.2, stationary phase cells) at 6000 rpm for 10 min at room temperature (Eppendorf, MiniSpin Plus). The supernatant was discarded, and the pellet re-suspended in 100 μ l of potassium phosphate buffer (50 mM, pH 7). The re-suspended pellet (0.7 mg mean dry weight) was pipetted onto the centre of a 13 mm diameter cellulose acetate membrane (0.2 μ m) (Sartorius, Italy) until the buffer had naturally filtered through. A secondary 25 mm diameter cellulose acetate membrane was then placed over the first to sandwich the cells in between. The bacterial membranes were then placed over a gas-permeable polyethylene membrane, and fixed onto the cap of the dissolved oxygen probe using a rubber *o*-ring. Each bacterial membrane was used for as long as the sensitivity of the sensor response persisted (up to 1 month). When not in use, the membranes were stored at 4 °C in potassium phosphate buffer (50 mM, pH 7) with 1 mM benzene.

2.5. Measurement scheme

The steady state baseline of the oxygen meter prior to the injection of standard solutions represents the endogenous respiration rate of the immobilised bacteria. Dissolved oxygen diffuses continuously through the dialysis membranes from the potassium phosphate buffer to the surface of the platinum working electrode of the oxygen probe, where it is reduced at a fixed potential of -600 mV versus a Ag/AgCl reference electrode. After sample injection, benzene passes into the flow cell and diffuses through the cellulose acetate membrane where it is assimilated by the immobilised bacteria. This causes an increase in bacterial respiration rate and oxygen consumption, which results in a decrease in the concentration of dissolved oxygen near the electrode surface. This is accompanied by a corresponding decrease in the output sensor signal until the injected sample starts to leave the flow cell and at which point the sensor signal starts to recover back to the original baseline. Thus the sensor response is determined by the height of the peak, which corresponds to the amount of dissolved oxygen consumed by the bacteria, and hence to the concentration of benzene injected.

All results are presented as a percentage decrease from the original steady state baseline of the oxygen meter, B_x/B_0 (%) where, B_x = sample/standard maximum sensor output signal, and B_0 = original baseline.

2.6. Air sampling

Benzene air samples were taken from an exposure room of controlled benzene concentration at the FSM institute of Pavia, Italy. Air samples were collected using 400/200 mg charcoal adsorption tubes (Supelco—Sigma, Milan, Italy) attached to pre-calibrated air pumps at a flow rate of 1 l min⁻¹. Benzene extraction was undertaken by solvent desorption using 5 ml of *N,N*-dimethylformamide for a period of 30 min

(with regular agitation). All sample eluents were diluted in potassium phosphate buffer (50 mM, pH 7) prior to injection into the FIA system for biosensor analyses.

3. Results and discussion

3.1. The compatibility of desorption solvents with the biosensor

Following the adsorption of benzene vapour on a charcoal air sampling tube, it is important to find a suitable method of desorption from the solid sorbent that is compatible with analysis by the biosensor. The biosensor operates in the liquid phase, hence the first point of consideration is the effective desorption of benzene using a solvent that is soluble in the water-based sample carrier stream (potassium phosphate buffer) of the FIA system. It is also necessary to dilute the sample eluent (containing the extracted benzene) in this buffer prior to injection into the FIA system, in order to create a benzene concentration within the detection range of the biosensor. Moreover, it is necessary to dilute the desorption solvent to a concentration which does not interfere with either the biosensor response, or viability of the bacterial cells. A range of desorption solvents were tested with the biosensor in order to find the most compatible combination. Table 1 highlights the solvents tested and their associated advantages and disadvantages.

Initial investigations with carbon disulphide (CS₂) proved that this solvent was not compatible for use with the biosensor. Firstly, CS₂ has only 0.3% solubility in water, thus rendering sample or standard dilutions in a water-based buffer unfeasible.

Furthermore, a 0.2% CS₂ concentration was found not to be completely miscible in the phosphate buffer even after 15 min, and was seen to affect the bacterial cells in such a way that no biosensor response to benzene was obtained after exposure to the partially diluted solvent. CS₂ was also seen to cause a diminution of the sensor response even without the presence of the bacterial cells, hence this would create difficulties in distinguishing sensor response readings to benzene and those to CS₂. This was also found to be the case with dimethylsulfoxide and acetonitrile, which are common solvents used for solid-phase extraction (Thurman and Mills, 1998). Methanol was also tested as an alternative solvent, but this required a silica solid phase due to methanol's poor desorption of benzene from charcoal. However, silica was found to be a poor sorbent for the collection of benzene vapours, having a higher affinity for water vapour. Furthermore, a decrease in the biosensor response to benzene was found with increasing concentration of methanol.

N,N-Dimethylformamide (DMF) was tested as an alternative desorption solvent for the extraction of benzene from charcoal (Johansen and Wendelboe, 1981). It has not been extensively used alone as a desorption solvent in the literature, but its desorption efficiency has been compared to that

Table 1
Desorption solvents tested with the biosensor and associated advantages and disadvantages

Solvent	Properties ^a	Advantages	Disadvantages
Carbon disulphide (CS ₂)	0.294% solubility in water	Best solvent for benzene extraction from a charcoal sorbent ^b	Found to be toxic to bacterial cells Found to be insoluble in water-based buffer at 0.2% concentration, hence sample dilution unfeasible
Dimethyl-sulfoxide (DMSO) ((CH ₃) ₂ SO)	Soluble in water	Alternative solvent for solid-phase extraction ^c Allows water-based sample dilution Not found to be toxic to bacterial cells over concentration range tested (0–20%)	Biosensor response to DMSO alone similar to that of benzene
Acetonitrile (CH ₃ CN)	Miscible with water	Alternative solvent for solid-phase extraction ^c Allows water-based sample dilution Not found to be toxic to bacterial cells over concentration range tested (0–20%)	Biosensor response to acetonitrile alone similar to that of benzene
Methanol (MeOH) (CH ₃ OH)	Miscible with water	Alternative desorption solvent from a silica sorbent ^b Allows water-based sample dilution Not found to be toxic to bacterial cells over concentration range tested (0–20%)	Decrease in biosensor response to benzene with increasing MeOH concentration Poor solvent for desorption of benzene from charcoal Required use of a silica sorbent, which has a higher affinity for water than benzene ^b
N,N-Dimethyl-formamide (DMF) (HCON (CH ₃) ₂)	Miscible with water	Alternative desorption solvent from a charcoal sorbent ^d Allows water-based sample dilution Not found to be toxic to bacterial cells over concentration range tested (0–20%) Negligible effect on biosensor response up to 2% concentration	Reported to be less effective for the desorption of benzene from charcoal (80% recovery with respect to 100% for CS ₂) ^d

^a Budavari et al. (1996).
^b Harper (2000).
^c Thurman and Mills (1998).
^d Johansen and Wendelboe (1981).

of CS₂, and it has been reported to be a good solvent for polar compounds, and a reasonable solvent for non-polar compounds (usually 80% recovery or more) (Harper, 2000). In the present work, DMF was found to be the most compatible solvent for use with the biosensor, being completely soluble in water, having negligible interference with the sensor response up to a 2% concentration, and having no toxic effects on the bacterial cells. Furthermore, DMF was found to have no effect on the sensor response without the presence of the bacterial cells.

3.2. The influence of DMF on the biosensor response

Prior to testing the efficiency of DMF for the desorption of benzene from charcoal, the effect of differing concentrations of this solvent on the biosensor response was investigated. Fig. 1 shows the effect of increasing DMF concentration on the sensor response to 0.1 mM benzene, and also includes the sensor response to DMF alone. It can be seen that 5 and 10% concentrations of DMF alone caused a decrease from

the sensor baseline of 14 and 32%, respectively, compared to a 1 and 2% DMF concentration, which caused a maximum decrease of 6% from the baseline. A maximum of 2% DMF was therefore selected as the highest concentration that could

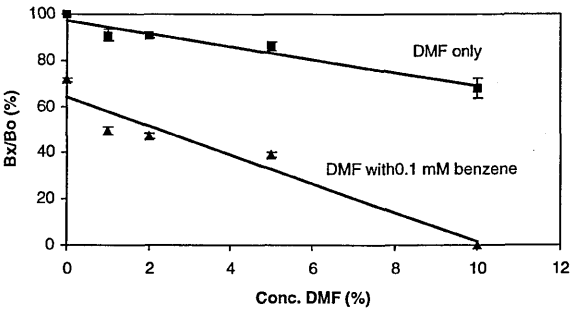


Fig. 1. The effect of increasing DMF concentration on the sensor response: (a) the effect of DMF only on the sensor response, (b) the effect of increasing DMF concentration on the sensor response to 0.1 mM benzene. B_x/B_o (%), where B_x = sample/standard maximum sensor output signal, and B_o = original baseline.

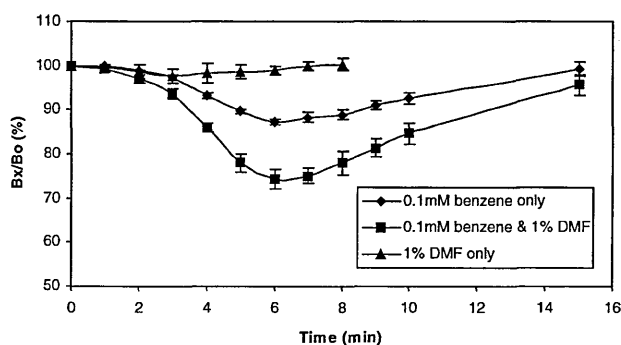


Fig. 2. The effect of 1% DMF on the sensor response to 0.1 mM benzene. B_x/B_0 (%), where B_x = sample/standard maximum sensor output signal, and B_0 = original baseline.

be used with the biosensor. This allows a maximum of 100 μ l of sample eluent (extracted benzene in pure DMF) to be diluted in 5 ml of phosphate buffer (which is the volume used to flush and entirely fill the 1 ml sample injection loop of the FIA system). Thus, during air sampling, it is important to collect a sufficient amount of benzene on the charcoal, to allow an eluent dilution of the above proportions (this is further influenced by the volume of DMF used during desorption, which is discussed in the following section). A larger analyte collection quantity also necessitates the use of larger air sampling tubes; hence a total of 600 mg of charcoal (400/200 mg for the primary and back-up sections, respectively) was used in the present work.

Fig. 2 shows the influence of a 1% concentration of DMF on the biosensor response to 0.1 mM benzene, in which a 15% increase in sensitivity was obtained with respect to benzene alone. This could be due to the increased solubility of benzene in the buffer with the presence of DMF, which has a solubility in water of just 0.2%. The lower vapour pressure of DMF with respect to benzene may also prevent some benzene evaporation into the headspace of the vial during standard solution preparations. Johansen and Wendelboe (1981) also reported a greater sample stability during storage over time in DMF, with respect to CS_2 . The increase in sensitivity of the biosensor response in the presence of DMF, required all calibration curves to be conducted from a benzene stock solution (10 mM) in DMF, which was diluted in phosphate buffer to the desired benzene (and DMF) concentration, to correspond with the air sample eluent benzene and DMF concentrations. A detection range between 0.025 and 0.15 mM benzene with a maximum DMF concentration of 2% gave a trend line equation of $y = -313x$ (y intercept 101) with an r^2 value 0.998 and a variation coefficient of 3.2% ($n = 17$). A lower detection limit of 0.01 mM was obtainable, though this was generally excluded from calibration curves due to the higher benzene eluent concentrations analysed after air sampling. The biosensor response time was 6 min, with a baseline recovery time between 10 and 15 min, depending on the concentration of benzene (as previously optimised, Lanyon et al., 2004).

Without the presence of the bacterial cells, no sensor response to DMF was obtained, indicating that the solvent alone does not effect the dissolved oxygen concentration. It is unclear if DMF can be degraded by the bacterial cells, but nevertheless it caused a decrease in the sensor signal with the presence of the bacteria, with a response time of between 3 and 4 min, with respect to 6 min for benzene (either alone or with DMF). The sensor response to DMF alone was more evident above 2% concentrations, but was undetectable when mixed with benzene, expressed as always in one peak at 6 min.

The lifetime of the same bacterial membrane over 8 days of exposure to a maximum of 2% DMF was investigated. This was based on daily calibration curves for benzene, based on dilutions of a 10 mM benzene stock solution in pure DMF. The sensor response was seen to maintain its sensitivity throughout the assay period, with a maximum variation coefficient of 3.2% ($n = 17$) (based on previously optimised membrane storage conditions when the biosensor was not in use, Lanyon et al., 2004).

3.3. DMF desorption efficiency

The efficiency of DMF as a solvent for benzene desorption from charcoal was investigated in the laboratory prior to the testing of air samples containing benzene. This involved injections of liquid benzene directly onto the primary sections of charcoal air sampling tubes, followed by the transfer of the charcoal into gas sampling vials for solvent desorption (following NIOSH air sample desorption procedures, Kennedy et al., 1995). For each experiment, 4.5 μ l of benzene was injected into the primary charcoal section, and DMF volumes between 2 and 10 ml were used to investigate desorption efficiency (benzene recovery). Each eluent was diluted in potassium phosphate buffer (between 1:250 and 1:50), prior to injection into the FIA system in order to obtain a theoretical 0.1 mM benzene concentration for each eluent (in the case of 100% desorption). Corresponding reference solutions containing 4.5 μ l of benzene over the same DMF volume range (without charcoal) were also made to represent 100% benzene recovery solutions (diluted in the same way prior to injection into the FIA system). The reference results were then compared with the results obtained from the charcoal eluents after desorption (calculated from the biosensor standard calibration curves). The results from these preliminary investigations showed a benzene recovery between 70 and 80%, over a DMF volume range between 2 and 10 ml (a 1 ml volume was not sufficient to immerse all of the charcoal), with a slightly improved recovery with increasing volume (Fig. 3) (an 80% recovery is in agreement with that obtained for benzene by Johansen and Wendelboe, 1981). However, in contrast to the increasing benzene recovery trend with the amount of DMF, a 10 ml desorption volume showed a slight decrease in benzene recovery. This could have been due to the larger vials used for the 10 ml volumes, which had a larger amount of headspace. Johansen and Wendelboe (1981) suggested that a distribution dilution of an analyte exists between

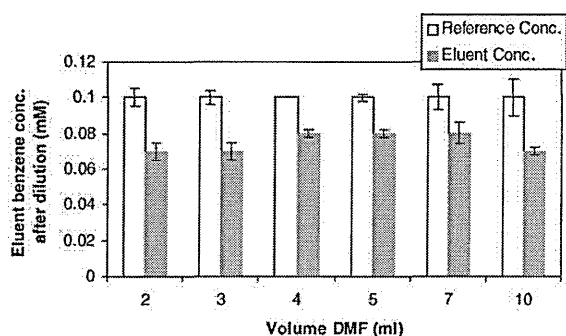


Fig. 3. The effect of increasing volume of DMF on the recovery of benzene from the charcoal sorbent. Reference concentrations based on benzene injected directly in the corresponding volume of DMF, representing solutions of 100% benzene recovery. Eluent concentrations based on the benzene obtained after charcoal desorption over the DMF volume range tested. All solutions diluted between 1:250 and 1:50 in potassium phosphate buffer prior to injection into the FIA system. Concentrations calculated using the biosensor standard calibration curves. Error bars based on triplicate desorptions.

the solvent and headspace of a vial, however if the analyte is more in favour of the headspace (such as benzene) this will result in a more negative calculation of the desorption efficiency.

These results shown in Fig. 3 were based on a total desorption time of 30 min, with 10 s of manual shaking every 5 min. Increased desorption time was seen not to improve benzene recovery beyond 80%, even after 20 h as previously tested by Johansen and Wendelboe (1981). Furthermore, the effect of vigorous agitation using a magnetic stirrer bar did not improve the recovery with respect to regular manual agitation. Moreover, the presence of the back-up section with respect to only the primary section of charcoal (a total of 600 or 400 mg of charcoal, respectively) was not seen to influence the desorption efficiency of DMF.

3.4. Interfering compounds

Typically a mixture of volatile compounds including benzene, toluene, ethylbenzene and xylene (BTEX compounds) will be present in the air of many industries or factories, depending on the type of processes undertaken there. Therefore the biosensor was tested with a mixture of BTEX compounds in DMF. Negligible sensor responses were obtained for each individual compound (toluene, ethylbenzene and xylene) and furthermore, the presence of all compounds together was seen not to affect the sensor response to benzene (results not shown).

3.5. Applicability of the biosensor for the analysis of benzene in air samples

The applicability of the biosensor for the analysis of benzene in air samples was investigated using a benzene exposure room of known concentration (analysed by gas chromatography).

Table 2

Exposure room benzene concentrations calculated from the biosensor results, compared with known exposure room concentrations analysed by gas chromatography

	Exposure room benzene concentration (ppm)	S.D.	CV (%)
Biosensor 100 l samples	9.1 (± 0.6)	0.8	9 ($n = 10$)
Known room concentration (GC)	7.6 (± 0.4)	0.4	5 ($n = 12$)
Biosensor 75 l samples	11.0 (± 0.8)	0.6	5 ($n = 8$)
Known room concentration (GC)	8.1 (± 0.4)	0.1	1 ($n = 8$)
Biosensor 50 l samples	9.8 (± 1.2)	0.9	9 ($n = 7$)
Known room concentration (GC)	8.2 (± 0.4)	0.2	2 ($n = 8$)
Mean room concentration calculated from biosensor results (all samples)	10.0 (± 0.9)	1	10 ($n = 25$)
Mean known room concentration (GC)	8.0 (± 0.4)	0.3	4 ($n = 28$)

Biosensor error based on that obtained from the standard calibration curves. Standard GC error for exposure room analysis = 5%. Biosensor S.D. based on variation in response between samples, GC S.D. based on fluctuations in control room concentration on day of sampling.

matography). Air samples were taken using charcoal adsorption tubes, which were attached to pre-calibrated air pumps (1 l min^{-1}) inside the exposure room. After the sampling period, the charcoal was transferred to a small vial and desorbed with 5 ml DMF, following the procedures used previously in the laboratory. For each sample, the theoretical benzene concentration of the eluent was calculated based on the known exposure room concentration, the total air sample volume, and the amount of DMF used for desorption. The eluents were then diluted accordingly to a concentration within the detection range of the biosensor (typically 1:50 in potassium phosphate buffer). For GC cross-analyses of the air samples, 5 μl of the eluent was injected into a gas-tight vial and allowed to evaporate for 30 min at 37°C prior to injection of the gas phase into the gas chromatograph.

Sample concentrations were calculated based on the trend line equation of the calibration curves conducted each day for the biosensor. The original concentration of the exposure room could then be calculated based on the eluent dilution, the DMF desorption volume, and the total volume of air sampled. Table 2 compares the exposure room benzene concentration calculated from the biosensor results with the known exposure room concentration during each sampling period (slight fluctuations in the exposure room concentration throughout the day were taken into account). For the air samples taken, the recovery of benzene from the charcoal after desorption was seen to be 100%, despite a lower recovery obtained for the laboratory desorption experiments as aforementioned. That is, the biosensor response was no less than that calculated for a 100% recovery for the theoretical eluent concentrations. Table 2 shows that for all air samples taken,

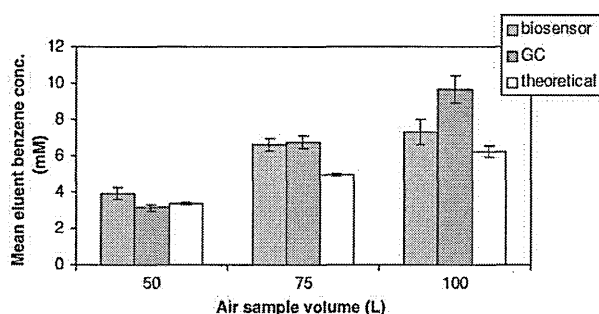


Fig. 4. The mean air sample eluent benzene concentrations calculated from the biosensor and GC results. Theoretical eluent concentrations based on known exposure room concentration (errors bars based on fluctuations), total volume of air sampled and DMF desorption volume. Biosensor and GC error bars based on 7, 8, and 10 samples for the 50, 75, and 100 L air volumes, respectively.

a higher exposure room benzene concentration was calculated from the biosensor results with respect to the known concentration analysed by the GC.

For the GC cross-analyses of the air samples taken using the charcoal tubes, similar results were obtained for both the biosensor and GC analysis of the 75 L air sample eluents, but both were higher than the calculated theoretical eluent concentration (Fig. 4). Similar concentrations were also obtained between the biosensor results and the calculated theoretical eluent concentration for the 50 L air samples, with a slightly lower concentration obtained for the GC analysis. The highest variation among the GC, biosensor, and theoretical eluent concentrations was found for the 100 L air samples.

Overall, the main difference in the results has been the higher benzene concentration obtained for the air samples taken using charcoal adsorption tubes, with respect to the known benzene concentration of the exposure room, which was analysed using air taken directly with a syringe (without requiring sample pre-concentration due to the high amount of benzene) and injected into the gas chromatograph. This difference is more evident for the 75 and 100 L air samples.

In summary, the results from these preliminary air samples have shown that the biosensor could be used as an early warning device for benzene concentrations in the workplace requiring further monitoring. The FIA system was easily transported to the institute in which these preliminary air samples were conducted, and the same bacterial membrane maintained a sensitive response over the 5 days of continual use.

4. Conclusions

An FIA-based bacterial biosensor has been developed in this work for the analysis of benzene in workplace air samples. Air sample collection and desorption based on a charcoal sorbent and dimethylformamide, respectively,

have shown to be the most compatible combination with the biosensor. A linear detection range between 0.025 and 0.15 mM benzene in a maximum concentration of 2% DMF was obtained, allowing the analysis of air containing between 3 and 16 ppm benzene, based on a 60-min sampling period. The biosensor response time was 6 min, and same bacterial membrane maintained a sensitive response over 5 days of continual use in an in situ location for air sample analyses. Furthermore, the biosensor response to benzene was not influenced by the presence of other benzene-related compounds in the same solution. The results from the air sampling section of this work have shown a correlation between the biosensor and gas chromatography results for the preliminary investigations conducted. Over all this paper has demonstrated that the *P. putida* ML2 biosensor has potential applications for the analysis of workplace air samples containing benzene with the added advantages over the conventional GC methods of low operation costs, ease of use, and portability for in situ measurements.

Acknowledgements

Many thanks to Sergio Ghittori at the FSM institute of Pavia for the use of the benzene exposure room, and for his advice on the air sampling section of this work. Sincere thanks also to Paola Zadra for all GC analyses of the air samples.

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APPENDIX 3

Poster presentation. XVIIth International Symposium of
Bioelectrochemistry and Bioenergetics, Florence, 19-24 June 2003.

The Development of an Amperometric Bacterial Biosensor for the Detection of Benzene

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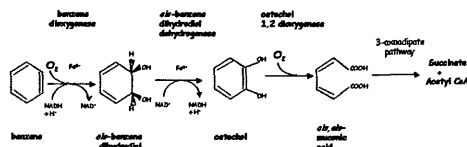
Abstract

The production and use of toxic compounds such as benzene, has caused many occupationally related deaths over the last century. An amperometric bacterial biosensor based on *Pseudomonas putida* ML2 has been developed, using a Flow Injection Analysis (FIA) system, for the detection of benzene. Whole bacterial cells are entrapped between two cellulose acetate membranes, and fixed onto a Clark-type dissolved oxygen electrode. Aerobic degradation of benzene by the bacteria causes a decrease in dissolved oxygen near the electrode surface, which results in a corresponding decrease in the sensor output current. The sensitivity, specificity, and lifetime of the bacterial membrane have also been investigated in this work.

Biosensor construction

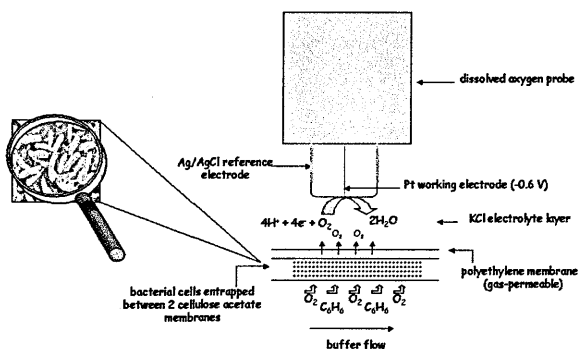
- Growth of bacterial culture in minimal medium with 0.1% (v/v) benzene (18h, 30°C, 150rpm)
- Immobilisation:
 - Centrifugation (6000rpm, 10 mins) of 1 ml of 18h bacterial culture (57×10^6 viable cells ml^{-1} , A_{660} 1.2)
 - Re-suspension of pellet in 1 ml sterile potassium phosphate buffer (50 mM, pH 7)
 - Filtration through 13mm diameter cellulose acetate membrane (0.2 micron)
 - Placement of secondary 25mm diameter cellulose acetate membrane (0.2 micron)
- Fixation of membranes onto oxygen probe with O-ring and immediate use on biosensor or storage at 4°C in a 1 mM benzene-buffer solution

Benzene catabolism by *Pseudomonas putida* ML2

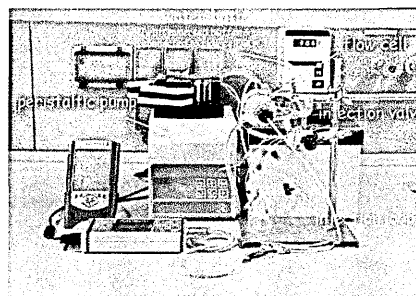


Mason, J.R. (1994). The induction and repression of benzene and catechol oxidising capacity of *Pseudomonas putida* ML2 studied in perturbed chemostat culture. *Arch. Microbiol.* 162: 57-62.

The bacterially-modified dissolved oxygen probe



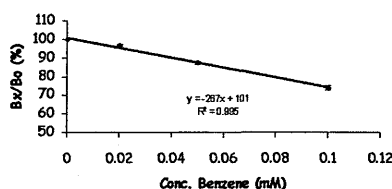
Apparatus



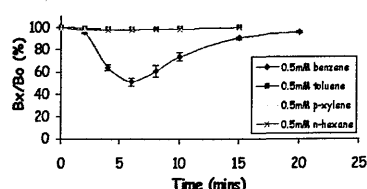
FIA measurement procedure

- Recording of stable current output (baseline representing endogenous respiration rate of bacteria) in FIA carrier stream (50 mM potassium phosphate buffer, pH 7; 0.5 ml min^{-1})
- Manual injection of benzene standard solution (1 ml injection loop)
- Recording of current decrease over time
- Peak height taken as sensor response
- Results presented as a percentage decrease from the original baseline

Calibration curve for benzene

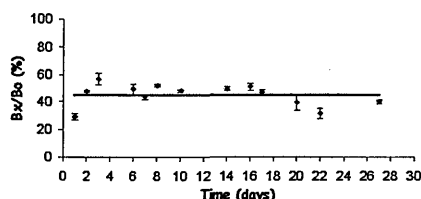


Interfering compounds



Membrane lifetime

(response over time to 0.5 mM benzene)
(storage at 4°C in 1 mM benzene-buffer when not in use)



Conclusions

- Specificity and linear detection range between 0.02 - 0.1 mM benzene
- Response and baseline recovery times of 6 minutes and 15 minutes, respectively
- Stable sensor response over several weeks
- Collection and analysis of air samples containing benzene currently being investigated

APPENDIX 4

Poster presentation. The Eighth World Congress on Biosensors, Granada,
24-26 May 2004.

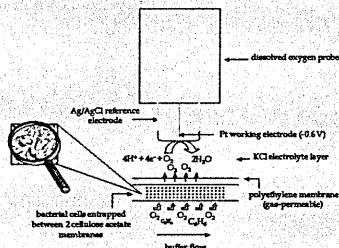
Benzene analysis in workplace air using an FIA-based bacterial biosensor

Y.H. Lanyon^{1,2}, G. Marrazza¹, S. Ghittori³, I.E. Tothill², M. Mascini¹
¹Florence University, Italy; ²Canfield University, U.K.; ³FSM Scientific Institute of Pavia, Italy.
y.h.lanyon.s01@canfield.ac.uk

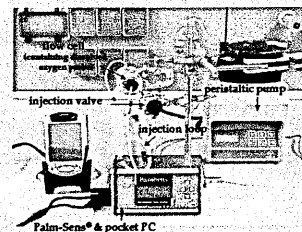
The presence of toxic volatile organic compounds (VOCs) such as benzene in workplace air has accounted for the death of many occupationally exposed workers over the last century. In this work, an amperometric bacterial biosensor based on *Pseudomonas putida* ML2 has been integrated into a flow injection analysis system for the detection of benzene. Benzene can be used by the bacteria as a sole carbon source, and its aerobic degradation can be measured using a dissolved oxygen electrode. The bacterial cells were immobilised between two cellulose acetate membranes and fixed onto a Clark dissolved oxygen probe, which was inserted into a custom-made flow cell. The applicability of the biosensor for the analysis of air samples containing benzene was investigated using a benzene exposure room of controlled concentration. Air samples were collected using charcoal adsorption tubes, and benzene extracted with solvent desorption using dimethylformamide (DMF). A correlation has been obtained between the biosensor and gas chromatography (GC) results for these preliminary air samples, and DMF has proved to be a compatible solvent for use with the biosensor, causing no interference in the sensor response or toxic effects on the bacterial cells. The results from this work have shown that the biosensor has potential for the detection of benzene in workplace air samples, with the added advantages over the conventional GC methods of low operation costs, fast and sensitive analyses, and portability for *in situ* measurements.

Biosensor Construction

- Growth of bacterial culture in minimal medium with 0.1% (v/v) benzene (18-h, 30°C, 150 rpm) (stationary phase cells)
- Centrifugation of 1 ml of 18-h bacterial culture (57×10^6 viable cells ml^{-1} , A_{600} 1.2)
- Re-suspension of bacterial pellet in 100 μl potassium phosphate buffer (50 mM, pH 7)
- Filtration through 13 mm diameter cellulose acetate membrane (0.2 micron)
- Placement of secondary 25 mm diameter cellulose acetate membrane (0.2 micron)
- Fixation of gas-permeable and bacterial membranes onto oxygen probe with O-ring and immediate use as biosensor or storage at 4°C in a 1 mM benzene-buffer solution



Apparatus



Biosensor Operation

- Recording of stable current output (baseline representing endogenous respiration rate of bacteria) in FIA carrier stream (50 mM potassium phosphate buffer, pH 7; 0.5 ml min^{-1})
- Manual injection of benzene standard solution or sample (1 ml injection loop)
- Recording of current decrease over time
- Peak height taken as sensor response
- Results presented as a percentage decrease from the original baseline, Bx/Bo (%) where Bx is the sample/standard maximum sensor output signal, and Bo is the original baseline

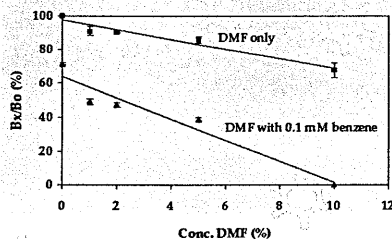
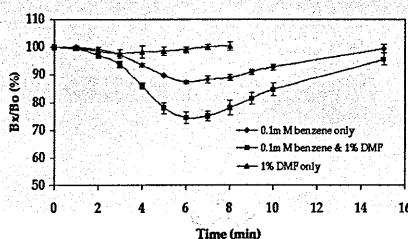
Biosensor Features

- Typical calibration curve slope statistics for a detection range between 0.025 – 0.15 mM benzene in a maximum concentration of 2% DMF: $y = -313x$ (y intercept 101), r^2 0.998, CV 3.2% (n = 17)
- Response time of 6 minutes, baseline recovery between 10–15 minutes
- Stable biosensor response to benzene using the same bacterial membrane over 8 days with continual exposure to 2% DMF (CV 3.2%, n = 17)

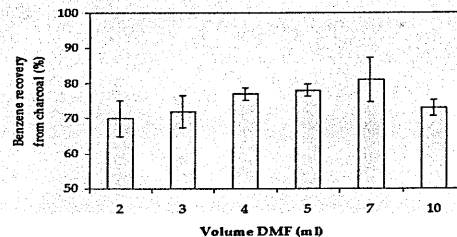
Air Sample Collection & Extraction

- Connection of charcoal air sampling tube to pre-calibrated air pump (1 L min^{-1}) for required amount of time
- Transfer of charcoal to vial for desorption procedure
- Addition of 5 ml DMF and manual shaking for 10 seconds every 5 minutes, for a total desorption period of 30 minutes
- Dilution of sample eluent in potassium phosphate buffer and injection into the FIA system
- Calculation of sample benzene concentration using pre-conducted calibration curves

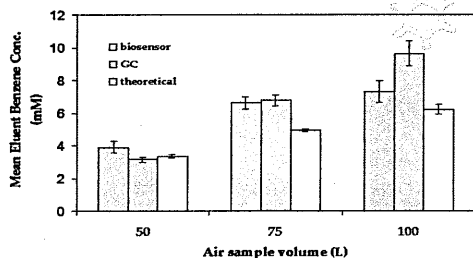
The Influence of DMF on the Biosensor Response



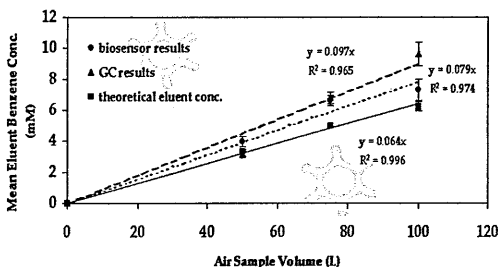
DMF Desorption Efficiency



Air Sample Results



Mean air sample eluent benzene concentrations calculated from the biosensor and GC results. Theoretical eluent concentrations based on known exposure room concentration (errors bars based on fluctuations), total volume of air sampled and DMF desorption volume. Biosensor and GC error bars based on 7, 8 and 10 samples for the 50, 75 and 100 L air volumes, respectively.



Exposure room benzene concentrations calculated from the biosensor results, compared with known exposure room concentrations analysed by gas chromatography. Biosensor error based on that obtained from the standard calibration curves. Standard GC error for exposure room concentrations = 5%.

	Exposure Room Benzene Conc. (ppm)	SD	CV (%)
Biosensor 100L sample Known room conc. (GC)	51 (±0.4) 74 (±0.4)	0.8 0.4	9.2 (n = 10) 5.0 (n = 12)
Biosensor 75L sample Known room conc. (GC)	11.0 (±0.8) 8.1 (±0.4)	0.6 0.1	5.1 (n = 9) 1.3 (n = 9)
Biosensor 50L sample Known room conc. (GC)	9.0 (±1.2) 8.2 (±0.4)	0.9 0.2	6.9 (n = 7) 2.3 (n = 9)
Mean room conc. calculated from biosensor results (all samples)	10.0 (±0.9)	1.0	10.0 (n = 29)
Mean known room conc. (GC)	8.9 (±0.4)	0.3	4.0 (n = 29)

Conclusions

- Correlation found between biosensor and GC results for air samples taken from controlled benzene exposure room
- Fast and sensitive analysis
- Low cost, easily constructed and operated
- FIA system easily transportable to *in situ* locations