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Organophosphorus Hydrolase-Based Assay for Organophosphate Pesticides

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We report a rapid and versatile organophosphorus hydrolase (OPH)-based method for measurement of organophosphates. This assay is based on a substrate-dependent change in pH at the local vicinity of the enzyme. The pH change is monitored using fluorescein isothiocyanate (FITC), which is covalently immobilized to the enzyme. This method employs the use of poly(methyl methacrylate) beads to which the FITC-labeled enzyme is adsorbed. Analytes were then measured using a microbead fluorescence analyzer. The dynamic concentration range for the assay extends from 25 to 400 μM for paraoxon with a detection limit of 8 μM. Organophosphorus insecticides measured using this technique included ethylparathion, methylparathion, dursban, fensulfothion, crotoxyphos, diazinon, mevinphos, dichlorvos, and coumaphos. This technique was used to measure coumaphos in biodegradation samples of cattle dip wastes and showed a high correlation ($r^2 = 0.998$) to an HPLC method.

Introduction

Organophosphorus (OP) compounds make up the largest class of insecticides currently used in industrial countries (*1*). These pesticides are widely used to control insects in a variety of diverse applications. For example, OP compounds are routinely applied to crops (*2*), such as cotton, rice, potato, corn, and wheat, turfgrass (*3*), and livestock (*4*) (i.e., used extensively in cattle dipping operations). Although these compounds are degraded under many environmental conditions, reports in the literature have expressed concern over exposure to nontarget organisms such as birds (*2*) and fish (*5*), as well as the potential for human exposure from sources such as fresh fruits and vegetables (*6*) and processed foods (*7*).

Laboratory-based methods which are commonly used for detection and measurement of OP pesticide residues include gas chromatography (GC), high-performance liquid chromatography (HPLC), and capillary electrophoresis (*8*). Although these methods can measure a wide variety of OP insecticides in a number of environmental matrices, they are typically expensive and time-consuming and are not well-suited to field applications or process control monitoring. In addition, laboratory-based methods which often require turnaround times of several weeks are not amenable to remediation and bioremediation process monitoring where rapid analysis times are essential.

The need for rapid and cost-effective field analytical methods for monitoring compounds such as OP pesticides has resulted in the development of many techniques, a number of which are bioanalytical in nature (*9*). These bioanalytical methods primarily include assays based on enzyme inhibition (*10*) and immunoassay (*11*). Enzyme-linked immunosorbant assays (ELISA) are quite sensitive to specific compounds such as ethylparathion or fenitrothion but, like most immunoassays, require multiple incubations and generate contaminated plates, tubes, etc. In addition, the characteristics of cholinesterase-based assays and immunoassays for OP pesticides are not well-suited to process control monitoring applications which require both inexpensive and rapid analyses.

Organophosphorus hydrolase (OPH) catalyzes the hydrolysis of a wide range of OP pesticides (*12*). OPH-based assays respond to OP compounds as enzyme substrates rather than inhibitors or antigens. Consequently, these assays can be reversible and require only the analyte of interest. As a result of these characteristics, OPH-based assays show considerable potential for development for process control applications which require rapid and repetitive analysis of a limited number of target compounds.

The optical assays typically used to measure OPH-catalyzed OP hydrolysis detect the increase in absorbance of the phenol leaving group (e.g., *p*-nitrophenol for paraoxon). The strategy we employed for the use of OPH-catalyzed hydrolysis to measure OP pesticides is based on a common product for a number of these compounds (i.e., H^+). The primary advantage for the use of the fluorescence assay format over the absorbance-based methods is that any OP substrate can be measured using the same detection mechanism. More specifically, each of the leaving groups from hydrolyzed OP pesticides have their own characteristic absorbance maximum and extinction coefficient, whereas, using the herein reported fluorescence-based assay, the fluorescein-reported pH can be used for any OP substrate. The measurement of immobilized OPH using the release of protons (i.e., by means of a pH electrode) has been recently reported (*13*) and is not unique to the herein reported method; however, the use of FITC-labeled OPH for detection of OP pesticides has not been previously reported. Presented in this report is the characterization of a versatile

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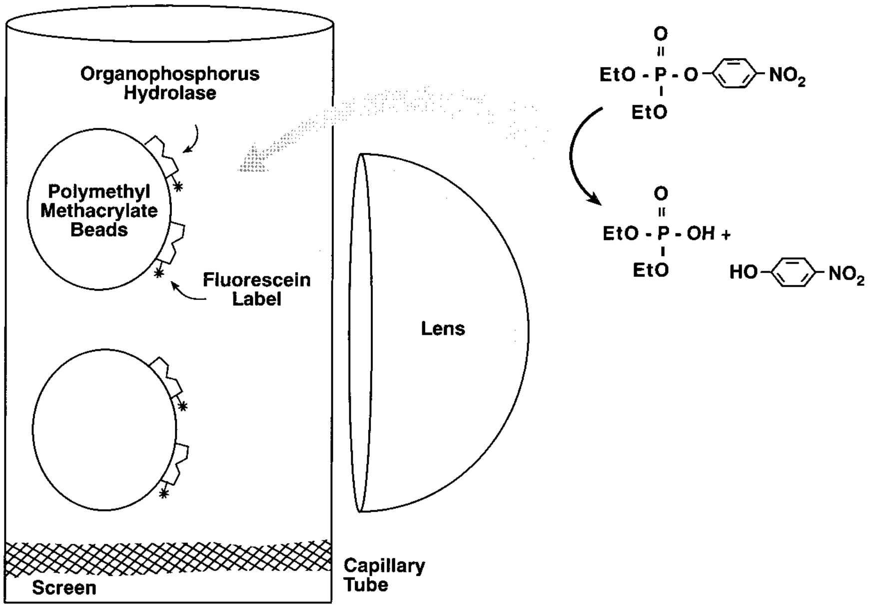

Figure 1. Biochemical schematic for the OPH assay run in the KinExA fluorescence analyzer.

fluorescence-based assay using the pH dependence of fluorescein isothiocyanate (FITC). This assay is also applied to the measurement of coumaphos in bioremediation samples of cattle dip wastes.

Experimental Section

Chemicals. Fluorescein isothiocyanate, *N*-(2-hydroxyethyl)piperazine-*N'*-(2-ethanesulfonic-*N*-[2-morholino]-ethanesulfonic acid) (HEPES), (2-morpholino)ethanesulfonic acid (MES), 2-[*N*-cyclohexylamino]ethanesulfonic acid (CHES), and *p*-nitrohenol were obtained from Sigma Chemical Co. (St. Louis, MO). Paraoxon, ethylparathion, methylparathion, dichlorvos, diazinon, fensulfothion, crotoxyphos, mevinphos, and coumaphos were obtained from Chem Service (West Chester, PA). Poly(methyl methacrylate) (PMMA) beads (100 μm diameter) were purchased from Bang's Laboratories (Carmel, IN). Dialysis membrane was purchased from Spectrum Medical Industries, Inc. (Bellefonte, PA). Chromatography packing materials, Sephadex G-150 and DEAE-50, were purchased from Pharmacia Biotech (Uppsala, Sweden). All other compounds and solvents used were of reagent grade. Coumaphos-containing cattle dip samples taken from various stages of the biodegradation cycle were a gift from Dr. W. Mulbry, Soil Microbial Systems Laboratory, U.S. Department of Agriculture, Beltsville, MD.

Instrumentation and Method. The KinExA fluorescence analyzer was purchased from Sapidyne Inc. (Boise, ID). Its features and basic operation have been previously described (*14*). For the OPH-based assay, FITC-labeled enzyme-coated beads were trapped on a nylon screen in a capillary which provided the optical flow cell in the filter-type fluorimeter. Because the quantum yield of FITC is pH-dependent, the catalytic activity of the enzyme (which releases protons during substrate hydrolysis) could be continuously monitored via the fluorescence of the FITC label (Figure 1).

The instrument software monitored the fluorescence once every second and displayed the data on the PC monitor. The fluorescence data were stored as voltage values in Excel, and intensities at specific times were used as an indicator of the local pH of the fluorescein label (*15*). The instrument flow system was operated under negative pressure using a syringe pump. The buffer flow was controlled during the assay cycle through the instrument hardware interface and PC-based software using a Windows environment. The assay procedure consisted of three parts: (i) noncovalent immobilization of FITC-labeled enzyme to the microbeads, (ii) sample handling and data collection, and (iii) data analysis. For preparation of the enzyme-coated beads, PMMA beads (200 mg) were incubated with 110 units (0.57 mg/mL) of OPH for 3 h at room temperature. The beads were washed 3 times with assay buffer by centrifugation and stored in 1 mL of assay buffer (100 μM each HEPES/MES; 100 mM NaCl, pH 8.0) at 4 °C until use. Prior to use, the enzyme-coated beads were diluted into the same buffer (1 mL of bead suspension + 19 mL of buffer). For experiments using different initial pHs or buffer concentrations, the beads were diluted into the appropriate assay buffer (see Results). For sample analysis, stock solutions of the OP substrates were prepared in methanol and diluted into assay buffer. Final methanol concentrations were 1% unless otherwise specified. Five samples along with the diluted beads were placed in the instrument reservoirs, and the sampling cycle was initiated using the specified assay buffer as the flow medium. Prior to the next run, the beads were ejected to waste and the fluidics system was rinsed.

For data handling, the fluorescence signal was determined using the following relationship:

$$FS = [1 - (S_C/S_B)]$$

where FS is the reported fluorescence signal, S_C is the 5 s signal average just prior to the continuation of the buffer flow (see Figure 2B), and S_B is the 5 s signal average just prior to the interruption of the buffer flow (see Figure 2C).

Purification of the OPH. The OPH, expressed in a recombinant strain of *E. coli* was purified as previously described (*13*). The purified enzyme showed a specific activity of 7250 U/mg.

OPH Absorbance Assay. Enzyme activity was determined by measuring the appearance of product which was released from paraoxon using the increase in absorbance at 400 nm for *p*-nitrophenol ($\epsilon_{400} = 17\,000$ M^{-1} cm^{-1}) (*12*). The initial concentration of paraoxon was 1

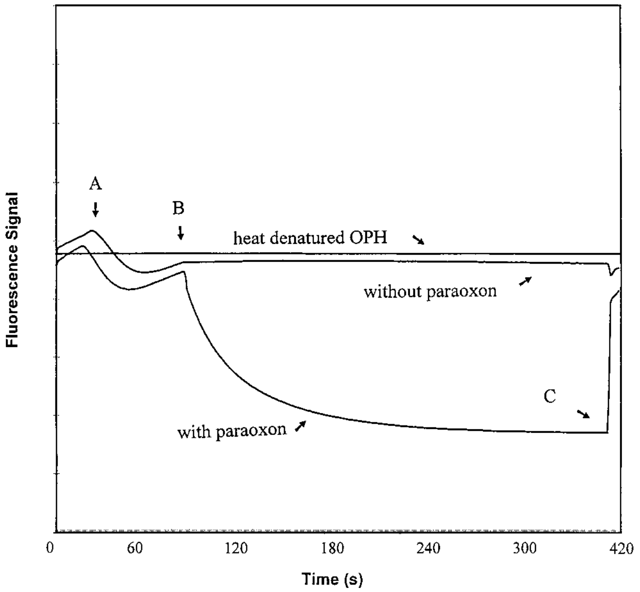

Figure 2. Representative tracings for FITC-labeled enzyme or heat-denatured FITC-labeled enzyme in the presence or absence of 100 μM paraoxon. Signal response upon (A) addition of substrate, (B) interruption of buffer flow through the bead pack, and (C) reinitiation of buffer flow. OPH was denatured by heating to 80 °C for 1 h. The flow buffer was HEPES/MES, 0.1 mM (each), pH 8.0; NaCl, 100 mM.

mM in 50 mM CHES buffer, pH 9.0. Absorbance was measured using a Varian model DMS 300 spectrophotometer.

Preparation of the FITC-Labeled Enzyme. FITC dissolved in dimethylformamide (DMF) (1 mg/mL) was added dropwise to the enzyme in PBS (pH 7.4) to a final mole ratio of 1:5 (OPH:FITC). The reaction mixture was incubated for 1.5 h at room temperature, then dialyzed against 1 L of phosphate buffer (100 mM, pH 7.4). The buffer was changed four times over a 12 h period. To determine the coupling ratio, the protein concentration was measured using the bicincroninic acid assay (Pierce) and the FITC concentration was determined by absorbance (extinction coefficient at 492 nm ϵ = 79 000 M^{-1} cm^{-1} was from (*16*)).

Determination of K_m Values for OPH and the FITC-Labeled OPH. For the determination of K_m values, the reaction mixtures were prepared containing paraoxon at concentrations ranging from 10 to 400 μM in 50 mM CHES buffer, pH 9.0. In each assay, 1.0 mL of reaction mixture was added to a disposable cuvette and then 10 μL of OPH solution containing either 1.7 or 3.4 U/mL was added, the sample mixed, and absorbance rate of change recorded. Complete analyses were generated for each enzyme concentration. Absorbance measurements were recorded every 0.1 min for 2.0 min. Each analysis was generated using 10 initial paraoxon concentrations. The data were transformed and analyzed using the Lineweaver–Burk reciprocal plot: $1/v$ vs 1/[S].

Extraction and Analysis of Coumaphos-Containing Biofilter-Treated Cattle Dip Vat Samples. Seven biofilter-treated cattle dip vat samples ranging in cuomaphos concentration from 0 to 1000 ppm were obtained from Dr. Mulbry, USDA. A chloroform extraction procedure was used for the OPH microbead assay in which 5 mL of sample suspension was combined with 5 mL of chloroform. Samples were mixed for 30 min by slow inversion followed by centrifugation (3000*g*, 5 min). One milliliter of the chloroform layer was removed, evaporated under a stream of N_2, and resuspended in assay buffer containing 20% methanol. Extraction efficiency was determined using a coumaphos standard in water.

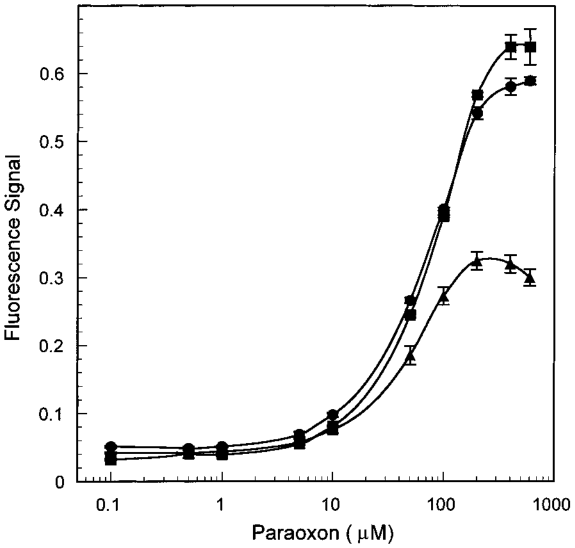

Figure 3. Affect of the initial pH on the OPH microbead assay response. Initial pH values were (■) 8.0, (●) 7.0, and (▲) 6.0. Assay buffer was HEPES/MES, 0.1 mM (each), and conditions (other than initial pH) were as described in Figure 2. Error bars represent SEM (n = 3).

A methanol extraction procedure was used for HPLC analysis in which 1 mL of the cattle dip vat suspension was added to 4 mL of methanol and mixed by vortex for 1 min, followed by centrifugation (3000*g*, 5 min). The samples were diluted (as required) into methanol:water (80:20) prior to HPLC analysis. HPLC was performed using a C-18 reverse phase column (5 μm particle size) with a mobile phase of acetonitrile:water (30:70) and a flow rate of 1 mL/min.

Results and Discussion

The observed molar coupling ratio of the FITC with OPH varied slightly from batch to batch with an average of 0.7 mol of FITC/mol of OPH. Although the enzyme contains nine lysine residues and a terminal amine (*12*), the protein was only labeled with an average of slightly less than one fluorescein per enzyme molecule. The labeling process resulted in about 50% loss of enzyme activity but had little effect on the apparent K_m value measured for paraoxon (i.e., K_m of unlabeled OPH, 60 μM; K_m of FITC-labeled OPH, 53 μM).

Microbead Fluorescence Assay. Upon introduction of the control buffer or buffered analyte to the bead pack (A), the fluorescence signal slightly decreased, in either the absence or presence of substrate (Figure 2). Interruption of the buffer flow (B) showed a decrease in the fluorescence, indicating a paraoxon-dependent drop in the pH within the local environment of the FITC-labeled OPH. This rapid drop in fluorescence was not observed in the absence of substrate or in the presence of substrate with heat-denatured enzyme. When the buffer flow was resumed (C), the fluorescence signals for all but the denatured enzyme increased to baseline levels. The fluorescence response was determined from the signal just prior to B and just prior to C.

Affect of Initial pH. Assay response curves for paraoxon using the microbead assay were generated using the HEPES/MES buffer system adjusted to initial pH values of 6.0, 7.0, or 8.0 (Figure 3). The fluorescence response curves were similar to each other with the exception of the curve for initial pH 6.0. In this case, the

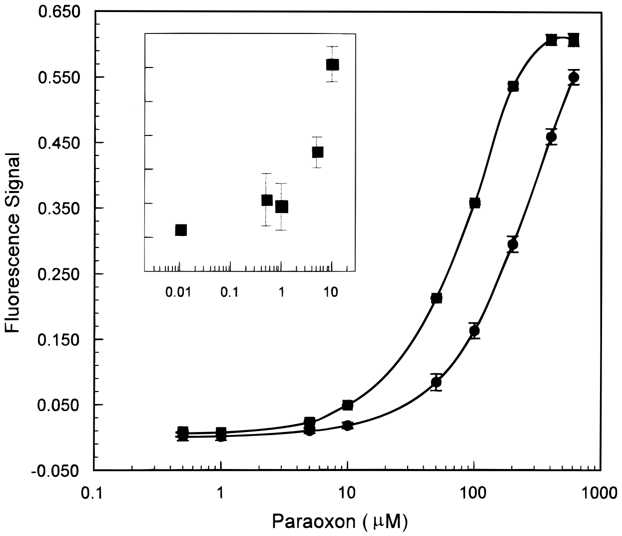

Figure 4. Calibration curves for paraoxon as a function of buffer concentration: (■) 0.1 mM, (●) 1 mM each HEPES/MES, initial pH 8.0. Inset shows low concentrations of paraoxon for the 0.1 mM buffer curve. Conditions (other than buffer concentration) were as specified in Figure 3. Error bars represent SEM ($n = 3$).

fluorescence signal was considerably lower for paraoxon concentrations above 50 μM. Several factors most likely contributed to this effect. The fluorescence quenching of FITC becomes nonlinear (i.e., the slope of the fluorescence vs pH becomes significantly lower) below 7.0 (*15*). The effect observed here, however, is also reported for an OPH-based enzyme electrode which shows a lower response to paraoxon at low pH values (*13*). This result suggests that other factors such as the lower activity of OPH at pH values below 6.5 (*17*) may also contribute to this effect.

Assay Optimization. The fluorescence-based assay was sensitive to the buffer concentration (Figure 4). The differences in response curves for two buffer concentrations (0.1 or 1 mM) were most pronounced between 40 and 400 μM paraoxon. The working range for the assay for both buffer concentrations was 25−400 μM for paraoxon. The detection limit determined using 3 times the SD was 8 μM for both buffer concentrations (Figure 4, inset, shows the lowest paraoxon concentrations for the calibration in 0.1 mM buffer). The working range and detection limit for the assay were consistent with the enzyme's K_m value for this substrate (*12*) and were also similar to those reported for an enzyme-electrode biosensor using this enzyme (*13*).

The relative fluorescence signal increased by about a factor of 10 for the nearly 100-fold dynamic concentration range of the assay. The standard error of the mean values, however, were relatively small and in many cases are obscured by the symbols (Figure 4).

Assay Response to Various OP Insecticides. Calibration curves for a number of OP pesticides were measured using this assay. Detection limits ranged from 8 μM for coumaphos and paraoxon to 50 μM for crotoxyphos (Table 1). Due to the limited solubility of many of these compounds in aqueous systems, 10 or 20% methanol was included in the assay buffer for some of these assays. These concentrations of methanol had little effect on the paraoxon calibration plot. Variability and reproducibility parameters of this assay were analyzed for paraoxon at a midrange concentration of 50 μM using the 0.1 M HEPES/MES buffer system. The assay variability represented as the coefficient of variation (CV) for $n = 10$ (run on the same day) was 2.9%. The reproducibility for the assay run on different days within a 3 month period showed a CV for $n = 3$ of 6%.

Table 1. Detection of OP Pesticides Using the OPH Microbead Assay

compound	detection limit (μM)	compound	detection limit (μM)
paraoxon	8	mevinphos	15
ethylparathion	12[a]	dichlorvos	25
methylparathion	20[a]	fensulfothion	40[a]
dursban	10[a]	crotoxyphos	50[a]
diazinon	15[a]	coumaphos	8[b]

[a] Buffer containing 10% methanol. [b] Buffer containing 20% methanol.

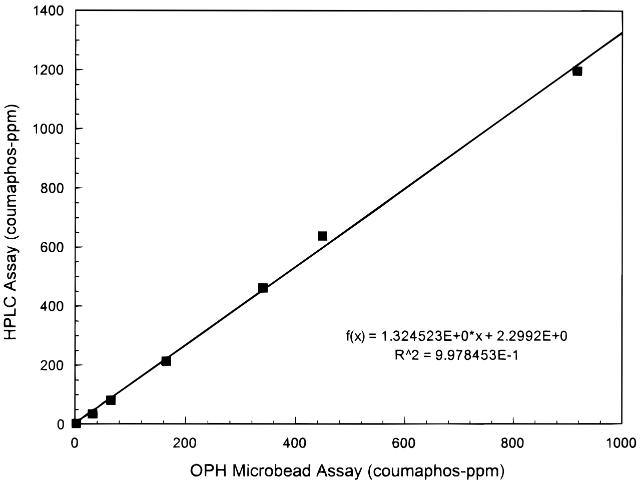

Figure 5. Comparison of OPH microbead assay to an HPLC method for analysis of coumaphos in biofilter-treated cattle dip waste samples. Extraction and analysis conditions are described in the Experimental Section. Slope = 1.32. Correlation coefficient $r^2 = 0.998$.

Measurement of Coumaphos in Biofilter Samples of Cattle Dip Wastes. The detection limits for this assay for the reported OP pesticides are higher than is typically required for many environmental screening applications. For example, immunoassay methods such as ELISA (*11*) and cholinesterase assays (*10*) show detection limits for paraoxon in the parts per billion range. There are a variety of circumstances, however, where the monitoring of relatively high concentrations of organophosphorus pesticides is required. For example, contaminated aqueous wastes from cattle dipping operations routinely reach concentrations of 2000 mg/L (*18*). The herein reported OPH microbead fluorescence assay was used to measure coumaphos from cattle dip vat wastes which had been subjected to treatment using a biofilter colonized by native bacteria from coumaphos-contaminated soils (*19*). Cycling the OP-contaminated waste through the biofilter-equipped agricultural storage tank resulted in a substantial decrease in coumaphos concentration over a 14 day period. The samples taken from various times during the process were analyzed by HPLC after methanol extraction or analyzed by the OPH-based microbead method following a chloroform extraction method. Data obtained with the enzyme-based method correlated well with those from the HPLC method (Figure 5). An 80% extraction efficiency (relative to the methanol extraction method used for HPLC) was calculated for the chloroform extraction method used for the OPH assay using coumaphos standard spiked solutions. Least-squares analysis yielded an r^2 value of 0.998 and a slope of 1.32. The negative bias for the OPH assay was most likely a reflection of the lower efficiency for the chloroform extraction (as compared to the methanol procedure used for HPLC analysis). The chloroform extraction was adopted for the OPH assay to reduce the transfer of buffering capacity from the biofilter sample media.

Conclusions

This report characterizes a rapid and versatile OPH-based assay for the measurement of OP insecticides. Coupling of this assay with the microbead fluorescence analyzer technology allows semiautomated sample handling and data analysis. This assay is relatively simple and versatile and requires only the addition of analyte in buffered solution. In addition, the assay can be used for measurement of OP insecticides extracted from bioremediation samples and can tolerate up to 20% methanol with no adverse effects.

The ability of this technique to detect but not differentiate among a number of insecticides may limit its use as a diagnostic tool. For bioremediation applications involving a mixture of OP pesticides, however, the complement of the various compounds would be known. In these cases, the total OPs might be interpreted and reported as paraoxon equivalents (i.e., the assay response for paraoxon). Response curves for individual compounds could be used to approximate the relative contributions from each compound. Due to its versatility and potential for automation, the basis of this assay may be particularly useful in the development of bioanalytical methods for bioremediation process control applications.

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