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Substrate Recycling Scheme for Tetrachloro-*p*-benzoquinone Using Bilirubin Oxidase and NADH: Application for Pentachlorophenol Assay

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A novel assay for tetrachloro-*p*-benzoquinone (TCBQ), the main oxidation product of pentachlorophenol (PCP), was developed using bilirubin oxidase (BOX) in the presence of excess NADH. TCBQ was easily and rapidly reduced by NADH to 1,4-tetrachlorohydroquinone (TCHQ), which was then recycled back to TCBQ by the enzyme. BOX exhibited no reactivity toward NADH while its catalytic activity for the oxidation of TCHQ was very high. Under an optimized condition (250 μM NADH, 0.3 U/mL BOX, and 25 mM sodium phosphate at pH 5.5), the rate of NADH consumption determined by measuring the absorbance decrease at 340 nm yielded a detection limit for TCBQ of 110 nM. Fluorescence detection of the NADH using a lower enzyme concentration (0.1 U/mL) with excitation and emission wavelengths of 345 and 450 nm, respectively, allowed for a TCBQ detection limit of 30 nM. PCP was oxidized to TCBQ with high yield using bis(trifluoroacetoxy)iodobenzene in 0.05 M trichloroacetic acid. Coupling this oxidation reaction to the BOX/NADH assay attained PCP detection limits of 170 and 50 nM using absorbance and fluorescence measurements, respectively. When tested on PCP-contaminated soil samples, the BOX assay compared very well with HPLC measurements.

Introduction

Chlorophenols constitute a major group of pollutants having been widely used as wood preservatives, pesticides, and herbicides (*1, 2*). They are also formed as byproducts of many industrial activities including chlorination of potable water and paper bleaching. In addition to their extreme toxicity, several chlorophenols are highly carcinogenic and considered priority pollutants by the U.S. Environmental Protection Agency (EPA). Pentachlorophenol (PCP), the most acutely toxic of all chlorophenol congeners, is a widespread contaminant of groundwater and surface water worldwide. Therefore, there is great interest in developing simple, fast, and accurate methods for measuring PCP in various environmental samples for the investigation of animal and human exposure.

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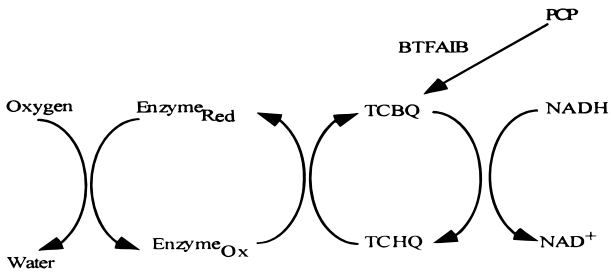
SCHEME 1



The use of enzymes in colorimetric assays or biosensor construction is one popular approach to achieve fast and accurate measurement, which could be useful for field screening during site characterization and remediation activities. There are several examples of biosensors and enzymatic procedures for phenolic compounds using a variety of enzymes such as tyrosinase (*3*−*13*), horseradish peroxidase (*14*−*16*), chloroperoxidase, and to a lesser extent, laccase (*17*). The tyrosinase or laccase-based sensors are only capable of measuring phenol, catechol, 4-chlorophenol, 2-amino-4-chlorophenol, and 2,4-dichlorophenol (*3*−*6, 13*). In addition, the detection limits of such enzyme-based sensors for the chlorophenols are not satisfactory (ca. 200 nM). 4-Chlorophenol is also the only chlorophenol detectable by the combined horseradish peroxidase and laccase sensors. Similarly, the substrate recycling assay using tyrosinase and NADH reported by Brown et al. (*13*) was only useful for phenol, catechol, 4-chlorophenol, and *p*-cresol. Although a combined chloroperoxidase/glucose oxidase-based sensor is able to detect several chlorophenol congeners, the system is not applicable for analysis of PCP and some other highly chlorinated phenols such as 3,4,5-trichlorophenol and 2,3,5,6-tetrachlorophenol (*18, 19*). In brief, depending upon the position and number of chlorines on the aromatic ring, various oxidation compounds can be generated in such a reaction: quinones, coupling products, or polymers. The oxidation product of PCP or these two chlorophenols was reported to undergo polymerization (*18, 19*), i.e., it was neither electroactive nor mediated glucose/glucose oxidase reaction. According to our knowledge, an enzymatic assay for PCP has not yet been developed.

This study describes a substrate recycling assay using bilirubin oxidase together with NADH for the determination of tetrachloro-*p*-benzoquinone (TCBQ), an oxidation product of PCP and 2,3,5,6-tetrachlorophenol (Scheme 1). It was reasoned that TCBQ could be reduced to its tetrachlorohydroquinone form (TCHQ) by NADH since quinones have been reported to oxidize NADH to produce NAD^+ in our laboratory (*13*). This TCHQ species was then reoxidized by bilirubin oxidase, a copper-containing enzyme, that has been shown to be capable of oxidizing several small organic compounds in the presence of oxygen (*20, 21*). As a result, a cycle for the substrate was established where one TCBQ molecule can consume many NADH molecules.

The substrate recycling assay could be quantitated by measuring the consumption of NADH (μM_{NADH}/min) versus the amount of TCBQ present in the sample using absorbance or fluorescence, the latter allowing for greater sensitivity. The resulting slopes of such calibration plots (μM_{NADH}/μM_{TCBQ}·min) serve as the referenced values for determining the concentration of TCBQ in unknown samples. The assay

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protocol was then demonstrated for analysis of PCP in contaminated soil by combining this substrate recycling scheme with a chemical oxidation technique using bis-(trifluoroacetoxy)iodobenzene (*22, 23*). It should be noted that this high-yield reaction was recently reported for the oxidation of various chlorinated phenols including PCP under mild conditions.

Experimental Section

Materials. Bilirubin oxidase (EC 1.3.3.5 from *Myrothecium verrucaria*, lyophilized powder: 15−65 U/mg of protein), laccase (EC 1.10.3.2 from *Rhus vernificera*, crude powder: 50 U/mg of solid), tyrosinase (EC 1.14.18.1 from mushroom: 2000 U/mg of solid), chloroperoxidase (EC 1.11.1.10 from *Caldariomyces fumago*, a purified suspension: 1000−2000 U/mg of protein), and NADH were obtained from Sigma (St. Louis, MO). Chlorophenols, monochlorobenzoquinone (MCBQ), 2,5-dichlorobenzoquinone (2,5-DCBQ), 2,6-DCBQ, tetrachloro-1,4-benzoquinone (TCBQ), bis(trifluoroacetoxy)-iodobenzene (BTFAIB), and the remaining chemicals were purchased from Aldrich (Milwaukee, WI). All other materials used were of the highest grade available, and deionized water was used in all stock solutions and buffers. Certified PCP-contaminated soil samples were obtained from Resource Technology Corp. (Laramie, WY). Other PCP-contaminated soil samples were obtained from several wood-preserving plants in the Quebec region.

Bilirubin Oxidase Reaction with Chloro-*p*-benzoquinones. The substrate recycling assay was monitored by following the rate of NADH consumption during the recycling assay. This was done spectrophotometrically by measuring the absorbance decrease at 340 nm (Beckman DU 640 spectrophotometer; Beckman, Fullerton, CA). The NADH consumption rate was also followed using fluorescence measurements with a Gilford Fluoro-IV spectrofluorometer (Gilford; Oberlin, OH). The excitation and emission wavelengths used were 345 and 450 nm, respectively, and the detector PMT voltage was set to +750 V. Unless otherwise indicated, all the assays were performed in 25 mM sodium phosphate at pH 5.5.

Pentachlorophenol (PCP) Oxidation. A protocol reported recently (*22, 23*) with a slight modification was used for the oxidation of PCP to tetrachloro-*p*-benzoquinone. The reaction was performed in 50 mM at pH 1.4 instead of in 100 mM trichloroacetic acid (TCA) at pH 1.1, using 500 μM BTFAIB since TCA was found later to inhibit the enzymatic reaction using bilirubin oxidase. The reduction of TCA from 100 to 50 mM, however, did not affect the PCP oxidation yield if the reaction was allowed to proceed for 1 h at room temperature with agitation and light protection. Notice that at pH 3 (*24*), the reaction was not complete until 2 h and the conversion yield from PCP to TCBQ was less than 70%. The reaction was stopped by the addition of 500 μM hydrogen peroxide, which destroyed any remaining BTFAIB. Notice that BTFAIB (*23*) displays an irreversible cyclic voltammogram with one reduction peak at +0.14 V (glassy carbon vs Ag/AgCl, 0.1 M tartrate buffer, pH 3.5). In the presence of 500 μM hydrogen peroxide, the reduction peak disappeared, an indication of complete BTFAIB destruction.

HPLC Determination of TCBQ and PCP. HPLC determination of TCBQ and PCP was performed using a Waters system (Waters, Milford, MA) consisting of a model 590 pump, a WISP 710B autosampler, and a model 481 LC spectrophotometer. The column used was a Supelcosil LC−PAH column (Supelco; Bellefonte, PA, 150 mm × 4.6 mm) containing C18 bonded silica (5 μm). The HPLC was run isocratically at a flow rate of 1 mL/min with a 50:50 mixture of acetonitrile and water containing 59 μL of 85% phosphoric acid/L as a mobile phase. The calibration was established between 2 and 50 μM PCP. The detection limit was ca. 1 μM, and the PCP retention time was about 20 min. Reproducibility of the peak height expressed as the coefficient of variation (standard deviation/mean) was ca. 5% of all measurements. The determination of PCP in contaminated soils was performed in triplicate.

Results and Discussion

Substrate Cycling Assay using Bilirubin Oxidase and NADH. The experimental data confirmed that NADH was easily and rapidly oxidized to NAD^+ by tetrachloro-*p*-benzoquinone (TCBQ) by following the NADH absorbance decrease at 340 nm. A series of experiments was then conducted to search for an enzyme with the capability of rapidly converting TCHQ to TCBQ to complete the recycling scheme. As a prerequisite, the enzyme must not react with NADH. Tyrosinase, laccase, and chloroperoxidase were initially tested in the substrate recycling assay in view of their widespread use in biosensor construction for phenolic compounds. However, none of these enzymes were suitable as tyrosinase and laccase resulted in a very unsatisfactory detection limit (ca. 1000 nM) and chloroperoxidase together with hydrogen peroxide was not applicable since this combination also oxidized NADH to NAD^+.

Bilirubin oxidase (BOX) was then tested in the recycling assay since both catechol and hydroquinone were reported to be substrates of BOX with *o*- and *p*-benzoquinone as their respective reaction products (*21*). Notice that the primary known function of this enzyme is the oxidation of bilirubin to biliverdin (*20*). BOX turned out to be the best with a high catalytic ability to convert TCHQ to TCBQ in the presence of oxygen whereas it exhibited no reactivity toward NADH in solution. When used in the recycling assay, the NADH consumption rate (μM_{NADH}/min) obtained by BOX was 10-fold higher than that of tyrosinase or laccase and twice that of chloroperoxidase. A series of experiments was then performed to estimate the maximal velocity (V_{max}) and the Michaelis−Menten constant (K_s) of BOX with respect to TCHQ as a substrate, and the oxidation of TCHQ to TCBQ was monitored at 290 nm. Notice that TCHQ was prepared by reacting TCBQ with zinc powder using a protocol described by Saby et al. (*23*). The resulting sample was filtered to remove the powder from TCHQ. The Hanes plot (9 data points, correlation coefficient of 0.97) provided a value of 2.02 mM_{TCBQ}/min and 0.057 mM for V_{max} and K_s, respectively.

In view of the instability of NADH at a pH below 5, the BOX assay was tested over a pH range of 5−8 (25 mM phosphate buffer) using four different chlorinated *p*-benzoquinones: monochlorobenzoquinone (MCBQ), 2,5-dichlorobenzoquinone (2,5-DCBQ), 2,6-DCBQ, and TCBQ. According to Saby et al. (*22, 23*), MCBQ is the oxidized product of 4-chlorophenol (2-CP), 3-CP, 2,4-dichlorophenol (DCP), or 3,4-DCP. The oxidation of either 2,5-DCP or 2,4,5-trichlorophenol (TCP) will produce 2,5-DCBQ whereas 2,6-DCBQ is the oxidized product of the following chlorophenols: 2,6-DCP, 3,5-DCP, 2,4,6-TCP, and 3,4,5-TCP. The oxidized product of 2,3-DCP or 2,3,4-TCP (i.e., 2,3-DCBQ) and trichlorobenzoquinone (the oxidized product of 2,3,5-TCP, 2,3,6-TCP, or 2,3,4,6-tetrachlorophenol) were not commercially available during the course of this study.

With respect to the NADH consumption rate, TCBQ assay was optimal at pH 5.5, and for the other chlorobenzoquinones (CBQs), it was in the range of pH 7−7.5 (Figure 1). A significant decrease in the assay response for TCBQ at pH above 6.5 was not completely unexpected due to the instability of this chloroquinone (*22, 23*). As also shown in this figure, the assay response toward TCBQ at pH 5.5 was at least 20−40-fold higher than that of other chlorobenzoquinones, which indicated a very high substrate activity of the enzyme for TCHQ. This type of behavior showed promise for the development of an assay specific for TCBQ at pH 5.5, an oxidation product of PCP. On the basis of the results obtained

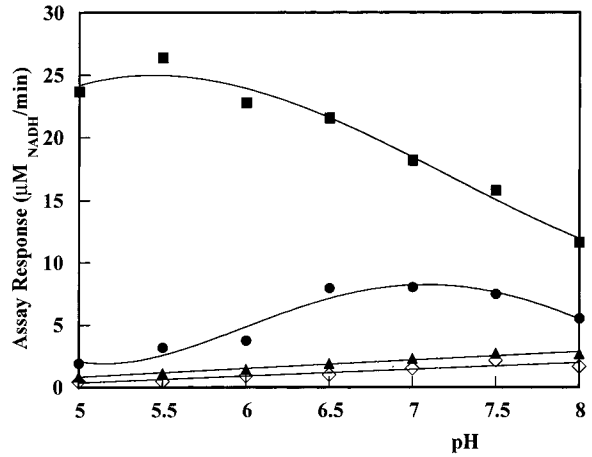

FIGURE 1. Effect of pH on the assay response for (▲)10 μM MCBQ, (◇) 10 μM 2,5-DCBQ, (●) 10 μM 2,6-DCBQ, and (■) 5 μM TCBQ. Each data point is an average of the triplicated results. 1 μM NADH = 0.0062 AU (absorption coefficient of NADH at 340 nm is 6200 M^{-1} cm^{-1}).

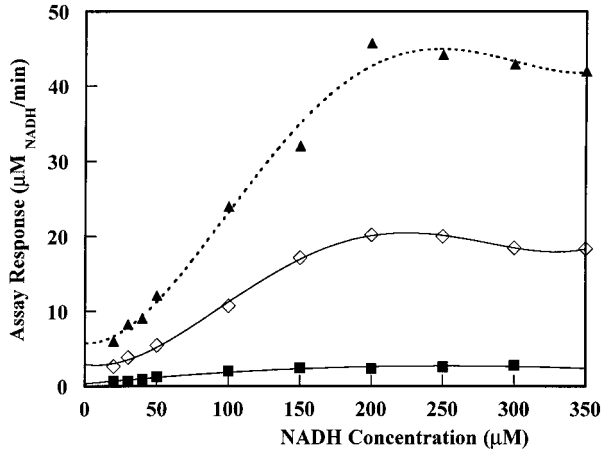

FIGURE 2. Effect of NADH concentration on the assay response: (■) 1 μM TCBQ, (◇) 5 μM TCBQ, and (▲) 10 μM TCBQ. The determination was performed in triplicate.

and due to the great interest in the detection and monitoring of PCP, the subsequent study focused on the development of an optimal assay procedure for TCBQ.

The effect of NADH concentrations was investigated using both absorbance and fluorescence measurements. In the case of the absorbance measurements (TCBQ = 0.05−10 μM), the assay response increased with increasing NADH concentration up to 200−250 μM (Figure 2). Beyond this level, the assay response was independent of NADH concentration. An NADH concentration higher than 250 μM could not be used and/or recommended because this would have given absorbance readings beyond the limit of linear detection of most spectrophotometers. Therefore, an NADH level of 250 μM was chosen in all subsequent experiments, allowing the assay to progress for a sufficient amount of time (1−20 min, depending on the TCBQ concentration) without the reaction rate being affected by any variation in the NADH concentration. The effect of increasing the BOX concentration for the absorbance experiment was also investigated and illustrated in Figure 3. Above 0.5 U/mL BOX, there was no further increase in the assay response for the absorbance experiments.

Selection of the NADH concentration for the fluorescence-based experiments was performed on the basis of the maximum NADH concentration that would allow for the maximum possible detector PMT voltage without giving

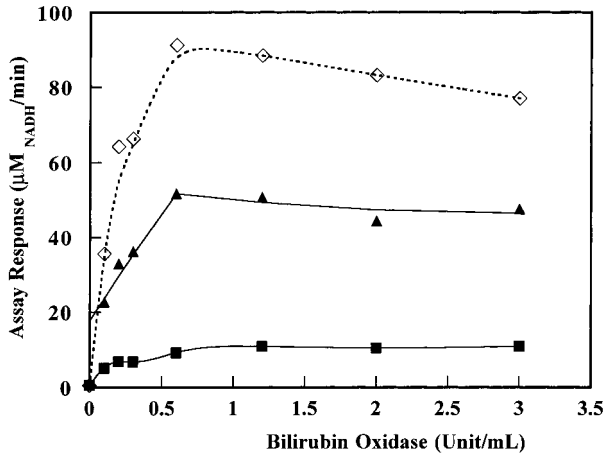

FIGURE 3. Effect of BOX concentration on the assay response: (■) 1 μM TCBQ, (▲) 5 μM TCBQ, and (◇) 10 μM TCBQ. Each data point is an average of the triplicates.

excessive background noise. In this case, the NADH concentration chosen was 2 μM with a PMT voltage of +750 V. There was an increase in the response assay with increasing BOX concentrations up to 0.1 U/mL. Beyond this enzyme level, the response was no longer dependent on the enzyme level (figure not shown). Therefore, a BOX level of 0.1 U/mL was chosen for fluorescence detection, and in an effort to conserve enzyme, a BOX level of 0.3 U/mL instead of 0.5 U/mL was chosen for the absorbance assay.

Assays for *p*-Chlorobenzoquinones. The four different chlorobenzoquinones were tested in the assay at the conditions optimized for TCBQ (pH 5.5, 250 μM NADH, 0.1−0.3 U/mL BOX) using both absorbance and fluorescence detection (Figure 4A,B). For the absorbance experiments, the slope of the TCBQ calibration line at the optimized conditions yielded an assay response (slope of the straight line) of 2.22 $\mu M_{NADH}/\mu M_{TCBQ}$·min. With respect to the absorption coefficient of NADH (6200 M^{-1} cm^{-1}), 1 μM NADH is equivalent to 0.0062 AU at 340 nm. When tested in the assay, the experimental data reconfirmed that MCBQ, 2,5-DCBQ, and 2,6-DCBQ displayed responses of 2.0%, 1.4%, and 7.1% of the value for the TCBQ calibration line, respectively. Such a result thus confirmed that the assay procedure was much more sensitive and selective to TCBQ.

Using fluorescence, the TCBQ calibration plot gave a response (slope of the straight line) of 1.14×10^{-2} RFU-(relative fluorescence units)/nM_{TCBQ}·min. In this case, MCBQ, 2,5-DCBQ, and 2,6-DCBQ gave responses of 12.3%, 2.9%, and 20%, respectively, of the TCBQ value. In view of the assay response for MCBQ and 2,5-DCBQ, the presence of 4-CP, 2-CP, 3-CP, 2,4-DCP, 3,4-DCP, 2,5-DCP, or TCP in the sample should not significantly affect the reliability of the recycling procedure for analysis of PCP. However, 2,6-DCP, 3,5-DCP, 2,4,6-TCP, and 3,4,5-TCP (2,6-DCBQ is an oxidized product of these chlorophenols) when present at an equivalent amount to PCP may contribute up to an error of 20% to the final reading.

The detection limits of the assay for the absorbance and fluorescence techniques were determined using their respective pooled sample standard deviations. This was accomplished by setting the detection limit response to three times the pooled standard deviation of the samples. These standard deviations were 0.103 μM_{NADH}/min for the absorbance studies and 0.083 RFU/min for the fluorescence experiments. These values were substituted into the regression equations for the standard curves for TCBQ and solved to give detection limits of 110 and 30 nM TCBQ for the absorbance and fluorescence techniques, respectively.

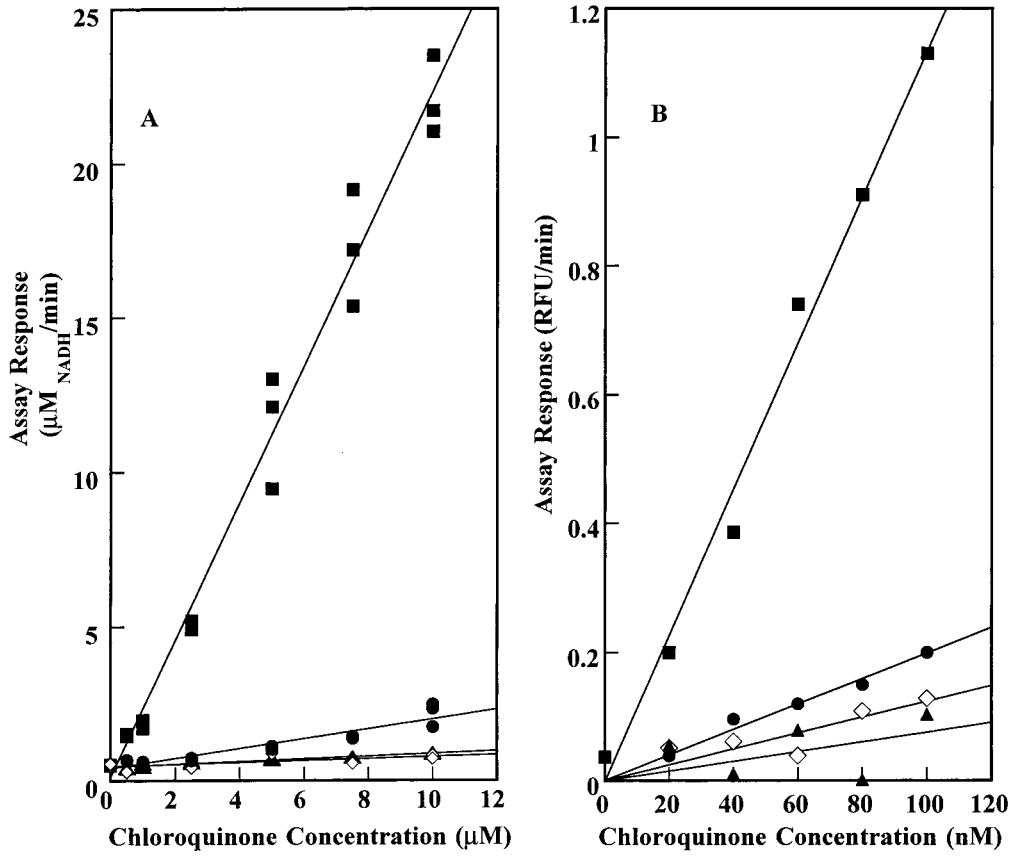

FIGURE 4. Assay of different CBQs using BOX. (A) Absorbance detection: (▲) MCBQ, (◇) 2,5-DCBQ, (●) 2,6-DCBQ, and (■) TCBQ; slope = 2.223 ± 0.135 at 95% confidence interval; intercept = 0.121; R^2= 0.984. (B) Fluorescence detection: (▲) MCBQ, (◇) 2,5-DCBQ, (●) 2,6-DCBQ, and (■) TCBQ; slope = 1.14 × 10^{-2} ± 1.6 × 10^{-3} at 95% confidence interval; intercept = 0.54 × 10^{-2}; R^2= 0.989.

Oxidation of Pentachlorophenol to TCBQ Using BTFAIB. In view of the difficulties encountered with the enzymatic/photochemical oxidation of the chlorinated phenols (*22*, *23*), the possibility of using a chemical procedure was explored as a complementary approach. Of the available methods for PCP oxidation, the use of BTFAIB has been shown to be rapid and reliable and to give high yields of TCBQ (*22*, *23*). Therefore, the oxidation of PCP (0.01−10 μM) was carried out in 0.05 M trichloroacetic acid using 500 μM BTFAIB. After allowing the reaction to proceed for 1 h with light protection and agitation, the reaction was stopped by the addition of 500 μM hydrogen peroxide. HPLC analysis showed that at least 95% of the PCP had been converted to TCBQ. It was also observed that both trichloroacetic acid and hydrogen peroxide exhibited extreme inhibitory effects on the activity of the enzyme and the stability of NADH. Therefore, it was necessary to remove these two chemicals after the oxidation of PCP to TCBQ. This cleanup procedure was facilitated through the use of Sep-Pak C18 cartridges (Waters; Milford, MA). The converted samples (20 mL) were loaded onto cartridges prewashed with acetonitrile and trichloroacetic acid. The cartridges contain very hydrophobic octadecylsilane packing; therefore, they will allow polar compounds such as hydrogen peroxide and TCA in the sample to pass through. The cartridges were then washed extensively with 25 mM NaH_2PO_4, pH 1.9, to remove any residual trichloroacetic acid and hydrogen peroxide. The bound TCBQ was then eluted from the cartridges using 1 mL of acetonitrile and made up to 20 mL with 25 mM NaH_2PO_4, pH 5.5. These eluted samples were expected to be free from both TCA and hydrogen peroxide and were then used directly in the BOX assay.

PCP Determination with the BOX Assay. The BTFAIB-treated PCP samples behaved much like the TCBQ samples in the BOX/NADH assay. The only difference between the two was that the converted PCP samples gave about 30% lower responses in the assay than their equivalent amount of TCBQ. The decrease in response could come from TCBQ loss at two points: First, during the BTFAIB oxidation there would not have been 100% conversion of PCP to TCBQ, or some of the TCBQ might have been further oxidized to another compound. As a matter of fact, about 5% PCP was reported to be oxidized to 1,2-tetrachlorobenzoquinone (*23*). HPLC analysis obtained in this study confirmed that about 95% of the PCP had been converted to TCBQ as mentioned previously. Second, some of the TCBQ could have been lost during the C18 cartridge cleanup step as verified by HPLC for analysis of the converted samples before and after the cleanup step. Repeated experiments confirmed the loss of 30% PCP during the combined oxidation and cleanup steps. The assay was calibrated for PCP by measuring the NADH consumption rate (μM_{NADH}/min) versus the amount of PCP present in the sample. The resulting slopes of such calibration plots ($\mu M_{NADH}/\mu M_{PCP}$·min) serve as the referenced values for determining the PCP concentration in unknown samples. Consequently, the detection limits for PCP using absorbance and fluorescence detection were established in the same manner as for TCBQ. The resulting limits were 170 and 50 nM for absorbance and fluorescence, respectively.

Analysis of Contaminated Soil Samples. Several PCP-contaminated soil samples including two certified soil samples (150−3000 ppm, μg of PCP/g of soil) were tested using the BOX assay by absorbance measurement. PCP was extracted from the soil samples using 10 mM NaOH. One gram of soil was contacted with 50 mL of the NaOH for 4 h at room temperature with strong agitation and light protection. Once the extraction was complete, the samples were centrifuged, and the sediment was removed. The remaining liquid was stored at 4 °C with light protection. When the soil

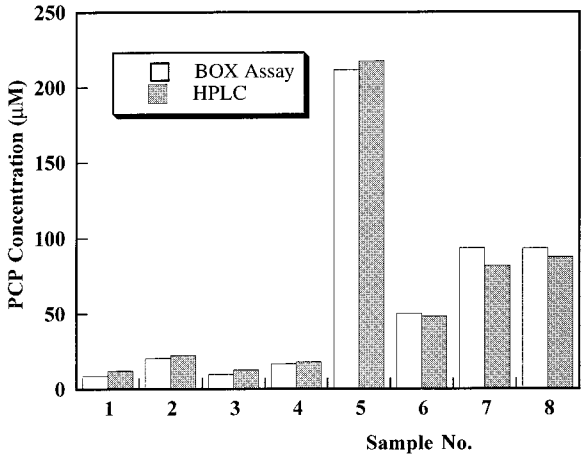

FIGURE 5. Comparison of HPLC detection and the BOX assay for PCP (μM). Each data point is an average of the triplicates. HPLC vs BOX: (0,0/0,0/12.2,8.7/22.3,20.4/12.7,9.8/18,17/217,211/81,93/87,94).

samples were used in the BTFAIB oxidation step, the various materials extracted from the soils did not have any effect on the conversion of PCP to TCBQ. Therefore, it was not necessary to have an additional sample cleanup step, greatly facilitating and reducing the amount of time required for sample preparation.

Comparing the BOX/NADH assay results for the soil samples with the HPLC analysis showed that there was very good agreement between the two methods (Figure 5), with no significant difference between the means of the two methods used ($\alpha = 0.05$). The PCP contamination level of 8 out of 10 extract samples tested ranged from 10 to 220 μM. The two other soil samples (nos. 9 and 10) contained no PCP as confirmed by both the enzymatic assay and the HPLC analysis. The certified samples (samples 7 and 8 from Resource Technology Corp., reference value 1109−1742 ppm of PCP/g of soil) gave an identical value of 93.5 μM with recycling assay, which is equivalent to 1150 ppm of PCP/g of soil. The values obtained by HPLC for these two samples were 1006 and 1078 ppm, respectively. A plot of the recycling assay and HPLC results against one another (figure not shown) gave a slope of 0.998 ± 0.062 at 95% confidence interval with a correlation coefficient (R^2) of 0.994, validating the applicability of the substrate recycling assay for measuring PCP in contaminated soil samples.

In brief, a substrate recycling assay for tetrachloro-*p*-benzoquinone was developed and optimized using the recycling of TCBQ between reactions with NADH and bilirubin oxidase. At pH 5.5 and with excess NADH (250 μM), absorbance monitoring of the NADH allowed for a TCBQ detection limit of 110 nM, while the fluorescence monitoring method gave a detection limit of 30 nM. PCP was effectively converted to TCBQ using BTFAIB with a high yield under mild conditions. This allowed for detection of PCP by the recycling assay, with detection limits of 170 and 50 nM for absorbance and fluorescence detection methods, respectively. The recycling assay has proven applicable for determining PCP in contaminated soils as the results obtained agreed well with the HPLC standard procedure. Together with a miniaturized spectrophotometer or fluorometer, this analytical procedure may be attractive for field analysis during site characterization and remediation activities. To improve the detection limit as well as reproducibility, bilirubin oxidase can be immobilized on an optical fiber to form an enzyme-based optical fiber biosensor. Efforts toward this goal are currently under way in our laboratory.

Acknowledgments

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