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Determination of Methyl Parathion in Water and Its Removal on Zirconia Using Optical Enzyme Assay

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Abstract A simple, miniaturized microplate chemiluminescence assay for determination of methyl parathion (MP) was developed in 384-microwell plates. Zirconia (ZrO_2) was added in microwell for adsorption of acetylcholinesterase (AChE). The developed assay is based on inhibition of AChE by MP. A good dynamic range 0.008–1,000 ng/mL was obtained for MP with limit of detection 0.008 ng/mL. Intrabatch and interbatch reproducibility for miniaturized assay was obtained with % RSD up to 3.07 and 5.66, respectively. In 384 well plate formats, 70 samples were simultaneously analyzed within 20 min with assay volume of 41.5 μL. The application of developed assay was extended for MP remediation. Column containing ZrO_2 was utilized for remediation where MP was selectively adsorbed. Under optimized condition, adsorption of MP on ZrO_2 was found to be 98–99% with 2-h contact time in real water samples. Adsorption of MP on ZrO_2 column followed by quantification using developed bioassay provides a novel approach to monitor remediation. The applicability of assay was successfully extended for determination of MP in water samples after removal through ZrO_2.

Keywords Optical enzymatic assay · Methyl parathion (MP) · Acetylcholinesterase (AChE) · Zirconia (ZrO_2) · Drinking water · Remediation · Inhibition

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Introduction

Organophosphate (OP) pesticides present a risk to human health and the environment because of their neurotoxicity and widespread usage [1]. OP contamination to various environmental compartments such as air and soil also eventually leads to runoff waste, which finally reaches underground water. Due to good water solubility, OPs infiltrate through soil into surface water and eventually cause damage to aquatic organisms. As estimated, only less than 1% of the pesticides applied would directly work on the pests, whereas the majority would be diffused into the environment [2]. OPs being highly used pesticides in the coastal agricultural region enter into marine environment through different pathways. Therefore, the impact of OP contaminants on coastal environment needs special attention [3]. Concerns about OPs are being raised because of their persistence, bioaccumulation and potential for toxicity in human and animals. The OPs are not rapidly degraded in natural waters. The reported hydrolytic half-life of extensively used OP, i.e., methyl parathion [MP] is 108 days [4]. There have been episodes of contamination of groundwater, surface water, and even bottled water with OP residue in developing countries [5]. Among the OPs, MP contributes major share in terms of its usage. The compound MP (*O*, *O*-dimethyl, *O*-4-nitrophenyl phosphorothioate) is an extremely hazardous insecticide and has been classified as restricted use Class 1A chemical by the World Health Organization [6]. Exposure to MP has been linked with adverse health effects on organ systems including cholinesterase inhibition, abnormalities in heart rate and headache, muscle weakness, insomnia, and dizziness [7]. The European Union has set maximum admissible concentrations of 0.1 μg/L for individual pesticides and 0.5 μg/L for total pesticide residues [8]. The Bureau of Indian Standards (BIS) has set permissible limit for total pesticide residues in drinking water as 1 μg/L [9]. Hence, for monitoring environment, sensitive detection techniques for trace analysis of MP are of immense importance.

Analysis of MP in surface and groundwater samples is routinely carried out using analytical technique such as gas and liquid chromatography–mass spectrometry. Biosensors are good alternative to these techniques as it requires minimum sample pretreatment and provides information concerning the toxicity of the sample rather than its concentration alone [10]. Among the various biosensors for OP determination, systems based on organophosphate hydrolases (OPH) [11] and acetylcholinesterase (AChE) [12, 13] contribute major share. MP residues are highly toxic and are powerful inhibitors of AChE, a vital enzyme involved in neurotransmission in the form of acetylcholine substitutes. In the presence of MP, serine hydroxyl moiety in the AChE enzyme active site is phosphorylated in a manner analogous to the acetylation of AChE. The serine hydroxyl group, blocked by a phosphoryl moiety, is no longer able to participate in the hydrolysis of AChCl decreasing enzyme activity [14]. Measurement of the enzyme activity before and after exposure to a target analyte quantitatively demonstrates MP concentration. The percentage of inhibited enzyme activity (I%) that results after exposure to the inhibitor is quantitatively related to the inhibitor concentration according to following equation:

$$\mathrm{I\%} = (\mathrm{A_{0-}A_i}/\mathrm{A_0}) \times 100$$

where A_0 is the activity in the absence of inhibitor, and A_i is the activity in the presence of inhibitor [10]. Recent reported work on MP biosensor has been more in to bioremediation of methyl parathion where Kumar et al. [15] reported colorimetric microbial bioassay in

microwell plate with sensitivity up to 4 μM. Leng et al. [16] reported fluorescent molecular biosensor using methyl parathion hydrolase with sensitivity up to 0.26 ng mL^{-1}. Similarly, Du et al. [17] reported covalent coupling of organophosphate hydrolase with sensitivity up to 1.0 ng/mL. For simultaneously analyzing large number of real samples, microwell plate-based assay has been reported using different combination of biocomponent and transducer system. Antibody-based assay coupled with fluorescence (FL) and chemiluminescences (CL) is reported with sensitivity up to 0.08 ng mL^{-1} [18, 19]. Good sensitivity up to 2 ng mL^{-1} was also reported by Weetal et al. using stabilized AChE and its subsequent analysis in microwell plate [20]. There is scarcity of literature on miniaturized high-throughput microwell plate assay for MP determination specially using enzyme inhibition assay. CL-based assay possesses higher sensitivity and minimal interference due to simple instrumental setup [21–23]. Herein, we demonstrate an improved, sensitive microtiter plate-based CL assay for the determination of MP in real water sample using free and adsorbed AChE. A novel application of the assay is demonstrated using zirconia (ZrO_2) in column for removal of MP. ZrO_2 has been reported as one of the most effective inorganic supports for adsorption of proteins as well as pollutants [24, 25]. The significance of ZrO_2 in remediation lies in its thermal stability, nontoxic nature, and strong affinity for phosphoric group. Applications of ZrO_2 have been reported in OP analysis which includes as sensing element for OP in electrochemical studies [24–27], as reusable cartridge in water purification [28, 29], and for adsorption of proteins [30–32]. We report determination of MP using highly sensitive enzyme inhibition assay in 384 well plates where ZrO_2 is utilized as novel material for AChE adsorption. Moreover, a novel simultaneous monitoring and remediation strategy is also demonstrated for MP removal, which may be extended to other analogous compounds.

Experimental

Reagents and Materials

Methyl parathion PESTANAL grade purity 99.8% was procured from Riedel-de Haën (Germany). Acetylcholinesterase (EC 3.1.1.7) from *Electrophorus electricus* (electric eel), choline oxidase (EC 1.1.3.17) from *Alkaligenes* sp., peroxidase (EC 1.11.1.7) from *Horseradish*, acetylcholine chloride (AChCl), choline chloride (ChCl), and luminol were purchased from Sigma Chemical Co. (USA). All other chemicals were of AnalaR or GR grade from Merck (Germany). Zirconium (IV) oxide (98.5%) and *O*-phenylenediamine dihydrochloride (OPD) were purchased from Fisher Scientific (USA). The reagents were prepared in 0.1 M phosphate buffer (PB) pH 7.4. A stock solution of 1 mg/L of MP was prepared by dissolving 1.0 mg of the standard pesticide in 1 ml of 5% ethanol in 0.1 M PB, pH 7.4. Working solutions of MP were prepared daily by appropriate dilutions of the stock solution with PB. Enzyme solutions were maintained at 4 °C using minicooler and without externally adding any enzyme stabilizer.

CL and colorimetric measurements were recorded using Multilabel Reader Victor™ X^4, Perkin Elmer (USA). Corning (USA) 384-microwell plate was used. Micropipettes, Eppendorf (Germany), were used. pH and conductivity were measured using digital pH and conductivity meter, model seven multi, Mettler Toledo (Switzerland). For validation, UV–VIS measurements were done in V-570 spectrophotometer, JASCO (Japan). Particle size analysis was done using Delsa™ Nano S submicron particle size analyzer, Beckman coulter (Switzerland).

Assay Protocol

Development of Assay in 384-Microwell Plate

Assay Using Free AChE AChE 0.5 μL (0.03 U) was kept in contact with 15 μL of MP (0.008–1,000 ng/mL) for 15 min in 384-microwell plates. The enzymatic reaction was carried further by adding 26 μL of reaction mixture containing AChCl (0.5 mM), ChOx (0.02 U), HRP (0.002 U), and luminol (1 mM). Since MP inhibits the AChE activity, conversion of AChCl to choline was reduced when measured in microwell plate. The numbers of photons emitted using free AChE with and without MP were counted.

Assay Using Zirconia-Adsorbed AChE (ZrO_2-AChE) One gram of zirconia powder was mixed with equal volume of 0.1 M PB pH 7.4 and AChE solution (3 U). It was shaken at room temperature for 2 h and kept at 4 °C for 2 h and then filtered. The filtrate along with stock enzyme solution was tested for enzyme activity using enzymatic assay as presented above. ZrO_2-AChE (≅0.03 U) was kept in contact with 15 μL of MP (0.008–1,000 ng/mL) for 15 min in 384-well plates. The enzymatic reaction was carried further by adding 26 μL of reaction mixture containing AChCl (0.5 mM), ChOx (0.02 U), HRP (0.002 U), and luminol (1 mM). Here, ZrO_2 is used as adsorbent for AChE. The numbers of photons emitted using ZrO_2-AChE assay with and without MP were counted.

Application of Bioassay for Monitoring MP Removal Using ZrO_2

Initially, batch kinetic experiments were carried out to evaluate the suitability of ZrO_2 for MP removal. Parameters such as amount of ZrO_2 and contact time for adsorption were evaluated. Based on the batch kinetic experiments, column studies were carried out to achieve the removal of MP on ZrO_2. The removal process was monitored by analyzing the column effluent using the developed bioassay. Column studies were performed in two steps: (a) adsorption of MP on ZrO_2 and (b) desorption of adsorbed MP fraction from column by Na_2CO_3. ZrO_2 was packed in plastic column using slurry pack method. Packed column of ZrO_2 was conditioned using sufficient 0.1 mM PB pH 7.4. Standard MP solution was kept in contact with ZrO_2 for 2 h. After adsorption, the column effluent was collected and kept aside as MP-fraction 1 for further analysis. Column containing adsorbed MP was eluted with 1 M Na_2CO_3. The eluted fraction was collected as MP-fraction 2. Both the MP-fraction 1 and MP-fraction 2 were simultaneously analyzed using bioassay. Schematic representing the MP removal process on ZrO_2 column integrated with bioassay is presented as Fig. 1. The attenuation of MP on ZrO_2 as ZrO_2-MP was investigated using microwell plate assay. A known amount of solid ZrO_2-MP was added in to the microwell plate. Subsequently, AChE was added followed by reaction mixture containing AChCl (0.5 mM), ChOx (0.02 U), HRP (0.002 U), and luminol (1 mM). The CL intensity was found proportional to amount of adsorbed MP on ZrO_2.

Real Sample Analysis

The presented bioassay was further tested for MP determination in drinking water, agricultural runoff water, and sea water. To preserve the chemical nature of sample and minimize interferences due to suspended solids, a simple one-step filtration strategy was adopted for recoveries of MP in spiked samples. Water samples were collected from the

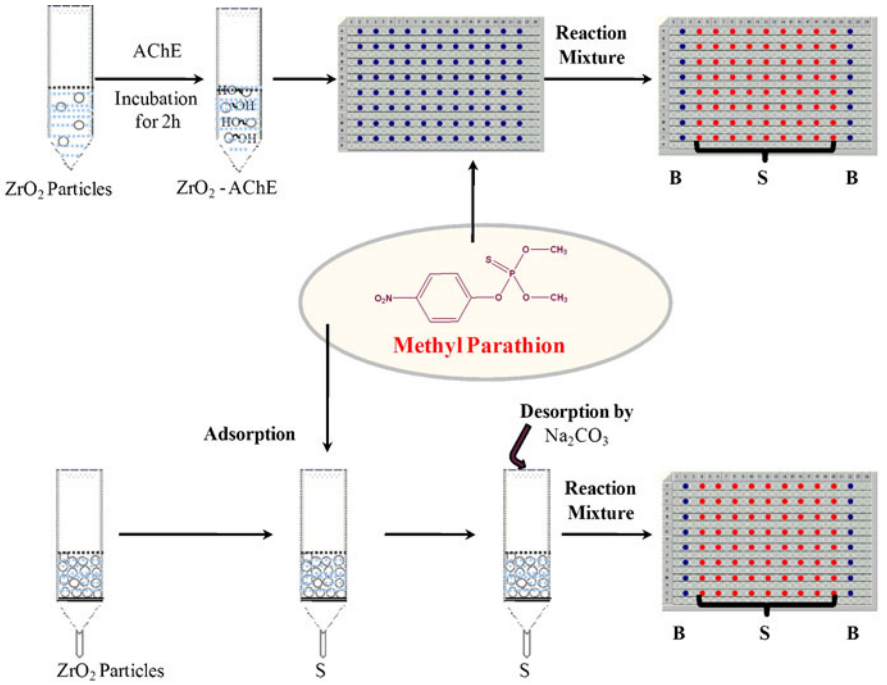

Fig. 1 Schematic representing ZrO_2-AChE assay in microwell plate and monitoring of MP adsorption on ZrO_2 column using CL bioassay

local source on the same day. The collected samples were filtered through 0.22-μm filter (Whatman, USA) and then diluted with freshly prepared PB in series of dilutions. The diluted samples were spiked with MP, and assay was carried out to study matrix effect and recovery studies.

Caution OPs are highly toxic and should be handled in a safety cabinet. Skin and eye contact and accidental inhalation or ingestion should be avoided.

Results and Discussion

Assay Using Free and ZrO_2-Adsorbed AChE in Microwell Plates

The CL method described earlier relies of coupled trienzyme reaction resulting in light emission that involves the enzyme AChE, ChOx, and HRP. Experimental parameters such as pH, ionic strength of PB, and temperature were optimized, and it was observed that AChE in trienzyme reaction gives optimum performance with 0.1 M PB, pH 7.4 at 30 °C. Adsorption of enzyme on ZrO_2 is reported to increase storage stability of enzyme [32]. The activities of the free and ZrO_2-AChE were measured in 384-microwell plates using earlier described protocol. An increasing CL emission was measured in presence of varying

concentration of AChE (0.0007–0.1 U) and 0.5 mM AChCl against fixed ChOx (0.02 U) and HRP (0.002 U) concentration. It was observed that under optimal conditions for a fixed substrate (AChCl), kinetics is controlled predominantly by the free enzyme concentration of AChE over the other two enzymes ChOx and HRP. The signal intensities resulting from trienzyme reaction involving various concentrations of free AChE and ZrO_2-AChE were also recorded and presented as Fig. 2. The slope of CL kinetic profile allows an easy evaluation of AChE activity. The quantitative evaluation of free and ZrO_2-AChE activity suggests that ZrO_2-AChE activity is more stable and reliable over free AChE assay. AChE concentration of 0.03 U was optimized for further experiments. Substrate concentration, i.e., AChCl, was optimized for optimal signal. Using experimental data and by Eadie–Hofstee plot, the value of K_M is found to be 0.25 mM.

Analytical Characteristics of Inhibition Assay for MP Determination

Analytical characteristics of inhibition assay are mainly dependent on incubation time, inhibitor concentration, concentration of enzyme, and substrate used. For ZrO_2-AChE inhibition, incubation time was optimized at 15 min. Inhibition studies were carried out for free and ZrO_2-AChE (0.03 U) using [MP] in the range 0.008–1,000 ng/mL with 15-min incubation time. As against free AChE, ZrO_2-AChE activity was found comparable and thus was used further as ZrO_2-AChE in monitoring MP removal. The achieved I% was plotted against the [MP] as shown in Fig. 3a. For MP determination using ZrO_2-AChE, linear range was found to be 0.01–1,000 ng/mL with limit of detection (LOD) 0.008 ng/mL

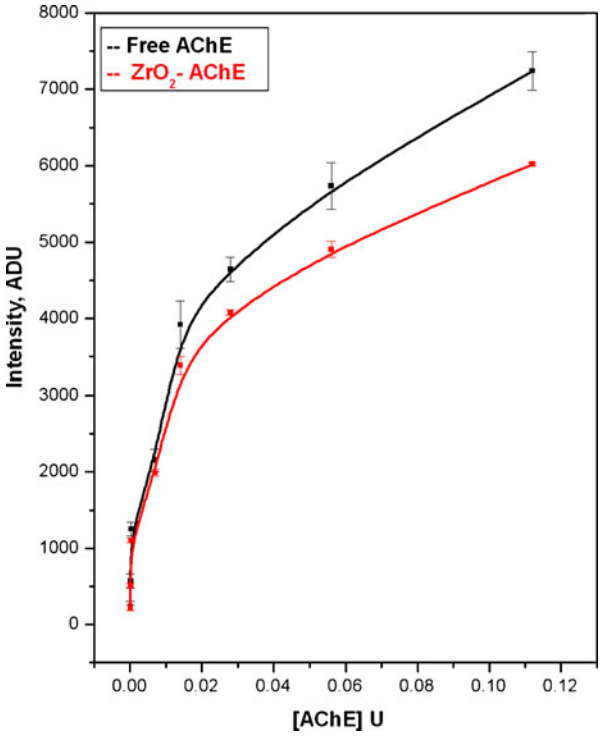

Fig. 2 Intensity profile for CL signal measured in presence of varying AChE concentration. Free AChE (*black line*) and ZrO_2-AChE (*red line*; 0.0007–0.1 U) with 0.5 mM AChCl, (ChOx 0.02 U, HRP 0.002 U) 0.1 M PB, pH 7.4 at 30 °C

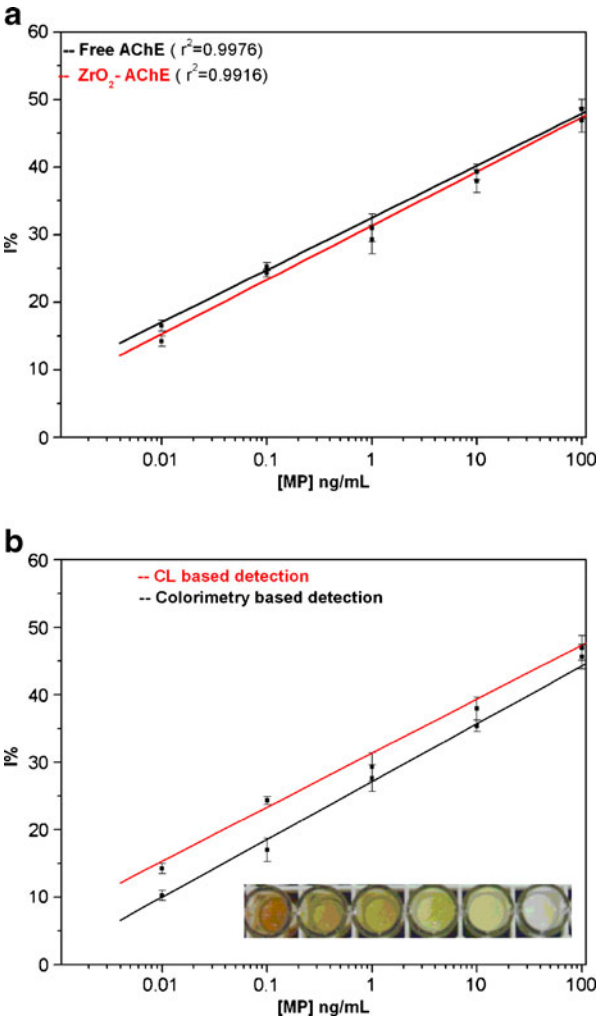

Fig. 3 **a** Calibration curve for MP obtained using trienzyme CL reaction. Free and ZrO_2-AChE were taken for inhibition studies. [MP] is represented in log scale. *Error bars* represent the standard deviation from the mean (n=3). **b** Comparative study of CL- and colorimetric based assay for MP determination

and limit of quantification (LOQ) 0.01 ng/mL. For free AChE, inhibition was also found linear in the range 0.01–1,000 ng/mL with LOQ 0.01 ng/mL and LOD 0.008 ng/mL. The regression coefficient obtained for both linear fit is r^2=0.9976 and 0.9916, respectively. Total assay inclusive of incubation step takes about 20 min with assay volume of 41.5 μL.

Statistical Analysis

As against the free AChE assay, I% observed for assay using ZrO_2-AChE was consistent and highly reliable. For reliability of ZrO_2-AChE assay, intra- and interday precision and accuracy studies were conducted. [MP] was spiked with 0.1, 1, and 10 ng/mL in PB. Variation in response for intraday (n=5) and interday (n=10) was calculated over the period of 10 weeks. The average calculated % RSD for intraday and interday assay was found to be 3.07 and 5.66, respectively. Results are presented in Table 1.

Table 1 Interday and intraday reliability of ZrO_2-AChE in trienzyme reaction performed in 384-well plate assay

[MP] ng/mL added	[MP] ng/mL found (mean ± SD)			
	Intraday (n=5)	% RSD	Interday (n=10)	% RSD
0.1	0.100±0.002	2	0.1000±0.0052	5.2
1.0	0.9910±0.032	3.22	0.9876±0.0646	6.60
10	9.45±0.3794	4.01	10.0026±0.5188	5.18

Validation Studies

Validation of developed CL-based assay on ZrO_2-AChE was performed in the following two ways: (a) using developed bioassay and (b) using conventional analytical system LC-MS/MS. For validation through bioassay, colorimetric measurements were recorded for same trienzyme system, whereas for LC-MS/MS, analysis was done through an external laboratory. A portion of the samples was used for analysis by developed bioassay, whereas another portion was simultaneously processed for external validation. In the colorimetric assay, OPD was used. Inhibition was carried out using ZrO_2-AChE in microwell plate, and colorimetric measurements were recorded at 490 nm. With OPD, we could visually differentiate [MP] corresponding to I%. The comparative study of CL-based assay and colorimetric assay is presented in Fig. 3b. I% achieved for different [MP] using CL and colorimetric study reveals that, although colorimetric technique can differentiate MP concentration visually, it exhibit lower sensitivity. For validation using standard analytical method LC-MS/MS, the analysis was done through an external certified analytical laboratory. The correlation was observed from both the techniques (data not shown). Extensive sample pretreatment was carried out for LC-MS/MS, whereas developed bioassay analyzed sample without much sample treatment.

Batch and Column Studies on ZrO_2 for MP Removal Using Developed Bioassay

ZrO_2 has been reported to be a promising material for MP removal. Particle size of the material is an important parameter for adsorption. Thus, characterization of ZrO_2 was carried out for particle size. In aqueous solution, the average diameter of the ZrO_2 particle was found to be 1.954 μm. For batch adsorption studies, pH, amount of adsorbent, and contact time were optimized. Maximum adsorption of MP on ZrO_2 using electrochemical sensor has been reported at pH 7 [27]. Thus, considering the compatibility of assay, pH 7.4 was selected for adsorption studies. The optimized experimental conditions for adsorption of MP in batch experiment are 0.1 M PB, pH 7.4, 0.2 g adsorbent with 2-h contact time. Increase in adsorption was observed with increase in contact time up to 2 h. The results obtained from batch experiments were utilized to perform column studies. MP stock solution in the range 0.01–1,000 ng/L was allowed to adsorb on ZrO_2 packed in column. Figure 4a shows measured I% by influent and effluent [MP] of ZrO_2 column. Significant decrease in I% was found in the effluent fraction. Table 2 summarizes the measured concentration of MP and % MP removal by developed CL-based assay after passing through ZrO_2 column. Concentration of MP was calculated corresponding to I% from inhibition curve (Fig. 4a), and difference (Δ I%) of influent and effluent MP solution was correlated with MP adsorbed on ZrO_2. Significant adsorption found helps to conclude that the ZrO_2 had effectively removed MP from influent sample.

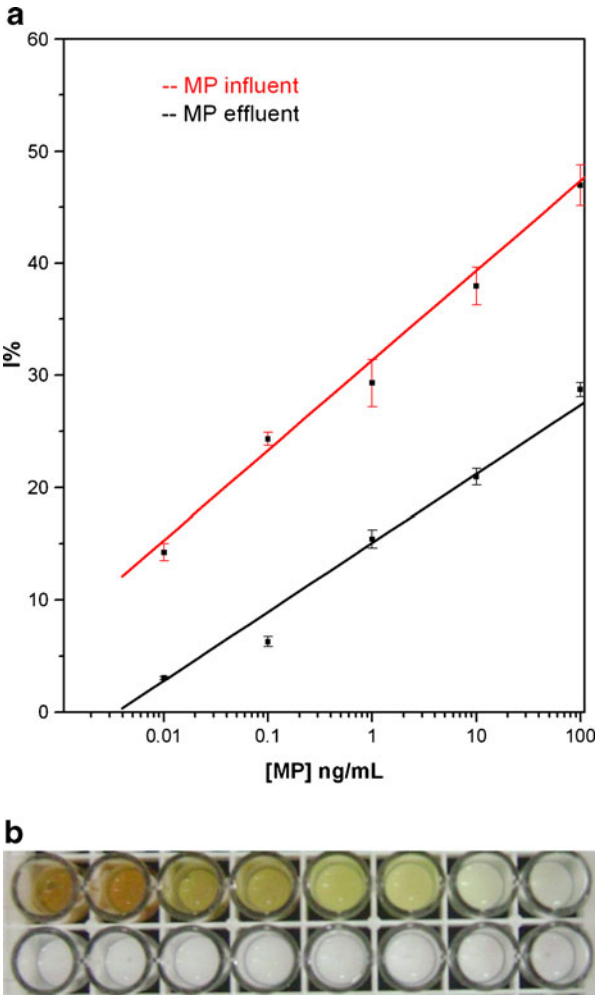

Fig. 4 **a** Inhibition obtained for influent and effluent MP on ZrO_2 column. Various MP concentrations were tested for adsorption on ZrO_2 (0.2 g) for 2 h, and *y*-axis represents inhibitions of AChE due to effluent and influent form ZrO_2 column. [MP] is represented in log scale. **b** Visual representing of the ZrO_2 adsorbed MP inhibition quantified using colorimetric assay. The samples are imaged in duplicate with different MP concentrations adsorbed

The presented ZrO_2-AChE assay can easily quantify MP samples greater than 1 ng/mL. To verify reliability and reproducibility of column studies using ZrO_2, the developed assay was repeated for MP adsorption. The average calculated % RSD for intraday and interday assay was found to be less than 7%. The interday assay was carried out by analyzing aliquots of MP (fraction 1 and 2) in triplicate.

Table 2 Determination of MP adsorption and %MP removal on ZrO_2 column using developed bioassay

[MP] ng/mL added	[MP] ng/mL after adsorption	% Removal by adsorption
1	0.018	98.20
10	0.080	99.20
100	0.526	99.47
1,000	6.195	99.38

Monitoring MP Attenuation on Zirconia

MP adsorbed on column was analyzed in microwell plate using colorimetric AChE inhibition assay. A known amount (0.2 g) of ZrO_2-MP from columns with different % MP removal was utilized for inhibition assay. To each well, AChE was added and incubated for 15 min. The activity was measured by addition of ChOx, HRP, OPD, and AChCl. In the first microwell, native zirconia was taken as control. A distinct inhibition was visualized in ZrO_2-MP samples as presented in Fig. 4b. Validation of adsorption and desorption was carried out spectrophotometrically.

Real Sample Analysis

The proposed method was applied to study MP analysis in real samples such as drinking water, agriculture runoff, and sea water in microwell plate. AChE activity was optimize in reagent blank, matrix match calibration, and finally spiked MP samples with 1 ng/mL MP. Conductivity of natural sea water is very high; thus, several dilutions of sea water were tested for matrix matching. Sea water diluted with PB in ratio 1:1,000 was optimized for further experiments. Recovery test was used to examine reliability and accuracy of developed assay. Spiked samples were analyzed in 384-well plates for recovery studies. The % recoveries for drinking water, agricultural runoff, and sea water were found to be 97–98%, 105.61%, and 102–103%, respectively, and presented in Table 3. Higher recoveries in some samples are due to the presence of trace concentration of MP already present in the water sample which may be either due to sampling site contaminated with MP or sampling during rainy season.

Conclusions

A highly sensitive and miniaturized optical bioassay in 384-well format for MP determination has been presented. The applicability of the assay in MP determination for real sample analysis is also demonstrated. The developed bioassay utilizes ZrO_2-adsorbed AChE with 15-min incubation time. The assay could measure MP with LOD 0.01 ng/mL with analysis time of 20 min. The MP measurements exhibited high intrabatch and interbatch reproducibility with % RSD up to 3.07 and 5.66. Good recoveries in the range 97–106% were recorded in spiked known samples. The assay has been extended to analyze simultaneously MP in water and its removal from contaminated water using ZrO_2 column.

Table 3 Recovery studies for MP in drinking water, agricultural runoff, and sea water samples using developed bioassay

Matrix	[MP] ng/mL added	[MP] ng/mL found	% Recovery
Drinking water-1	0.1	0.097±0.01	97.00
Drinking water-2	1	0.98±0.10	98.00
Agricultural runoff	1	1.05±0.61	105.61
Sea water-1	1	1.03±0.86	103.86
Sea water-2	1	1.02±0.45	102.45

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