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Highly Sensitive and Selective Amperometric Microbial Biosensor for Direct Determination of *p*-Nitrophenyl-Substituted Organophosphate Nerve Agents

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We report herein a whole cell-based amperometric biosensor for highly selective, highly sensitive, direct, single-step, rapid, and cost-effective determination of organophosphate pesticides with a *p*-nitrophenyl substituent. The biosensor was comprised of a *p*-nitrophenol degrader, *Pseudomonas putida* JS444, genetically engineered to express organophosphorus hydrolase (OPH) on the cell surface immobilized on the carbon paste electrode. Surface-expressed OPH catalyzed hydrolysis of the *p*-nitrophenyl substituent organophosphorus pesticides such as paraoxon, parathion, and methyl parathion to release *p*-nitrophenol, which was subsequently degraded by the enzymatic machinery of *P. putida* JS444. The electrooxidization current of the intermediates was measured and correlated to the concentration of organophosphates. The best sensitivity and response time were obtained using a sensor constructed with 0.086 mg dry weight of cells operating at 600 mV applied potential (vs Ag/AgCl reference) in 50 mM citrate−phosphate pH 7.5 buffer with 50 μM $CoCl_2$ at room temperature. Under optimum operating conditions the biosensor measured as low as 0.28 ppb of paraoxon, 0.26 ppb of methyl parathion, and 0.29 ppb parathion. These detection limits are comparable to cholinesterase inhibition-based biosensors. Unlike the inhibition-based format, this biosensor manifests a selective response to organophosphate pesticides with a *p*-nitrophenyl substituent only, has a simplified single-step protocol with short response time, and can be used for repetitive/multiple and on-line analysis. The service life of the microbial amperometric biosensor was 5 days when stored in the operating buffer at 4 °C. The new biosensor offers great promise for rapid environmental monitoring of OP pesticides with nitrophenyl substituent.

Introduction

The extreme toxicity, broad-spectrum activity, and low cost of organophosphorus compounds (OPs) have made them popular as pesticides, insecticides, and chemical warfare agents (*1*−*3*). Organophosphates with a *p*-nitrophenyl substituent, such as methyl parathion and parathion, are extensively used as agriculture pesticides in the United States and around the world. While use of pesticides is very important for higher agriculture yields, release of these hazardous chemicals into our environment poses an increasing ecological and health risk. Consequently, there is considerable interest in highly sensitive, selective, rapid, reliable, field-deployable, and cost-effective analytical tools/devices for detection of organophosphates. Analytical techniques of chromatography, immunoassay, and enzyme biosensors based on inhibition of cholinesterase (ChE) activity are the most commonly used methods. Although sensitive, these methods are time consuming, expensive, and suitable only for single use and require multiple washing steps, making them unsuitable for on-site/field monitoring (*4*−*7*).

Biosensors based on organophosphorus hydrolase (OPH), an enzyme that has been shown to hydrolyze a wide range of organophosphorus pesticides (*8*−*10*), have been reported (*1*, *11*−*13*). Since OPH utilizes OPs as substrates rather than inhibitors, as is in the case of cholinesterase, these biosensors are direct, have simple single-step protocol, and are reversible as compared to the inhibition-based format. Consequently, OPH-based biosensors demonstrate considerable potential for applications requiring repetitive/multiple and on-line analysis. As an alternative to purified enzyme, whole cells have also been applied as biological transducer. Several examples of microbial-based biosensors, including OPs, have been reported and reviewed (*13*−*19*). While these biosensors provided simple, rapid, and direct monitoring of OP compounds, either the detection limit or selectivity limited their applications.

Several PNP-degrading microorganisms have been isolated from PNP-contaminated waste sites (*20*−*22*). These organisms mineralize PNP while releasing nitrite and consuming oxygen. One example is *Pseudomonas putida* JS444, which mineralizes PNP through benzoquinone, hydroquinone, and hydroxymuconic semialdehyde to tricarboxylic acid cycle intermediates while releasing nitrite and consuming oxygen (*20*). Recently, we genetically engineered this microorganism to endow it with organophosphorus hydrolase activity, enabling the simultaneous degradation of OPs such as paraoxon, parathion, methyl parathion, fenitrothion, ethyl *p*-nitrophenol thiobenzenephosphonate (EPN), and PNP (or 3-methyl-4-nitrophenol), the product of OPH-catalyzed hydrolysis of these OPs. Combining this microorganism with a Clark-type dissolve oxygen electrode we constructed a selective, rapid, low-cost, simple, and portable microbial biosensor for monitoring PNP-substituted OPs (*23*). While suitable for on-line monitoring of the detoxification/remediation process for aqueous wastes generated by the producers and consumers, the detection limit was not adequate for environmental monitoring. In this paper we report the construction, optimization, characterization, and evaluation of an amperometric microbial biosensor based on the genetically engineered *P. putida*, harboring both OP hydrolyzing and PNP degradation capabilities, as the biological sensing element integrated to a carbon paste amperometric transducer for a simple yet selective, sensitive, rapid, and cost-effective microbial biosensor suitable for on-line determination of OPs with PNP substituent. The new

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amperometric microbial biosensor was 3 orders of magnitude more sensitive than the oxygen electrode-modified microbial biosensor using the same bacterium and comparable to cholinesterases inhibition-based biosensors. This high sensitivity along with high selectivity, repetitive/multiple and on-line analysis, simple single-step protocol, rapid response, and low cost make this biosensor ideal for environmental applications.

Materials and Methods

Reagents. $FeCl_3$, $ZnSO_4 \cdot 7H_2O$, and $Na_2MoO_4 \cdot 2H_2O$ were obtained from VWR (San Diego, CA). Citric acid, lactic acid, glucose, sucrose, fructose, galactose, glycerol, sodium acetate, $MgSO_4 \cdot 7H_2O$, KH_2PO_4, $Na_2HPO_4 \cdot 7H_2O$, yeast extract, graphite powder, mineral oil, and $CaCl_2$ were purchased from Fisher Scientific (Tustin, CA). PNP, 2-nitrophenol, 3-nitrophenol, phenol, *p*-chlorophenol, 3-methyl-4-nitrophenol, 2,4-dinitrophenol, and paraoxon were bought from Sigma-Aldrich (Milwaukee, WI). Coumaphos, diazinon, sevin, sutan, atrazine, parathion, and methyl parathion were acquired from Supelco Inc. (Bellefonte, PA). LB broth was purchased from Becton Dickinson (Sparks, MD). NH_4Cl and $CoCl_2$ were acquired from J. T. Baker (Phillipsburg, NJ). All solutions were prepared in distilled deionized water.

Bacterial Strains and Growth Conditions. The details of the recombinant PNP-degrader *P. putida* JS444 expressing OPH on the cell surface and growth conditions used in this study have been described elsewhere (*24*). In brief, *P. putida* JS444 carrying pPNCO33 was inoculated into LB medium with 50 μg/mL kanamycin and incubated overnight on a gyratory incubator shaker at 30 °C and 300 rpm. Subsequently, cells were inoculated ($OD_{600} = 0.1$) in minimal salts medium supplemented with 1 mM IPTG, 0.2 mM PNP, 0.1% yeast extract, and 50 μg/mL kanamycin and incubated at 30 °C and 300 rpm until the yellow color of PNP disappeared. At this time additional PNP (0.2 mM) was added and the sequence repeated for three more times. The cells were harvested using a refrigerated centrifuge (Beckman Instruments, CA) at 4 °C, followed by washing with buffer (50 mM pH 7.5 citrate−phosphate buffer with 50 μM $CoCl_2$) twice. The pellet was resuspended in the same buffer and stored in a refrigerator overnight until use.

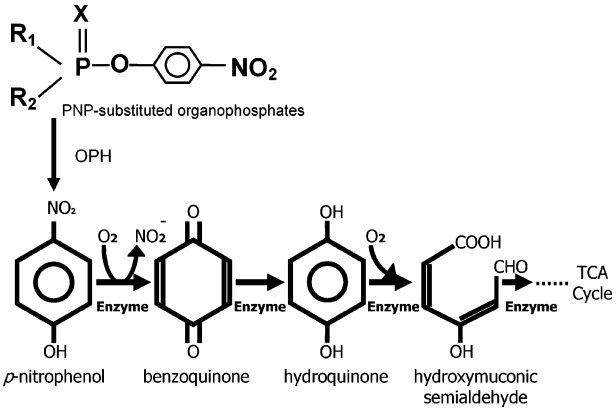
Assembly of Microbial Biosensor. The carbon paste was prepared by mixing 75% (w/w) graphite powder and 25% (w/w) mineral oil. The paste was subsequently packed firmly into the electrode cavity (3 mm diameter and 1 mm deep) of a Kel-F sleeve (Bioanalytical System Inc., Lafayette, IN) and polished to a smooth shiny finish by gently rubbing over a weighing paper to make the carbon paste electrode (CPE).

A predetermined amount of the cell suspension, based on the desired cell loading, was slowly dropped on a 25 mm diameter, 0.4 μm pore size Nucleopore polycarbonate membrane (Whatman, NJ) with slight suction. The cell-retaining membrane was then placed on the top of the carbon paste electrode and held in place by a rubber O-ring. The electrode was kept at 4 °C till use.

Electrochemical Cell Setup and Measurement. Amperometric measurements were performed using a Bioanalytical Systems (BAS) voltammetric analyzer (model LC-4C) coupled to a chart recorder (model BD112, Kipp and Zonen, Holland). All experiments were conducted using a three-electrode electrochemical cell (10 mL volume with a working volume of 4 mL) inside a Faraday cage (BAS, model C2 cell stand) with a Ag/AgCl reference electrode (BAS, MF 2063) and a platinum wire auxiliary electrode (BAS, MF 1032).

All measurements, unless stated otherwise, were performed by applying a potential of +0.6 V (vs the Ag/AgCl reference) to the working electrode and allowing the transient background current to decay to a steady-state value prior to addition of 10−20 μL of OP dissolved in pure methanol. A stirred solution was employed to provide convective transport.

SCHEME 1. Proposed Pathway for Degradation of PNP-Substituted OP Nerve Agents[a]



[a] R_1 and R_2 can be ethoxy or methoxy, and X can be O or S.

Results and Discussion

Optimization of Microbial Biosensor Operating Conditions. *P. putida* JS444 isolated from PNP-contaminated soils degrades PNP using a series of enzymes. On endowing with OPH activity, the new bacterium was able to degrade PNP-substituted organophosphate nerve agents such as paraoxon, parathion, methyl parathion, EPN, and fenitrothion and the OPH-catalyzed hydrolysis product of these OPs, PNP (or 3-methyl-4-nitrophenol), simultaneously (*24*). The engineered bacterium degraded these organophosphates through a series of intermediates that include electroactive compounds benzoquinone and hydroquinone (Scheme 1). While the former can be electrochemically reduced, the later can be oxidized, generating electroreduction and electrooxidation currents, respectively. To establish the optimum operating potential for the biosensor, a hydrodynamic voltammetry study was performed for the genetically engineered *P. putida* JS444-modified CPE (Figure 1A) using 1 μM paraoxon. As shown, the oxidation current for paraoxon increased sharply up to +0.6 V and leveled off thereafter. An applied potential of +0.6 V (vs Ag/AgCl reference electrode) corresponding to the maximum oxidation current was used in subsequent work. The lower applied potential of the electrode and the excellent response to 1 μM paraoxon make this amperometric biosensor more selective (due to reduced potential for oxidation of interferents) and sensitive than the earlier OPH-based amperometric biosensors operating at +0.85 V (*11*).

The amount of cells immobilized on the transducer surface plays an important role in the microbial biosensor response. As shown in Figure 1B, the biosensor response initially increased and then decreased with higher loading of cells at the transducer surface. The observed trend was in accordance with several reported amperometric microbial biosensors (*11*). The initial increase is attributed to an increased catalytic activity of enzyme(s) responsible for biocatalysis, while the subsequent decrease at higher cell loading is due to the transport resistance of OP compounds and oxygen to the cells embedded deeper in the immobilized layer. A cell loading of 0.086 mg dry weight corresponding to the maximum current output was used in subsequent work.

Figure 1C shows the pH profile for the microbial biosensor response. As shown, the biosensor response was maximal at pH 7.5. The optimum activities of the enzymes involved in the biological transduction, OPH and *p*-nitrophenol oxygenase (the first enzyme involved in PNP oxidation of *P. putida* JS 444), were achieved at pH 8.5 (*11*−*13*) and 7.5−8 (*22*),

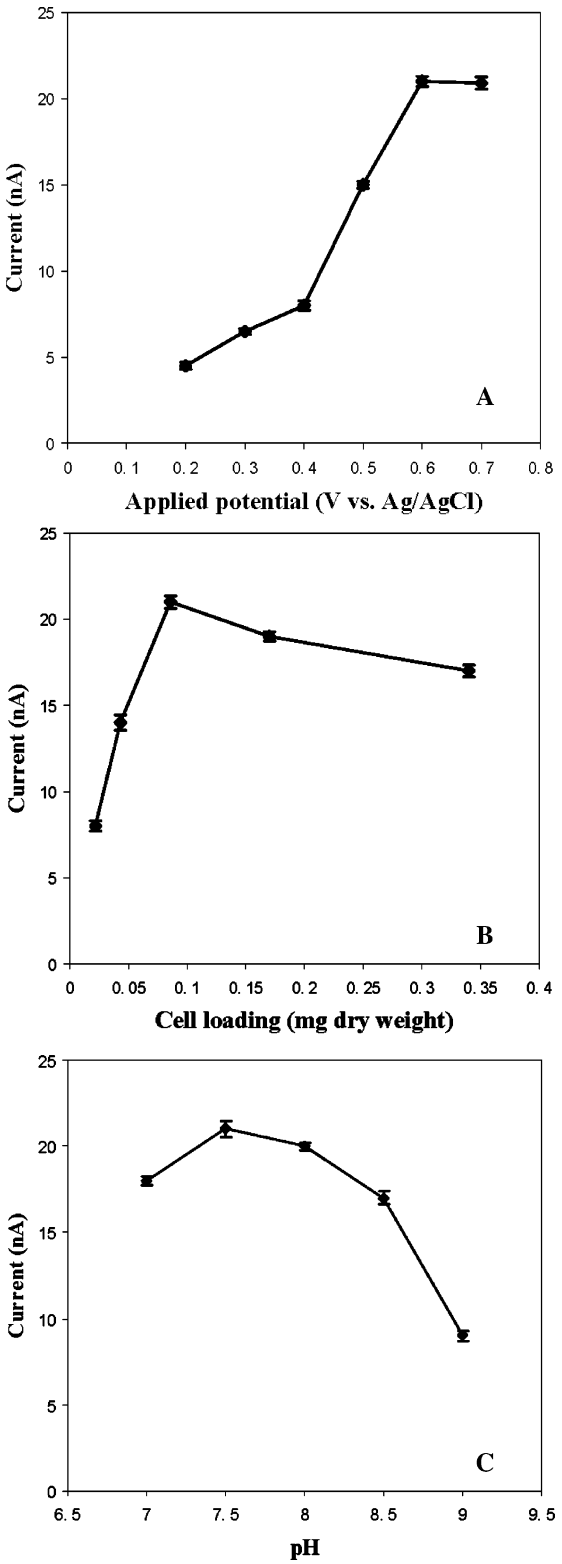


FIGURE 1. Optimization of biosensor performance parameters. (A) Hydrodynamic voltammogram of engineered *Pseudomonas putida* JS 444-modified carbon paste electrode for 1 μM paraoxon in 50 mM pH 7.5 citrate−phosphate buffer with 50 μM $CoCl_2$ at room temperature. (B) Effect of cell loading on biosensor response to 1 μM paraoxon in 50 mM pH 7.5 citrate−phosphate buffer with 50 μM $CoCl_2$ at room temperature. Operating potential: +0.6 V vs the Ag/AgCl reference. (C) Effect of pH (50 mM citrate−phosphate with 50 μM $CoCl_2$) on biosensor response to 1 μM paraoxon with 0.086 mg cell loading at room temperature. Operating potential: +0.6 V vs Ag/AgCl reference. Data are given as mean ± SD for three measurements.

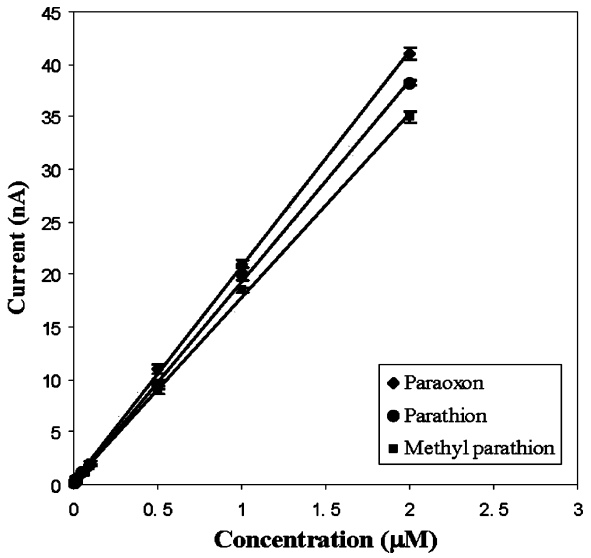


FIGURE 2. Calibration plots for paraoxon, parathion, and methyl parathion. Operating conditions: 50 mM pH 7.5 citrate−phosphate buffer with 50 μM $CoCl_2$ at room temperature with 0.086 mg cell loading, applying +0.6 V to working electrode vs the Ag/AgCl reference. Data are given as mean ± SD for three measurements.

respectively. The optimum pH at 7.5 suggested that PNP oxidation enzyme is the rate-limiting step in the series reactions of OPH-catalyzed hydrolysis of paraoxon and the subsequent oxidation of the resulting PNP (*24*). The pH of 7.5 was used for subsequent studies.

Analytical Characteristics. By operating the microbial biosensor at the optimized conditions determined above, the dependence of biosensor response to different concentrations of paraoxon, methyl parathion, and parathion is shown in Figure 2. The plots were linear up to 2 μM with sensitivities (slope) of 20.50 nA per μM ($R^2 = 0.9995$), 19.13 nA per μM ($R^2 = 0.9995$), and 17.57 nA per μM ($R^2 = 0.9994$) for paraoxon, parathion, and methyl parathion, respectively. The limit of detection (LOD), determined as 3 times the standard deviation of the signal for buffer (blank), was 0.28 ppb (1 nM) of paraoxon, 0.26 ppb (1 nM) of methyl parathion, and 0.29 ppb (1 nM) parathion. These values are almost 200-fold lower than the microbial biosensor based on dissolved oxygen consumption using the same *P. putida* engineered cells (*23*) and 20−100 fold lower than the OPH-based amperometric biosensor based on measurement of PNP electrooxidation (*11*−*13*). More importantly, these detection limits are comparable to or better than the values for cholinesterases (ChE) inhibition-based biosensors (0.01−100 ppb) (*4*, *5*). This should make the biosensor an ideal analytical tool for sensitive detection of organophosphates in the environment without any sample preconcentration.

The biosensor selectivity was evaluated against a range of compounds. As shown in Table 1, there was no interference from other commonly used pesticides, belonging to three different groups, such as triazine (atrazine and simazine), carbamates (sutan and sevin), and OP without the *p*-nitrophenol substituent (coumaphos and diazinon). This is a benefit over ChE-based biosensors, which are unable to differentiate between organophosphates from other neurotoxic compounds (*4*) and the potentiometric OPH-based biosensors that are unable to differentiate between subclasses of OPs (*11*, *25*). Additionally, the low applied potential (0.6 V vs Ag/AgCl) enhances the sensor selectivity against other phenolic compounds as compared to earlier OPH-based amperometric biosensors that operate at 0.85 V (vs Ag/AgCl) (*11*). The biosensor also responded to 3-methyl-4-nitrophenol, a hydrolysis product of fenitrothion, thus extending the

TABLE 1. Amperometric Microbial Biosensor Selectivity

concentration (μM)	compound	current (nA)
1	paraoxon	21
1	coumaphos	0
1	diazinon	0
1	sutan	0
1	sevin	0
1	atrazine	0
1	simazine	0
1	2-nitrophenol	0
1	3-nitrophenol	0
1	4-nitrophenol	21
1	phenol	2.5
1	*p*-chlorophenol	3
1	3-methyl-4-nitrophenol	20.5
1	2,4-dinitrophenol	5
1000	glucose	0
1000	sucrose	0
1000	fructose	0
1000	galactose	0
1000	glycerol	0
1000	sodium acetate	0
1000	lactic acid	0

applicability of the present microbial amperometric biosensor to this OP.

Because nonspecific cellular responses to substrate(s) and intermediates of microbial catabolism can limit the selectivity of microbial-based biosensors, it was necessary to evaluate interference from sugars and organic acids. As shown in Table 1, no interference from sugars such as glucose, sucrose, lactic acid, and sodium acetate was observed.

The response of the amperometric microbial biosensor was reproducible as demonstrated by the low relative standard deviations ($n = 4$) of 4.40%, 4.24%, and 4.52% to 1 μM paraoxon, methyl parathion, and parathion, respectively, for the same electrode. Additionally, there was excellent electrode-to-electrode reproducibility as characterized by the low relative standard deviations of 4.55%, 5.78%, and 4.22% in the response of three microbial biosensors prepared at different times using different batches of cells to 1 μM paraoxon, methyl parathion, and parathion, respectively.

To evaluate the biosensor accuracy, paraoxon concentration determined by the biosensor was compared to an independent spectrophotometric assay based on the measurement of PNP (at 412 nm) formed by the OPH-catalyzed hydrolysis. As shown in Figure 3, there was excellent agreement between the two methods (slope = 0.96 and $r^2 =$ 0.9996), indicating excellent accuracy and reliability of the biosensor.

Unlike ChE-based biosensors, the protocol for the new amperometric biosensor is simple, direct, and single step. The analysis is rapid, requiring less than 5 min, and the same sensor can be used up to 20 times without loss of response, demonstrating the suitability for on-line/real-time monitoring. In contrast, the ChE inhibition-based biosensors are indirect, require multiple steps, and are significantly slower, analysis time ranges anywhere from 15 min for a disposable type (where enzyme regeneration step is excluded and hence unsuitable for on-line/real-time monitoring purposes) to 5 h for a reusable type (*26*, *27*).

The long-term storage stability of the biosensor was investigated by evaluating the response of the same sensor to 0.1 μM parathion when stored at 4 °C in pH 7.5, 50 mM citrate−phosphate buffer with 50 μM $CoCl_2$. The sensor response was stable for a period of 5 days and then dropped sharply (data not shown). The precipitous drop in the activity is hypothesized to be a result of the depletion of the NAD(P)H in the resting/nongrowing cells (*28*), which is required for *p*-nitrophenol degradation (*20*, *24*). Addition of NAD(P)H to the storage buffer, however, could not reactivate the biosensor response, probably because the whole cell could not intake NAD(P)H from outside.

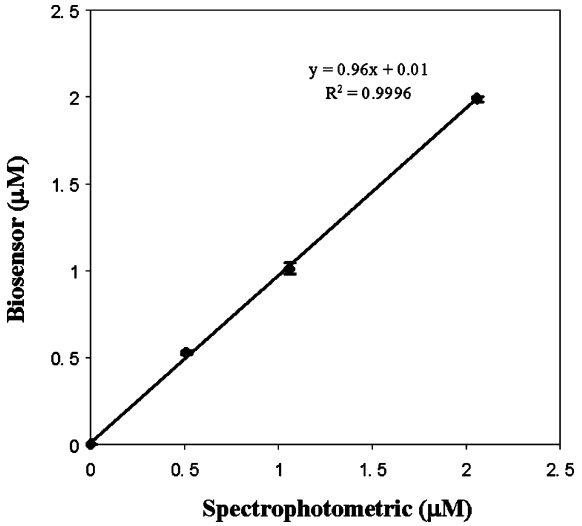


FIGURE 3. Accuracy of amperometric microbial biosensor for paraoxon. Operating conditions: 50 mM pH 7.5 citrate−phosphate buffer with 50 μM $CoCl_2$ at room temperature with 0.086 mg cell loading, applying +0.6 V to working electrode vs the Ag/AgCl reference. Data are given as mean ± SD for three measurements.

TABLE 2. Evaluation of Matrix Effect in Lake Water from Lake Elsinore, CA

paraoxon concentration (μM)	biosensor response in operating buffer (nA)	biosensor response in lake water (nA)
0.5	11	9.5
1	21	20
2	41	42

Testing with Contaminated Lake Water Samples. Biosensor selectivity was also evaluated against naturally occurring compounds in real sample. The microbial biosensor was applied to measure paraoxon spiked in lake water from Lake Elsinore, CA, after the lake water was filtered through a 0.22 μm membrane and the pH was adjusted from 9.2 to 7.5. As shown in Table 2, the biosensor response was similar to that observed in the synthetic samples, demonstrating there was no interference from phosphorus, nitrogen, chlorophyll, and several metal ions present in the water of Lake Elsinore, CA (*29*), and the applicability of the microbial biosensor for organophosphate pesticide-contaminated environmental samples.

In summary, an amperometric microbial biosensor combining genetically engineered *P. putida* JS444 with a carbon paste electrode amperometric transducer with a sensitivity and limit of detection comparable to cholinesterase inhibition-based biosensors was developed. The high sensitivity combined with excellent selectivity, direct determination, simple and single-step protocol, rapid response repetitive/multiple use (necessary for on-line/remote monitoring), and low cost are the many advantages of the newly developed sensor over the inhibition assay format. The endowment of OPH activity and its genetic immobilization on the cell surface of a PNP degrader *P. putida* (1) eliminated the need for purified OPH, (2) eliminated the need for immobilizing a second biological transducer and an additional immobilization chemistry, and (3) alleviated the diffusional transport resistance of the cell membrane. While the applicability of the biosensor has been illustrated for paraoxon, methyl parathion, and parathion, it will also be valid for other PNP-substituted OP pesticides, fenitrothion and EPN. Additionally,

combination of the present biosensor with the OPH-based potentiometric biosensor reported earlier will enhance the information content of a mixed analyte sample (*30*).

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