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# A sensitive, rapid and inexpensive way to assay pesticide toxicity based on electrochemical biosensor

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ABSTRACT

We reported a rapid toxicity assay method using electrochemical biosensor for pesticides, *Escherichia coli* (*E. coli*) was taken as a model microorganism for test. In this method, we adopted ferricyanide instead of natural electron acceptor $O_2$, and then microbial oxidation was substantially accelerated. Toxicity assays measured the effect of toxic materials on the metabolic activity of microorganisms. The current signal of ferrocyanide produced from the metabolism was proven to be directly related to the toxicity, which could be amplified by ultramicroelectrode array (UMEA). The ratio of the electrochemical signals, recorded in the presence and absence of toxin, provided an index of inhibition. Accordingly, a direct toxicity assessment (DTA) based on chronoamperometry was proposed to detect the effect of toxic chemicals on microorganisms. 3,5-Dichlorophenol (DCP) was taken as the reference toxicant, its IC50 was estimated to be 8.0 mg/L. Three pesticides were examined using this method. IC50 values of 6.5 mg/L for Ametryn, 22 mg/L for Fenamiphos and 5.7 mg/L for Endosulfan were determined and in line with EC50 values reported in the literature. Atomic force microscopy (AFM) was also used for morphology characterization of *E. coli* induced by three pesticides. These results confirmed the present electrochemical method used is reliable. In addition, the electrochemical method is a sensitive, rapid and inexpensive way for toxicity assays of pesticides.



## 1. Introduction

Pesticides are widely used every year for control of pests in the environment, and may exert a significant effect, especially on aquatic ecosystems. Enforcement of environmental standards demands accurate and cost effective monitoring techniques. Bioassay tests have been used to establish the toxicity levels of compounds for aquatic organisms. Many types of bioassays are available, and the test organisms incorporated in these assays include: plants, invertebrates and fish [1]. Unfortunately, many of these tests are time consuming, expensive and are no longer ethically acceptable. There is a move towards using the simplest practical forms of life as the test organism. Hence, using microorganisms for direct toxicity assessment (DTA) testing is attractive. The tests can be based on a broad range of responses, are fast, less labored and less expensive than that with higher trophic level indicators. Much work has been invested in developing new analytical tools that incorporate the specificity of biological assays to reflect organisms/ecosystem health, while still retaining the scope of traditional chemical assays. The present paper reports a combined method using toxicity tests and electrochemical method, in order to evaluate the toxic impact of pesticides.

The MICREDOX method developed by Lincoln technology, is a rapid catalytic microbial based-method in which the natural co-substrate, oxygen is substituted by a synthetic co-substrate or mediator [2]. The method is characterized by high levels of biocatalyst (microorganisms) and mediator in order to facilitate the reaction. The microbially reduced mediator accumulates as a product registering the amount of bioconversion. Electroanalytical techniques, either bulk electrolysis or chronoamperometry at microelectrode, are used to measure the quantity of reduced mediator and give a direct measure of the microbially catalysed substrate oxidation.

To date, MICREDOX has been demonstrated using three microorganisms (*Escherichia coli*, *Bacillus subtilis* and *Pseudomonas putida*) for toxicity assessment of DCP [3]. Pasco et al. [4] also used MICREDOX to study differences in the inhibitory properties of various chlorinated phenol compounds. Recently, Teasdale and co-workers used ferricyanide-mediated respiration bioassays to quantify stimulatory and inhibitory effects on *E. coli* populations [5]. Especially, they have developed a two-step approach which could substantially improve the assay sensitivity [6].

The successful development of cell-based biosensors for the detection of environmental pollutants requires the selection of

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a sensitive biocatalyst and a transducer capable of detecting the metabolic perturbations caused by the pollutant. Chronoamperometry at microelectrode was used to measure quantity of the reduced mediator as it offers the advantages of simple and low cost operation, reproducibility and real time analysis [7–9]. The use of microelectrode amperometry offers steady state current in less than 10 s [10]. Especially ultramicroelectrode array (UMEA), effectively amplify the signal from the total limiting currents of multi individual ones to 3,5-dichlorophenol (DCP). The ability to monitor changes in the cellular metabolic state, in real time or at selected intervals following exposure to an environmental or chemical pollutant, enables rapid detection of cellular perturbation, and can provide the basis for developing a successful toxicity-screening test.

Here, we took *E. coli* as a model microorganism that works as biocatalyst. Various samples, such as 3,5-dichlorophenol (DCP), Ametryn, Fenamiphos and Endosulfan were chosen as toxicants. In this study, amperometry incorporating ferricyanide as a redox probe was employed to determine the whole effects of toxicity on *E. coli* respiration rather than testing all the potential single compounds. Especially ultramicroelectrode array (UMEA), effectively amplifying the signal from the total limiting currents of multi individuals, was used to distinguish a little change in toxicity [11]. Finally, the limiting currents were directly converted to inhibitory percentage by simple signal treatment. 50% inhibiting concentration (IC50) values determined using the electrochemical method are reliable and can be compared with other methods. In addition, the effect of pesticides on the morphology of cells (*E. coli*) was monitored by atomic force microscopy (AFM). We found that Endosulfan caused the greatest impact on *E. coli*, Ametryn in the middle and Fenamiphos was the lowest. This result is consistent with the result obtained with that of works reported previously.

## 2. Experimental

### 2.1. Chemicals and materials

Ametryn PESTANAL® (98.5% purity) was purchased from Sigma–Aldrich, Fenamiphos PESTANAL® (92.5% purity) and Endosulfan PESTANAL® (97.5% purity) were purchased from Dr. Ehrenstorfer GmbH. *E. coli* DH 5 α was obtained from Beijing Dingguo Changsheng Biotechnology Co., Ltd. DCP was taken from Aldrich. Glucose and glutamic acid were purchased from Sigma. Peptone and yeast extract were from OXOID Ltd.

All chemicals used in this study were of analytical grade, and all solutions were prepared with deionized water, unless otherwise indicated. Stock solutions of the pesticides were prepared in distilled water, when a compound had a low solubility in water, 0.5% (v/v) of dimethyl sulfoxide (DMSO) was used to dissolve it [12]. The standard GGA solution (150 mg/L glucose and 150 mg/L glutamic acid) was prepared according to American Public Health Association (APHA) standard methods. The Luria–Bertani (LB: 10.0 g/L tryptone, 5.0 g/L yeast extract, and 10.0 g/L NaCl) broth was adjusted to the desired pH with 1 mol/L HCl and sterilized in high-pressure steam at 120 °C for 20 min. $K_3[Fe(CN)_6]$ was freshly prepared before use.

### 2.2. Cultivation of microorganism

*E. coli* was used as a test microorganism and maintained on nutrient agar plates at 4 °C. Bacterial cultures were grown aerobically in LB substrate at 37 °C with a shaker (220 rpm for 10 h) to the stationary phase. The microorganisms were harvested by centrifugation at 4500 rpm for 10 min at room temperature, then washed twice with PBS and resuspended in PBS. The concentration of cells was adjusted to an absorbance value of 20.0, measured at 600 nm using a Cary 500 Scan UV-vis-NIR spectrophotometer and the cells were stored at 4 °C until required. The bacterial suspension was used for the experiments on the day of harvesting.

### 2.3. Toxicity assay

The toxicants were examined at several different concentrations. All solutions were pre-purged for 15 min using oxygen-free nitrogen at 37 °C in a water bath. Ten mL sample mixture for incubation containing 45 mM potassium ferricyanide, standard GGA solution, toxicant at the appropriate level and cell suspension with the absorbance value of ~5.0 were incubated anaerobically at 37 °C for 60 min. Control incubation contained PBS in place of the toxicant, positive and negative controls refer to the presence and absence of GGA substrate, respectively. To terminate the reaction, solutions were withdrawn and centrifuged at 10,000 rpm for 10 min. The supernatant solutions were maintained without oxygen and then taken for analysis.

### 2.4. Signal detection

Chronoamperometry at microelectrode array was used to measure the quantity of reduced mediator produced during the microbial incubation. The UMEA fabricated by six pieces of 25 μm single Pt ultramicroelectrode was used as a working electrode, a Pt gauze as auxiliary electrode and Ag/AgCl (saturated KCl) as reference electrode. The working electrode was polished using 0.05 μm α alumina slurry and thoroughly washed before use. All electrochemical measurements were conducted using a CHI 832B electrochemical workstation (Chen Hua, Shanghai).

The working electrode was poised at 450 mV and the anodic limiting current obtained within 10 s after imposition of the applied potential was recorded. For each toxicant concentration, the anodic limiting currents were converted to equivalent inhibitory percentage values following Eq. (1):

$$\text{inhibition\%} = \left(1 - \frac{i_{\text{lim(sample)}} - i_{\text{lim(s-control)}}}{i_{\text{lim(p-control)}} - i_{\text{lim(n-control)}}}\right) \times 100\% \quad (1)$$

where $i_{\text{lim(sample)}}$: output of limiting current at a appropriate concentration of sample and standard GGA solution; $i_{\text{lim(s-control)}}$: output of limiting current at a appropriate concentration of sample without GGA solution; $i_{\text{lim(p-control)}}$: output of positive control current; $i_{\text{lim(n-control)}}$: output of negative control current.

Eq. (1) is derived from the previous work by Pasco et al. [13], in which, the effects of toxicant on bacterial endogenous respiration were negligible.

### 2.5. Morphology characterization

*E. coli* cells were grown aerobically at 37 °C overnight on LB substrate by shaking incubation (220 rpm). When *E. coli* grew to its mid-log phase after 4–5 h, different volumes of pesticides were added into the cultures with final concentrations of 5 mg/L. The cell suspensions with pesticides shaken at 220 rpm were incubated for 1 h at 37 °C under aerobic condition. The control samples were prepared as the same way but used sterile deionized water (SDW) instead of pesticides. Then each sample was drawn out and washed with SDW for three times. The samples were placed on freshly treated cover slides and allowed to dry for ~30 min. All images were obtained from a tapping mode atomic force microscope (AFM, SPI3800N-Spa 400, Seiko Instrument). A rectangular Si cantilever/tip with a spring constant of 17 N/m and a resonance frequency of ~150 kHz were used. The scan speed was set at 0.7 Hz and final resolution was 256 × 256 pixels. Each scan resulted in a

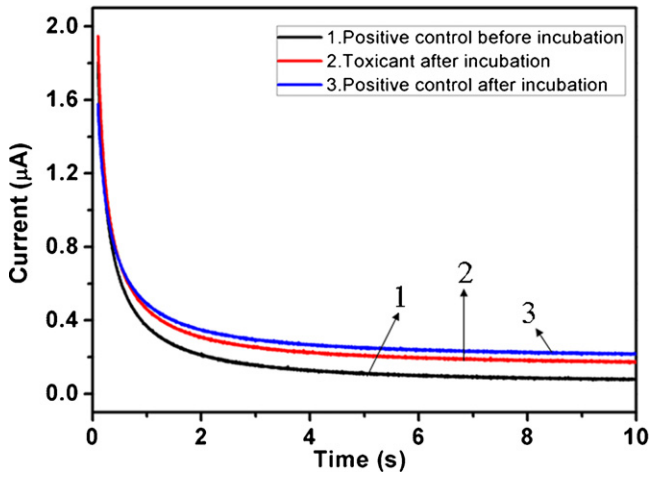


**Fig. 1.** *i–t* curves record electrochemical signals at Pt UMEA (diameter 25 μm, six pieces) before and after 1 h incubation of positive control, and the effect of 4 mg/L DCP on *E. coli*.

topography image and a phase image simultaneously. The height scale of cell was depicted as shades of gray, with bright area being nearer to the tip in topography images. The elasticity scale of cell was given as shades of gray, and whiter area showed more elastic in phase image.

## 3. Results and discussion

### 3.1. The optimum reaction conditions

In previous studies, chronoamperometry and coulometry had been widely employed in the BOD detection [14]. Morris et al. found the optimum temperature was 37 °C, this result was in accord with the behavior of *E. coli*, which was known to have maximum metabolic and respiratory activity at 37 °C. The resultant analytical signal curve (GGA–endog) showed a rapid early increase by 1 h, followed by a slower increase that eventually levelled off after 3 h [8]. Based on the study of pH influences, *E. coli* was found to display excellent activity at pH 5.0–7.0 [11]. The final concentration of 45 mM ferricyanide was adopted because of higher concentration of ferricyanide was harmful on *E. coli* [15].

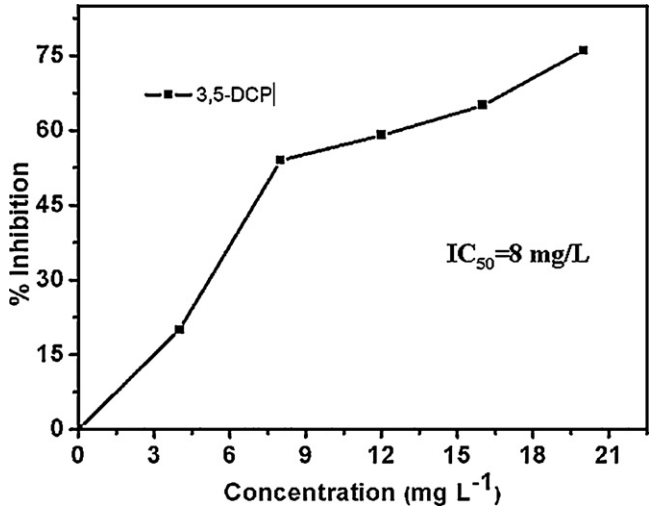


**Fig. 2.** Inhibitory curves of *E. coli* at different concentrations of DCP. Replicate samples containing 45 mM ferricyanide, standard GGA solution and cells suspension with the absorbance value of ~5.0.

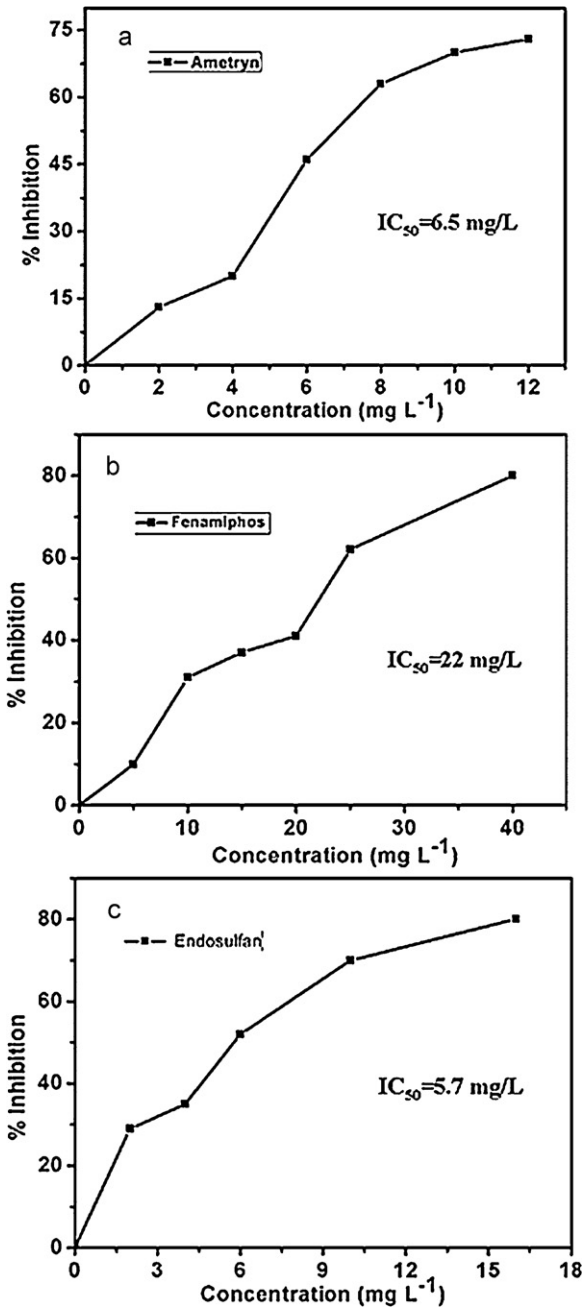


**Fig. 3.** Inhibitory curves of *E. coli* at different concentrations of Ametryn (a), Fenamiphos (b) and Endosulfan (c) are plotted. Replicate samples containing 45 mM ferricyanide, standard GGA solution and cells suspension with the absorbance value of ~5.0.

### 3.2. Toxicity assessment

#### 3.2.1. The toxicity of DCP

DCP was chosen as the reference toxicant as its toxicity has been widely studied using different approaches. The OECD 209 activated sludge respiration inhibition test (ASRIT) [16] also advised the use of DCP as a reference toxicant. Fig. 1 shows the variation of *i–t* curves under the same potential 450 mV. As expected, the steady and strong limiting current occurred at electrode less than 10 s and thus toxicity could be rapidly determined and distinguished sensitively even in a small difference of toxicant concentration variation. Fig. 2 shows the inhibitory curve of *E. coli* at different concentrations of DCP after 60 min incubation. Inhibition quotients for *E. coli* were calculated according to Eq. (1). When DCP was less than 4.0 mg/L, it

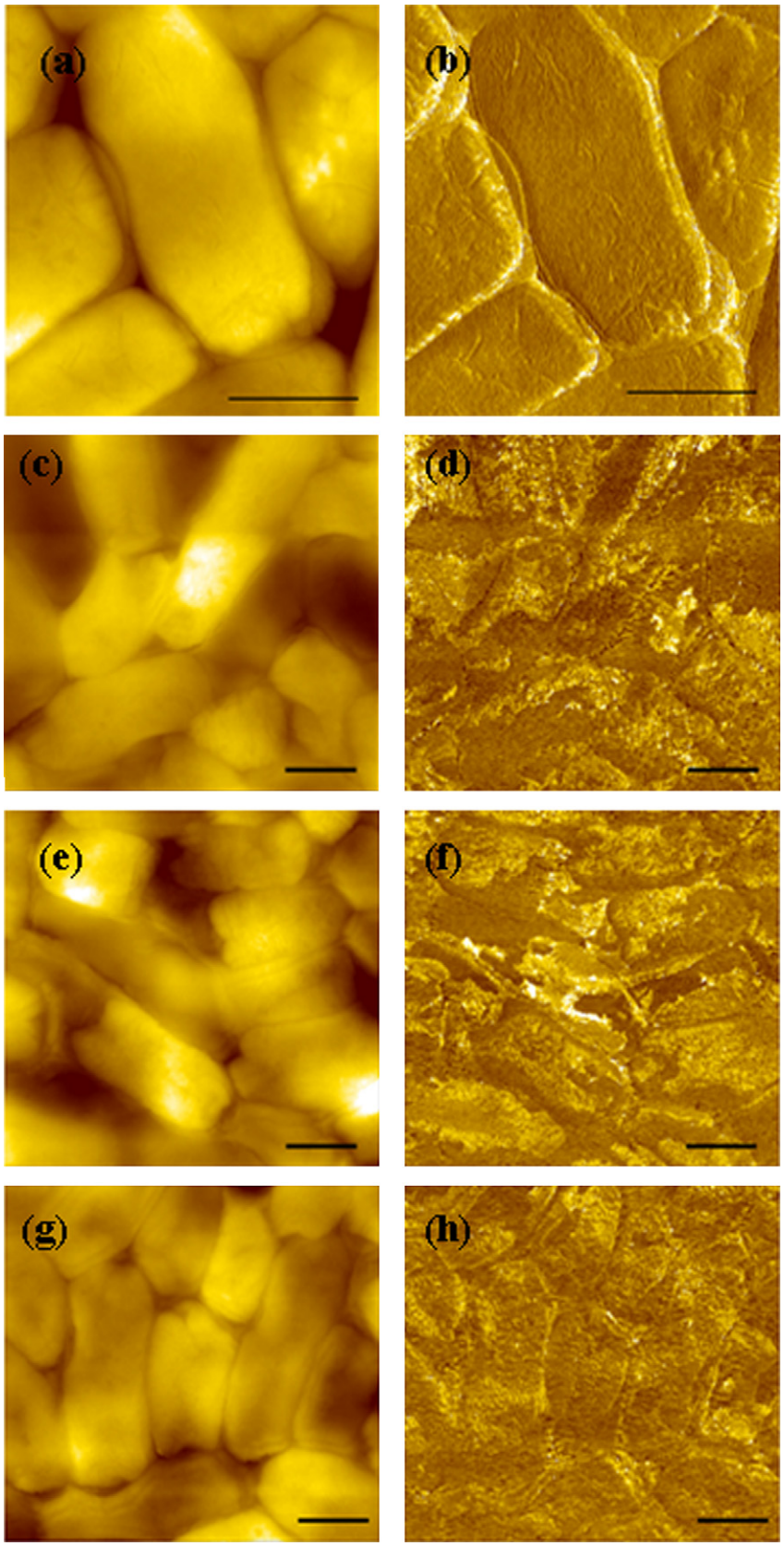


**Fig. 4.** AFM images of *E. coli* treated with three pesticides for 1 h. (a), (b) no pesticides, control; (c), (d) 5 mg/L Fenamiphos; (e), (f) 5 mg/L Ametryn; (g), (h) 5 mg/L Endosulfan. (a), (c), (e) and (g) are topography images; (b), (d), (f) and (h) are their phase images, respectively. Scale bar of all images are 1 μm.

**Table 1**
Comparison of electrochemical method IC50 values to other DTA assays.

| Toxicants | DTA assay (IC50 mg/L) | | |
|---|---|---|---|
| | Toxalert *V. fischeri* | Electrochemical method *E. coli* | Cellsense *P. putida* |
| Endosulfan | 5.63 | 5.7 | 0.928 |
| Fenamiphos | 31.63 | 22 | 3.38 |
| Ametryn | 18.64 | 6.5 | 2.21 |

has caused a small reduction in respiration. However, there was a significant fall in respiration when toxicant concentrations were at or greater than 8 mg/L. In this investigation, IC50 of DCP for 60 min incubation was more sensitive than that of Santos reported, such as activated sludge and *Vibrio fischeri* Cellsense with 9.8 mg/L and 37.5 mg/L [16], respectively. But our result was less sensitive than that of the standard Microtox® with 3.2 mg/L [17].

*3.2.2. The toxicities of pesticides*

Toxicities of Endosulfan, Fenamiphos and Ametryn to *E. coli* using the electrochemical method were examined, respectively. The pesticides studied were organochlorine, organophosphorus and a triazine pesticide, all of them were widely used around the world. From the selected compounds, Endosulfan is priority pollutant and used as an insecticide in a wide variety of food crops including fruits, vegetables and cereals. In many countries the use of Endosulfan is restricted due to acute toxicity towards aquatic animals and has been replaced by organophosphorus pesticides. Among the most common ones, Fenamiphos is one of the most prominent nematicide. The group of organophosphate compounds is nowadays the most widely used group of insecticides in the world. As many other triazines, Ametryn is a selective herbicide used on corn and potatoes for general weed control. The main problem presented for this pesticide is its persistence in groundwater. It moves both vertically and laterally in soil due to its high water solubility.

Considering the electrochemical method used, we chose these pesticides, showing no influence on electrochemical signal of the mediator (ferricyanide) based on their electrochemically inactive properties. The pesticide interferes the electronic interchange between the probe and the transducer when it is electrochemically active in the working potential window. Fig. 3 shows the inhibitory curves of *E. coli* exposed to three pesticides ((a) Ametryn, (b) Fenamiphos, and (c) Endosulfan) with different concentrations, respectively. Accordingly, IC50 values of 6.5 mg/L for Ametryn, 22 mg/L for Fenamiphos and 5.7 mg/L for Endosulfan were determined with 60 min incubation. The lowest IC50 value corresponded to Endosulfan, followed by Ametryn, indicating their high acute toxicity. The Fenamiphos showed the highest IC50 value represented the lowest acute toxicity among them. The compound which showed the highest toxicity was the organochlorine pesticide due to inhibition of the cellular response.

*3.3. Comparison with other DTA assays*

In order to demonstrate IC50 values determined using the electrochemical method is reliable, three pesticides were investigated and comparing the results with that of published data by other methods. Table 1 reports the IC50 values to Fenamiphos, Ametryn and Endosulfan as 22 mg/L, 6.5 mg/L and 5.7 mg/L, respectively. Obviously, Endosulfan was the most toxic, Ametryn in the middle and Fenamiphos was the lowest one of the three pesticides. As is known, the Toxalert® assays incorporate bioluminescent *V. fischeri*, with toxic responses being quantified by a change in the intensity of light emissions. The bioluminescent marine organism *V. fischeri* is also an Internationally Approved Toxicity Testing Standard (DIN 38412T34), and the EC50 values to Ametryn, Fenamiphos and Endosulfan were obtained as 18.64 mg/L, 31.63 mg/L and 5.63 mg/L, respectively. The results gotten by the two methods are comparable, where Toxalert® assay is not suitable for measurement of turbid solutions due to the decrease of light intensity. In contrast, measurements by the electrochemical method are not disrupted by turbidity, even when measuring suspensions, which is an advantage, especially for wastewater samples. Although Cellsense assay using *P. putida* immobilized on screen printed electrodes shows more sensitive response to the toxicants, but the time spend for treatment and immobilization is too long and was shown to be less reproducible than electrochemical method [16].

*3.4. Morphological characteristics of E. coli induced by pesticides*

AFM is a very helpful tool for imaging biological process at the nanometer level. Since its invention, AFM has been used more and more extensively to probe biosystems. The sample preparation process of AFM is simple, which effectively reduces artifacts on samples in comparison with other nanoscale determination [18]. The evidence of toxicity on bacteria can be obtained through atomic force microscopy (AFM), which directly observes the morphological alterations of cells before and after exposure to toxin [15,19,20]. In this study, AFM was used to characterize the morphology variation characteristics of *E. coli* induced by three pesticides, respectively. Normal bacterial images (Fig. 4(a) and (b)) show that untreated cells had a typical *E. coli* rod-shape and relatively smooth surface.

In contrast, AFM images show that with the addition of three pesticides, *E. coli* had surface morphology changes (Fig. 4(c)–(h)). We found the roughness of the bacterial surface increased and some obscure patches appeared on the bacterial surface. The cell wall disappeared and led to the lysis of the bacteria. All bacterial surfaces seemed very rough and a small amount of cytoplasm remained. However, we also observed that the survived cells that remained intact cytoplasmic membrane, and only convex patches were obviously shown on the envelope. *E. coli* was stimulated by pesticides and resulting in the appearance of a concave part. Fig. 4(c), (e), and (g) is topography images of *E. coli* treated with Fenamiphos, Ametryn and Endosulfan, respectively. It should be noted that the obscure patches in the image was gradually increasing (a < c < e < g), which showed the destruction of toxicant on *E. coli* was growing (Fenamiphos < Ametryn < Endosulfan).

## 4. Conclusions

We reported a rapid toxicity assay method using electrochemical biosensor for pesticides. It is well suited to a simple method of toxicity measurement. The UMEA used, effectively amplifying the current signal of ferrocyanide increased from a zero background, which provided a significant advantage over other tests. The obtained IC50 (60 min) for DCP is 8.0 mg/L, it is lower than that of other electrochemical methods. Three pesticides (Endosulfan, Fenamiphos and Ametryn) were investigated using this method. IC50 values of 6.5 mg/L for Ametryn, 22 mg/L for Fenamiphos and 5.7 mg/L for Endosulfan, were determined, respectively. They are in line with EC50 values reported in the literature. We also investigated the variation in morphology of *E. coli* induced by pesticides. AFM images clearly revealed that the compact patches were induced on the bacterial surface under the stimulation of pesticides. This result is consistent with that of other method reported. However, an extension of this work in future needs to be assessed under different conditions of mediators and biological systems. In general, the electrochemical method described has shown great potential as

an initial toxicity-screening tool. The ease of use, real time analysis and good correlation to reported EC50 values are particularly encouraging.

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