

Microbial biosensor for *p*-nitrophenol using *Moraxella* sp.

Priti Mulchandani^a, Yu Lei^a, Wilfred Chen^a, Joseph Wang^b, Ashok Mulchandani^{a,*}

^a Department of Chemical and Environmental Engineering, University of California, Riverside, CA 92521, USA

^b Department of Chemistry and Biochemistry, New Mexico State University, Las Cruces, NM 88003, USA

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Abstract

A novel microbial biosensor/electrode for highly selective, sensitive and rapid quantitative determination of *p*-nitrophenol (PNP) was developed using PNP-degrading organisms immobilized in a membrane on a dissolved oxygen electrode. *Moraxella* sp. specifically oxidizes PNP while consuming oxygen. A change in oxygen concentration was determined by a Clark oxygen electrode and correlated to the PNP concentration. The sensor signal and response time were optimized with respect to the buffer pH, temperature, time of cell growth and weight of cells immobilized. The best sensitivity and response time were obtained using a sensor constructed with 0.3 mg of cells and operating in pH 7.5, 20 mM phosphate buffer. Using these conditions, the biosensor was used to measure as low as 14 ppb (0.1 μ M) of PNP extremely selectively without interference from structurally similar compounds, such as phenol, nitrophenols and chlorophenols. The biosensor had very good storage and multiple use stability when stored in the operating buffer at 4 °C.

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1. Introduction

p-Nitrophenol (PNP), because of its toxicity (LD₅₀ 247 mg kg⁻¹ oral and 920 mg kg⁻¹ through the skin [1]), is a US Environmental Protection Agency priority pollutant [2] and the European Commission (EC) has set a limit of 0.1 ppb in drinking water [3]. It is a breakdown product as well as a chemical intermediate in the production of organophosphorus pesticides (methyl parathion, parathion, fenitrothion and EPN) and analgesic paracetamol (*N*-acetyl-*p*-aminophenol) [1,3–12]. Because it is carcinogenic [13], mutagenic [14–17] and cyto- and embryotoxic to mammals [18–20], there are urgent needs for innovative an-

alytical tools/devices to facilitate its detection in case of an accidental release during these industrial activities.

Current EPA recommended analytical technique for PNP detection/monitoring is based on gas chromatography coupled with electron capture or mass spectrometry detector [21,22]. Some recently developed techniques are based on liquid chromatography [23–28] or capillary electrophoresis [29,30] in conjunction with electrochemical [24,25], diode-array [27,28] or mass spectrometry detector [23,26] and immunoassay [3,31,32]. Although extremely sensitive, the previous techniques are time-consuming, expensive, require skilled operators and are not suitable for on-line or field monitoring [3].

Biosensor technology based on enzyme or micro-organism is well suited for rapid, cost-effective, sensitive, selective, and on-line/field monitoring [33].

* Corresponding author. Tel.: +1-909-787-6419;
fax: +1-909-787-5696.
E-mail address: adani@enr.ucr.edu (A. Mulchandani).

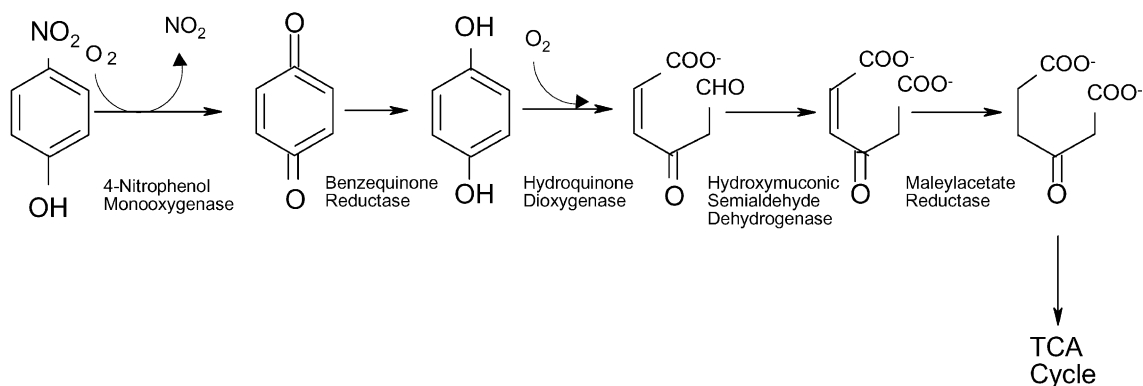


Fig. 1. Proposed pathway for PNP degradation [34].

This paper reports the development, characterization and evaluation of a simple, selective, rapid and cost-effective microbial biosensor for PNP based on the integration of a PNP degrading microorganism *Moraxella* sp. and a dissolved oxygen electrode.

Moraxella sp., isolated from activated sludge plant, degrades PNP through benzoquinone, hydroquinone, γ -hydroxymuconic semialdehyde, maleylacetate and β -keto adipate to tricarboxylic acid (TCA) intermediates while releasing nitrite and consuming oxygen (Fig. 1) [34,35]. Correlating the oxygen consumption to PNP concentration provides the basis of this very simple, sensitive, selective and rapid microbial biosensor for PNP.

2. Experimental

2.1. Materials

Yeast extract, phenol, Na_2HPO_4 , NaH_2PO_4 , K_2HPO_4 , and KH_2PO_4 were purchased from Fisher Scientific (Tustin, CA, USA). Tryptic soy broth was acquired from Becton Dickinson (Sparks, MD, USA). PNP, 3-nitrophenol and 2-nitrophenol were obtained from Aldrich (Milwaukee, WI, USA). NH_4Cl was purchased from J.T. Baker (Phillipsburg, NJ, USA). Polycarbonate membrane (Nucleopore, 50 nm pore size, 25 mm diameter disc) was obtained from Whatman (Clifton, NJ, USA). All solutions were prepared in distilled deionized water.

2.2. Microorganism and culture conditions

Moraxella sp. isolated from activated sludge was received from Dr. Jim C. Spain (US Airforce Research Laboratory, Tyndall Airforce Base, FL). *Moraxella* sp. stock culture maintained on tryptic soy broth was inoculated into tryptic soy broth and incubated overnight on a gyratory incubator shaker (Innova 4000, New Brunswick Scientific, Edison, NJ, USA) at 30°C and 300 rpm. Subsequently, these cells were inoculated ($\text{OD}_{600} = 0.1$) in pH 7.2 minimal salt medium (3.73 mM K_2HPO_4 , 1.25 mM KH_2PO_4 , 1.4 mM NH_4Cl , 0.4 mM $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ and 0.02 mM $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$) supplemented with 0.4 mM PNP and 0.2% yeast extract. The cells were incubated at 30°C and 300 rpm until the yellow color of PNP disappeared in approximately 5 h. At this time, additional PNP (0.4 mM) was added and the sequence repeated for three more times over 1.25 h. The cells were harvested using a refrigerated centrifuge (Model J21, Beckman Instruments, CA, USA) at 4°C , followed by washing with buffer (20 mM sodium phosphate pH 7.5) twice. The pellet was resuspended in appropriate volume of buffer to obtain a suspension of known cell concentration (determined using dry weight and optical density calibration plot generated for this organism). Cells were stored in refrigerator until use.

2.3. Microbial electrode assembly

A known and desired weight of cell suspension in buffer was dripped onto a 25 mm diameter $0.05 \mu\text{m}$ pore size polycarbonate membrane with slight suction.

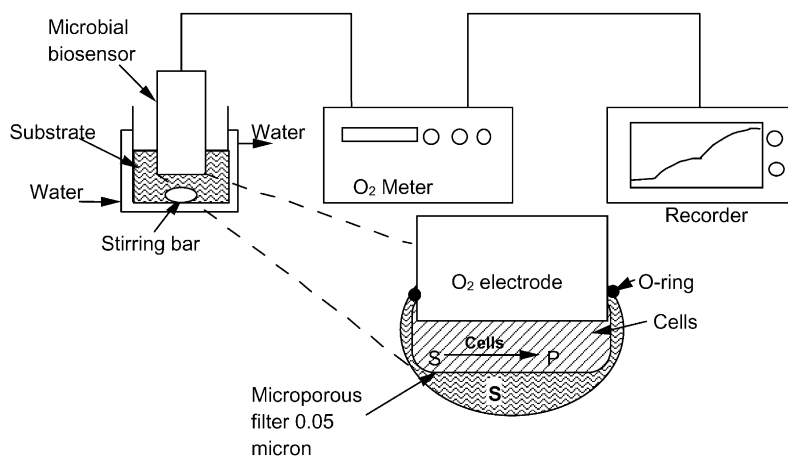


Fig. 2. Schematic of experimental set-up.

The cells retaining membrane was then attached to the surface of the Teflon membrane of the dissolved oxygen electrode (Model 5332, Yellow Springs Instrument, Yellow Springs, OH, USA) and held in-place by a rubber O-ring. The cells were thus immobilized (entrapped) between the two membranes.

2.4. Experimental set-up and measurement

The experimental set-up used in the study (Fig. 2) consisted of a 10 ml temperature controlled detection cell in which the microbial electrode described earlier (Section 2.3) was inserted through a hole in the rubber stopper, a circulating water bath to maintain the temperature, a magnetic stirrer, an oxygen monitor (Model 5300, Yellow Springs Instruments) and a chart recorder.

The measurements were made in 3 ml of pH 7.0, 20 mM sodium phosphate buffer that was saturated with oxygen by bubbling air using sparger until an equilibrium/steady state response of 100% dissolved oxygen was established. At this time, 20–30 μ l of a known concentration of PNP solution was added and the changes in dissolved oxygen were recorded.

3. Results and discussion

3.1. Optimization of operating conditions

The response of a microbial biosensor is a function of the time of adaptation of the cells on PNP, amount of

cells immobilized on the transducer, operating buffer pH and temperature. Experiments were performed to investigate the effect of these variables on the oxygen consumed to 0.05 mM of PNP.

3.1.1. Effect of time of adaptation of cells on PNP

Spain and Gibson [34] noted that the oxidation of PNP in *Moraxella* sp. is enhanced by pre-exposure to PNP, indicating inducible gene expression of the enzymes involved in PNP catabolism. However, there is no report on the relationship between the adaptation period and the degree of the activity enhancement. To investigate the effect of period of adaptation of the cells on PNP (after inoculating to minimal medium with PNP and subsequent PNP additions) on the biosensor response, electrodes were prepared using constant amount of cells harvested after different incubation periods. As shown in Table 1, oxygen consumption increased from 23% after the first adaptation period on PNP and reached a plateau around 27.5% after three more PNP additions to the cell culture. Additionally, the time required for the cells to consume most of the added PNP (indicated by the disappearance of the yellow color attributed to PNP) shortened from 5 to 0.6 h (Table 1) with virtually no cell growth on PNP. Cells generated after four repeated additions of 0.4 mM PNP were used for biosensor construction.

3.1.2. Effect of cell loading

The effect of biomass loading on the surface of the oxygen electrode is shown in Fig. 3. As expected

Table 1
Effect of *Moraxella* sp. adaptation time on PNP

Adaptation time (h)	Biosensor response ^a (% O ₂ consumed)	Cell density ^a (A ₆₀₀)
0	0	0.11
5	23 ± 1.2 ^b	0.57 ± 0.02 ^b
5.7	26.5 ± 1.4	0.58 ± 0.03
6.3	27.5 ± 2	0.61 ± 0.03

^a Data are average of three measurements.

^b Mean ± S.D.

the response initially increased with cell loading and then after reaching a maximum decreased. This trend, which is similar to that reported for other microbial biosensors [36–38], is attributed to the initial increase of the catalytic activity with increasing cell amount followed by increased mass transfer resistance due to increased thickness of the biomass layer. A cell loading of 0.3 mg dry weight was used in the subsequent work.

3.1.3. Effect of pH

The catalytic activities of the enzymes involved in PNP catabolism are a function of the pH. As shown in Fig. 4, the microbial biosensor response was maximal between pH 7–7.5. This is in good agreement with the

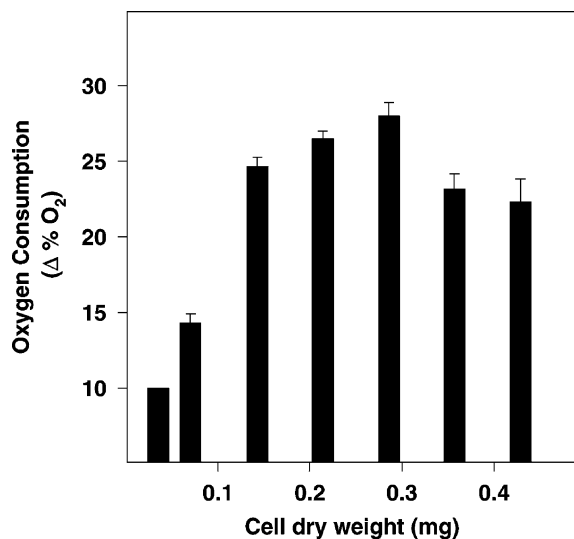


Fig. 3. Effect of cell loading on biosensor response to 0.05 mM PNP in 20 mM pH 7.5 phosphate buffer at 20 °C. Each point represents an average of three measurements and the error bars represent ±1 S.D.

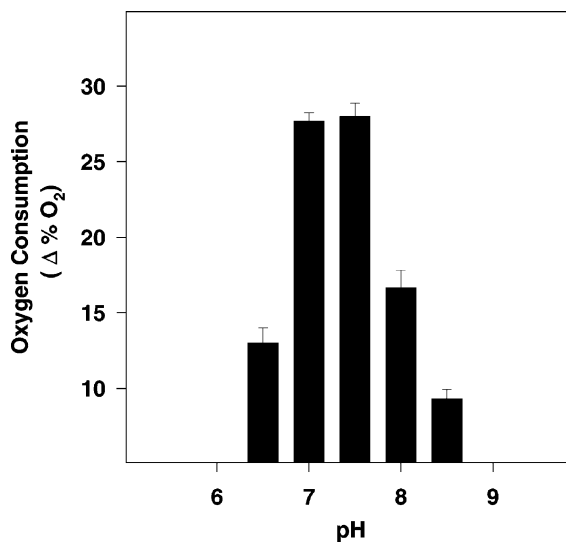


Fig. 4. Effect of pH (20 mM phosphate) on biosensor response to 0.05 mM PNP with 0.3 mg cell loading at 20 °C. Each point represents an average of three measurements and the error bars represent ±1 S.D.

pH optimum of 7.5–8, reported by Spain and Gibson [34] for maximum activity of nitrophenol oxygenase, the first enzyme involved in PNP oxidation pathway of *Moraxella* sp. The pH of 7.5 was used for subsequent studies.

3.1.4. Effect of temperature

Fig. 5 shows the effect of temperature on the response of the microbial biosensor. As shown, response increased with temperature up to 37 °C and then decreased with further temperature increase. The initial increase in the rate is attributed to the increase of both the enzyme reaction and mass transport rates. The decrease in the rate at higher temperatures is due to enzyme denaturation. Although 37 °C was determined to be the optimum temperature for the biosensor operation, subsequent experiments were still performed at room temperature, 20 °C. This was done in order to overcome the problem of evaporative losses during the course of the experiment and ease of operations. Additionally, although not determined experimentally, because the solubility of oxygen is an inverse function of the temperature, operating at higher temperature will reduce the linear and dynamic ranges of the biosensor.

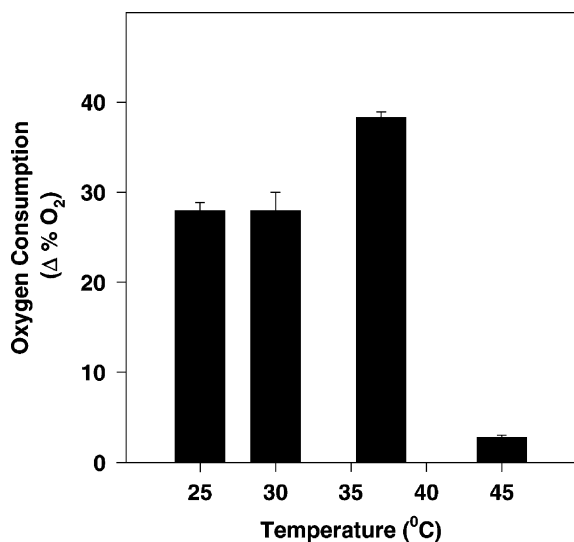


Fig. 5. Effect of temperature on biosensor response to 0.05 mM PNP in 20 mM pH 7.5 phosphate buffer with 0.3 mg cell loading. Each point represents an average of three measurements and the error bars represent ± 1 S.D.

3.2. Analytical characteristics

3.2.1. Calibration

Fig. 6 shows the calibration plot for PNP using the microbial biosensor (the plot was prepared from the sensor steady-state response data). The plot is linear up to 0.05 mM (6.95 ppm) with a sensitivity (slope) of 0.54% oxygen consumed per μM PNP.

The lower detection limit (defined as three times the standard deviation of the response obtained for a blank) of the present microbial electrode for PNP is 0.1 μM (14 ppb). This is one–two orders of magnitude higher than that the EC regulation of 0.1 ppb [3] and LOD for immunoassays [3,31]. This will therefore limit the applicability of the present sensor for environmental monitoring. For any such application of the present biosensor, off-line sample preparation involving extraction and sample concentration will be necessary. The present microbial electrode, however, will be ideal for selective on-line monitoring wastewater generated during production and consumption of the

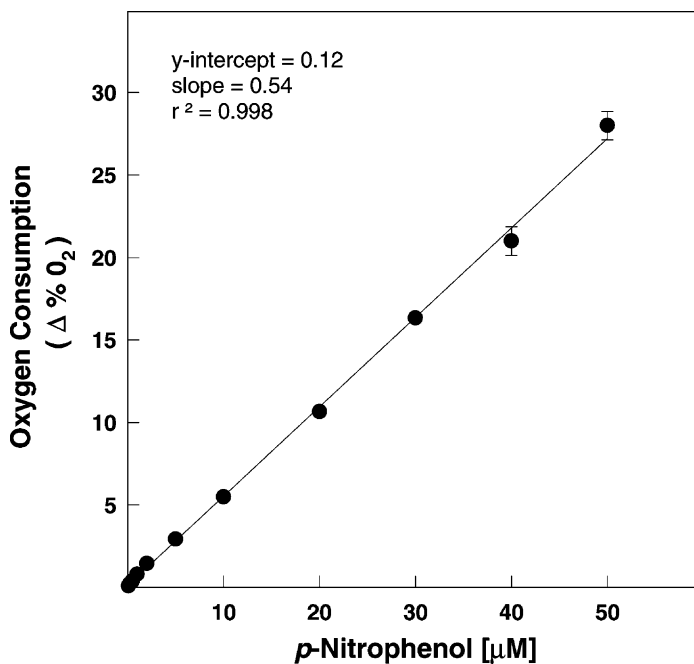


Fig. 6. Calibration plot for PNP microbial biosensor: operating conditions, 20 mM pH 7.5 phosphate buffer at 20 °C with 0.3 mg cell loading. Each point represents an average of three measurements and the error bars represent ± 1 S.D.

PNP and chemical or biological methods for treatment of PNP contaminated wastewaters. Additional advantages of the present microbial biosensor when compared to other techniques are the low cost (does not require expensive antibodies, trained personnel and instrumentation) and short assay time.

3.2.2. Selectivity

The microbial biosensor selectivity was evaluated against a range of compounds (Table 2). As shown in Table 2, even the molecularly similar compounds such as phenol, 2-nitrophenol, 3-nitrophenol and 4-chlorophenol, did not interfere. This high degree of selectivity is a significant advantage of other methods of determination of phenolic compounds such as, amperometry [39].

Non-specific cellular responses to substrate(s) and intermediates of microbial catabolism generally limit the selectivity of microbial biosensors. As illustrated in Table 2 sugars such as glucose, sucrose, fructose and galactose and salts of acetic and citric acids at 100-fold higher concentrations do not interfere with the OPH-specific response of the cells. There was, however, a very small degree of interference from suc-

Table 2
Microbial biosensor selectivity

Compound	Concentration (mM)	Biosensor response (% O ₂ consumed)
Sucrose	5	0
Galactose	5	0
Fructose	5	0
Phenol	0.05	0
2-Nitrophenol	0.05	1
3-Nitrophenol	0.05	1
4-Chlorophenol	0.05	0
Sodium acetate	5	0
Sodium citrate	5	0
Sodium succinate	0.05	1.33
PNP	0.05	28

cinic acid, an intermediate in the TCA cycle. Although the biosensor selectivity for this compound was evaluated, it is expected that the biosensor will perform adequately since this compound is not commonly present in samples of interest.

3.2.3. Response time and stability

The detection of PNP with the new microbial biosensor is simple, direct, single step and rapid. The

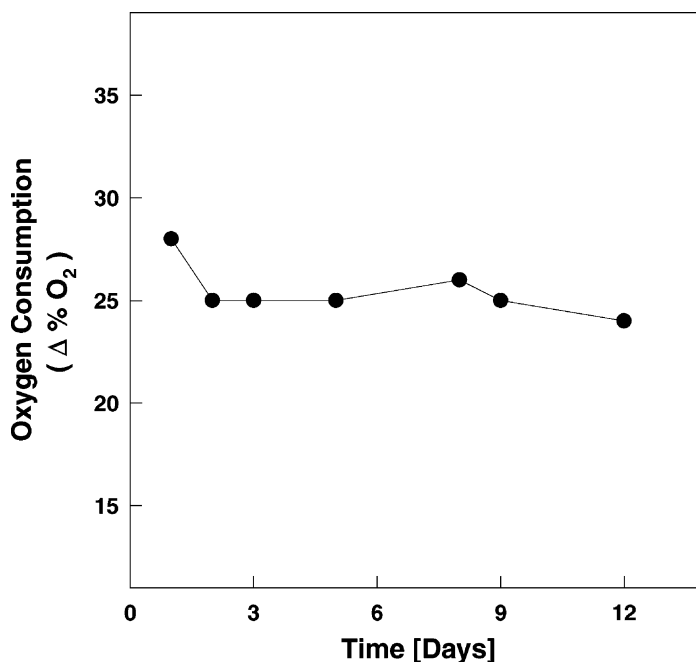


Fig. 7. Stability of microbial biosensor: response to 0.05 mM PNP in 20 mM pH 7.5 phosphate buffer at 20°C with 0.3 mg cell loading.

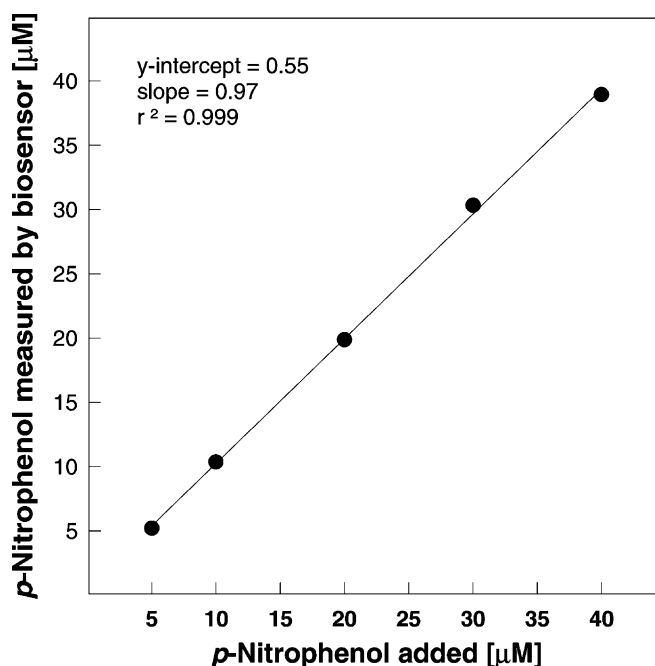


Fig. 8. Accuracy of microbial biosensor: operating conditions, 20 mM pH 7.5 phosphate buffer at 20 °C with 0.3 mg cell loading.

analysis time for each sample was approximately 3 min, which is significantly shorter than the hours required for immunoassay.

For a sensor to be suitable for on-line process monitoring application, it should have excellent multiple use and long-term storage stability and short response time. The long-term storage and the multiple use stability of the microbial electrode was investigated by evaluating the response of the same electrode to PNP and storing it back at 4 °C 20 mM phosphate pH 7.5 buffer. As the results show (Fig. 7), the microbial electrode was very stable, retaining over 90% of its original response, for the 2 weeks period during which it was used a total of 20 times.

3.2.4. Precision and accuracy

The low relative standard deviation of 4.77% ($n = 8$) for 0.05 mM PNP demonstrates the high precision of analysis. Additionally, a very low relative standard deviation of 4.64% ($n = 5$) in the response of five microbial biosensors prepared at different times from cells grown in different lots, demonstrate an excellent reproducibility from electrode-to-electrode.

Simulated samples representing PNP contaminated water were analyzed using the microbial biosensor and compared to the concentration determined by measuring absorbance at 405 nm spectrophotometrically. The good agreement (Fig. 8) between the two methods indicate the sensor is very accurate and reliable.

4. Conclusions

In conclusion, a *Moraxella* sp. modified oxygen electrode for the direct, rapid and selective measurement of PNP was developed. The sensor had excellent stability, precision, accuracy, short response time and selectivity for PNP over other structurally similar compounds. The method is very simple to use and is low cost, does not require expensive antibodies, trained personnel and expensive instrumentation.

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