Analytica Chimica Acta 924 (2016) 21–28

Contents lists available at ScienceDirect

Analytica Chimica Acta

journal homepage: www.elsevier.com/locate/aca

Development of a mediated whole cell-based electrochemical biosensor for joint toxicity assessment of multi-pollutants using a mixed microbial consortium

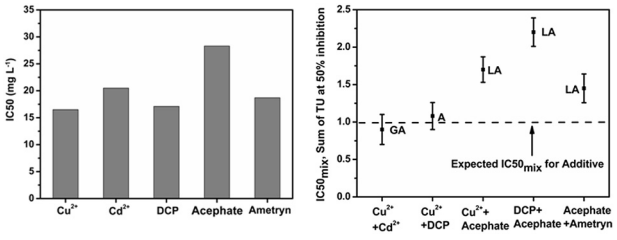
Guanyue Gao [a, b], Jun Qian [a], Deyu Fang [a, b], Yuan Yu [a], Jinfang Zhi [a, *]

[a] Key Laboratory of Photochemical Conversion and Optoelectronic Materials, Technical Institute of Physics and Chemistry, Chinese Academy of Sciences, Beijing 100190, PR China
[b] University of Chinese Academy of Sciences, Beijing 100049, PR China

HIGHLIGHTS

- In present work, a multi-species microbial biosensor with improved sensitivity and broadened detection range was developed.
- The joint toxicity effect of binary toxicants was analyzed to provide a reference for biotoxicity assessment of wastewater.
- A simple and low cost biosensor was fabricated, providing a reliable way for early risk warning of acute toxicity.

GRAPHICAL ABSTRACT



ARTICLE INFO

Article history:
Received 24 November 2015
Accepted 10 April 2016
Available online 20 April 2016

Keywords:
Mediated cell-based electrochemical biosensor
Mixed microbial consortium
Toxic unit
Joint toxicity

ABSTRACT

Since most risk assessment for toxicants is based on individual single-species test, the deduction of such results to ecosystem evaluation is afflicted with uncertainties. Herein, we successfully developed a p-benzoquinone mediated whole-cell electrochemical biosensor for multi-pollutants toxicological analysis by co-immobilizing mixed strains of microorganism, including *Escherichia coli* (gram-negative bacteria), *Bacillus subtilis* (gram-positive bacteria) and *Saccharomyces cerevisiae* (fungus). The individual and combined toxicities of heavy metal ions (Cu^{2+}, Cd^{2+}), phenol (3,5-dichlorophenol) and pesticides (Ametryn, Acephate) were examined. The experimental results showed that the order of toxicity for individual toxicant was ranked as Cu^{2+} > 3,5-dichlorophenol (DCP) > Ametryn > Cd^{2+} > Acephate. Then the toxic unit (TU) model was applied to determine the nature of toxicological interaction of the toxicants which can be classified as concentration additive ($IC50_{mix}$ = 1TU), synergistic ($IC50_{mix}$ < 1TU) and antagonistic ($IC50_{mix}$ > 1TU) responses. The binary combination of Cu^{2+} + Cd^{2+}, Cu^{2+} + DCP, Cu^{2+} + Acephate, DCP + Acephate, Acephate + Ametryn were analyzed and the three kind of joint toxicity effects (i.e. additive, synergistic and antagonistic) mentioned above were observed according to the dose-response relationship. The results indicate that the whole-cell electrochemical biosensor based on mixed microbial consortium is more reasonable to reflect the joint biotoxicity of multi-pollutants existing in real wastewater, and combined effects of toxicants is extremely necessary to be taken into

* Corresponding author. Key Laboratory of Photochemical Conversion and Optoelectronic Materials, Technical Institute of Physics and Chemistry, Chinese Academy of Sciences, Beijing 100190, PR China.
E-mail address: zhi-mail@mail.ipc.ac.cn (J. Zhi).

http://dx.doi.org/10.1016/j.aca.2016.04.011

account in ecological risk assessment. Thus, present study has provided a promising approach to the quality assessment of wastewater and a reliable way for early risk warning of acute biotoxicity.

1. Introduction

Recent decades have witnessed extensive industrialization and farming development, simultaneously, with insufficient waste management policies, the aquatic ecosystems are suffering severe pollutants damage, causing a dramatic drop in world fresh water resources and exerting a potential harm to people's health. To implement proper regulation over the emission of industrial effluent and agricultural sewage, an efficient and reliable monitoring system is demanded for acute wastewater toxicity assessment [1,2]. To achieve that, a wide range of laboratory models of biotoxicity analyzers have been invented by using plant tissues [3], invertebrates [4], fish [5] and some bacterial species [6–8] as test organisms. The sensing scheme of these methods mainly relies on monitoring certain physiological changes in the living organism under the effect of an environmental stimulus. However, many of these methods require several days or even longer time to be completed, involving complex and tedious procedures, and also need highly skillful personnel to obtain reproducible results. These disadvantages, especially the long duration time, make these methods unsuitable for real-time detection or on-line measurement. Consequently, there is a great challenge for developing effective testing techniques, which can provide rapid, sensitive, simple and low-cost measurements to assess total biotoxicity of the targeted water.

Microorganism incorporated electrochemical biosensors could meet these demands because of their short life cycle and instant response to toxicants, and therefore were rapidly adapted to toxicological studies for assessing organisms or ecosystem health [4,9–12]. The main advantages offered by electrochemical biosensors over conventional analytical techniques are the possibility of portable, miniaturized and working on-line, in addition, their ability to measure pollutants in complex matrices with minimal sample preparation also attracted considerable attention [13]. Thus, for toxicants detection, cell-based biosensors (CBB) are emerging as unique alternatives to other analytical methods [14]. To fabricate the CBB devices, the living cells incorporate both prokaryotic (bacteria) and/or eukaryotic (yeast, invertebrate and vertebrate) cells are directly integrated onto the biosensor platform [15]. The main advantages using the CBB are: (a) the cells were immobilized on biosensor, unlike a system with cells in the free state, and the immobilized biosensor can easily be stored and transported, thus, it is convenient to be applied *in situ*; (b) the immobilization of cells on the sensing platform will increase the stability of biosensor, and will definitely enhance the reproducibility of the method [16]. Moreover, unlike enzyme and immuno-based detection systems where the specificity of the biocatalyst is exploited, the CBB can respond to a wide range of targets and are considered as particularly suitable for the application in biotoxicity testing and environmental monitoring where the source and ingredient of the toxicants or pollutants cannot be predicted. Additionally, mediated electrochemical biosensors have the superiority of instant response and no interference from the turbidity of samples, even when measuring suspensions.

To create a cell-based biosensor, the test microbe is the key element by which the signal information is captured. At present, most biotoxicity assessment methodologies use single-specie microorganism as the recognition element to estimate water quality. However, since the real wastewater sample is a complicated contaminative system which contains variety of pollutants, the traditional single-species microbial sensors are not capable enough to detect the total biotoxicity of wastewater samples objectively. Moreover, in previous reported researches, various strains bacteria were used to develop biosensors, their sensitivity is different and the reproducibility is poor for different kind of pollutants. To reflect the authentic condition of the aquatic environment, multi-species microbes are applied to the bioassay systems and expected to provide a more realistic evaluation of water biotoxicity [17–20]. There were several studies based on the multi-species-test, such as activated sludge [21], which has a broad detection range and more convincing detection results, but there are also significant differences between the bacterial flora of different activated sludge in composition and structure, leading to the test results not comparable between laboratories. For all of these reasons, we developed a microbial biosensor based on mixed consortium microbes for wastewater biotoxicity analysis. To the best of our knowledge, mixing different microbes into the same matrix film, and modifying electrodes by the mixed microbes biofilm for wastewater biotoxicity assay has not been reported yet.

On the other hand, toxicants are often found as mixtures in the environments, but detection methods are normally designed to estimate the biotoxicity of a single pollutant. In joint toxicity tests, the common used method to determine the combined effects is the toxic unit (TU) approach. In the TU model, the concentrations of the various toxicants presented in the mixture are expressed as TU, the fractions of the EC50 or IC50 values. The joint effect of the toxic pollutants is then judged by comparing the real IC50 values of the mixture ($IC50_{mix}$) with the corresponding sum of toxic units [22]. The joint effects of binary toxicants on a specific organism could be less than, equal to, or greater than that calculated from the sum of the individual chemicals, so-called "antagonistic effect", "additive effect" and "synergistic effect", correspondingly [23]. Therefore, joint toxicity experiments of the mixed toxicants can reflect the genuine hazard of contaminated environments much better than experiments in which the effect of a single toxicant is tested [24].

In this study, we primarily measured and evaluated the toxicities of heavy metal ions (Cu^{2+}, Cd^{2+}), DCP and pesticides (Ametryn, Acephate) separately as well as their mixtures, by using a multi-species microbial whole-cell sensing system. The microorganism includes *Escherichia coli* (gram-negative bacteria), *Bacillus subtilis* (gram-positive bacteria) and *Saccharomyces cerevisiae* (fungus). Results from the individual toxicity tests revealed that the order of toxicity for the five toxicants ranked as Cu^{2+} > DCP > Ametryn > Cd^{2+} > Acephate. In addition, results of the binary experiments implied that different kinds of toxicants may have interaction in the wastewater system, which will change their toxic effects on microorganism. The results indicate that this multi-species microbial whole-cell biosensor can be efficiently used to assess the joint effects of toxicant mixture and reflect the genuine biotoxicity of real wastewater samples. Thus, our studies have provided a promising approach to the quality assessment of wastewater and a reliable way for early risk warning of acute biotoxicity.

2. Experimental

2.1. Chemicals and reagents

$CuSO_4 \cdot 5H_2O$, $Cd(NO_3)_2 \cdot 4H_2O$, 3,5-DCP, Acephate, Ametryn, glucose and p-benzoquinone were provided by Beijing Lanyi Chemical Products Co., Ltd., China. Peptone, beef extract and yeast extract were obtained from Beijing Aoboxing Bio-tech Co., Ltd., China. All chemicals were of analytical grade and used as received without further purification. All solutions were prepared using deionized water obtained from Millpore Mill-Q purification system (M-Q water, >18 MΩ cm). A cocktail of respiratory substrates (sodium lactate, sodium succinate and glucose each at 10 mM) in 0.85% saline were prepared for resuscitating and monitoring the biosensor. p-Benzoquinone (BQ) was used as mediator and BQ stock solution with desired concentration was wrapped with aluminum foil and kept at 4 °C refrigerator for further use. All toxicants (Cu^{2+}, Cd^{2+}, DCP, Acephate, Ametryn) in desired concentration were freshly prepared in Milli-Q water from high-concentration stock solutions. Stock solutions were freshly prepared or kept at 4 °C. Low solubility compounds were dissolved through the assistance of 0.5% (v/v) dimethyl sulfoxide (DMSO) [25]. The three kinds of real wastewater samples were taken from garbage-treatment plant, electroplating factory and laboratory respectively. The biotoxicity of above three real wastewater samples were tested without further treatment.

2.2. Cultivation of microorganisms

E. coli (ATCC25922), *S. cerevisiae* (S288C) and *B. subtilis* (CGMCC 1.1086) were inoculated from China General Microbiological Culture Collection Center (CGMCC). *E. coli* and *B. subtilis* were cultured in 100 mL autoclaved Nutrient Broth solution (10 g L^{-1}peptone, 3 g L^{-1}beef extract, and 5 g L^{-1}NaCl) for 16 h and 24 h at 37 °C, respectively. A 100 mL autoclaved Yeast Extract Peptone Dextrose medium (YEPD, 1% yeast extract, 2% peptone, 2% glucose) was inoculated with a colony of *S. cerevisiae* and grown aerobically at 30 °C for 24 h on a rotary shaker (200 rpm), this allows the cells to reach the exponential-log growth phase. The microorganisms were harvested by centrifugation at 5000 rpm for 10 min at room temperature, then washed twice with PBS and resuspended in PBS. The final concentration of *E. coli*, *B. subtilis* and *S. cerevisiae* were adjusted to an absorbance value of 20.0 at 600 nm (OD_{600}), measured by Secoman Uvikon UV–Vis spectrophotometer. The cell suspensions with desired concentration were stored at 4 °C until required. The bacterial suspension was used for the experiments on the day of harvesting.

2.3. Preparation of multi-species microbial biofilm

The *E. coli*, *S. cerevisiae* and *B. subtilis* cell suspension with desired concentration were mixed in designated ratio to form the multi-species microbial mixture and kept at 4 °C refrigerator before use. The biofilm was prepared as following procedure: 100 μL 10% (w/v) PVA (degree of saponification 98%, degree of polymerization 2400) solution, 10 μL 1.0 M Na_2SO_4 aqueous solution and 40 μL 0.8% (w/v) sodium alginate solution were mixed adequately. Then 50 μL as prepared cell suspension was added to the mixed solution above. The resulting mixture with all three kinds of cells was spun to the surface of a polyethylene terephthalate (PET) plastic sheet. The PET sheet coated with cells was further dripped in a 2% (w/v) $CaCl_2$ solution and crosslinked for 15–20 min. A PVA-alginate microbial biofilm was formed after above procedure and peeled off from the PET sheet template. The biofilm prepared was washed thoroughly with PBS and stored at 4 °C until use [7].

2.4. Electrochemical measurements and biotoxicity assay

The electrochemical experiments were performed with a potentiostat/galvanostat (Model 263 A, Princeton, USA). The electrochemical measurements were carried out in a biofilm reactor (BFR), including a platinum sheet working electrode, platinum wire auxiliary electrode, and Ag/AgCl (saturated KCl) reference electrode. The platinum sheet electrode is of 1 cm × 1 cm size and polished by 30–50 nm α-alumina powder each time before use. The polished platinum sheet electrode was ultrasonic washed by nitrate, ethanol and distilled water successively. The working electrode was modified by attaching an appropriate size of biofilm prepared above on the polished platinum electrode, the modified working electrode was fixed on the reactor by the O-ring.

The biosensor signals were monitored in a stirred vial containing 10 mL pH 7.0 respiratory substrates solution and the BQ mediator was used to divert electrons from the respiratory system of the immobilized microbes to the platinum sheet electrode. The chronoamperometric experiments were performed at 300 mV with respect to an Ag/AgCl (sat' KCl) reference electrode at room temperature. The electric current was monitored and the data were displayed in real-time as current against time plots. After a stabilization period about 5 min, 100 μL of BQ solution was added to the monitoring system with the final concentration of 0.4 mM. Target toxicants were added into the vial shortly after the second period of stabilization was achieved. After adding a toxicant sample, the current was decreased significantly because of their detrimental effect to metabolic activities of microorganisms. The toxicity of the sample can be determined by measuring current changes of the BQ-mediated respiration chain activity. BQ can be reduced to hydroquinone (HQ) during cell respiration, and the resultant HQ can diffuse to the platinum sheet electrode and reoxidized to BQ at the electrode surface. The resulting current is proportional to the metabolic status of the cell. For each toxicant concentration, the anodic currents were converted to equivalent inhibitory percentage values according to Eq. (1):

$$\text{Inhibition}\% = (1 - I_2/I_1) \times 100\% \quad (1)$$

Where I_1 is the steady-state current before adding the toxicant, I_2 is the steady-state current after adding the toxicant.

2.5. Joint toxicity tests

Joint toxicity experiments were conducted according to TU approach [26]. In the TU model, the toxicants concentrations in the mixture are expressed as TU, which was calculated as fractions of their half maximal inhibitory concentration IC50. The sum of TU in the mixture can be expressed by the Eq. (2)

$$\sum \text{TU}_i = \sum_{i=1}^{n} \frac{c_i}{\text{IC}x_i} \quad (2)$$

Where n is the number of toxicants in the sample, c_i is measured concentration of the individual toxicant in the sample, and ICx_i is the concentration of ith sample component that cause x% inhibitory effect magnitude.

In joint toxicity tests, toxicants were mixed in different toxic units. The mixture were prepared by adding the appropriate amount of each toxicant at the same concentration as in their individual experiments, but their toxic units are varies. Joint toxicity experiments were composed of five binary mixtures: Cu^{2+} + Cd^{2+},Cu^{2+} + DCP, Cu^{2+} + Acephate, DCP + Acephate and Acephate + Ametryn, which represent the combination of binary metal ions, metal ion and phenol, metal ion and pesticide, phenol

and pesticide and two pesticides, respectively.

2.6. The biotoxicity assay and composition determination of real wastewater samples

The biotoxicity of three real wastewater samples (landfill wastewater, electroplating wastewater and laboratory wastewater) were estimated by chronoamperometry, the *i*-t curves were recorded to observe the real-time signal changes in the toxicity detection procedure. The inhibition rate was calculated according to Eq. (1), and the biotoxicity level of three real water samples was determined. The composition analysis of real water samples were performed by inductively coupled plasma atomic emission spectrometry (ICP-AES) (Varian 710-ES ICP Optical Emission Spectrometer). The chemical oxygen demand (COD) values of these three samples were measured by the closed reflux colorimetric method with spectrophotometer (HACH DRB 200).

3. Results and discussion

3.1. Feasibility assay of multi-species microbial biosensor to acute biotoxicity assessment

Previous reports [7,8,30] indicated that single microorganism specie as recognition element have a defect of especially sensitive to certain group pollutants while no response to others. For instance, *E. coli* have an instinct response to heavy metal ions, but for the organic pollutants, their acute toxicity response is relatively low. Whereas, *B. subtilis* have an exactly opposite sensitivity response by contrast to *E. coli*. Thus, to realize the accurate detection of biotoxicity of all types of toxic substances, we proposed a methodology to mix three different microorganism species together to prepare a mixed microbial consortium biosensor. The microorganisms include *E. coli* (gram-negative bacteria), *B. subtilis* (gram-positive bacteria) and *S. cerevisiae* (fungus). To prove the feasibility of proposed approach, the toxicity of 10 mg L^{-1} Cu^{2+}, 10 mg L^{-1} DCP and 25 mg L^{-1}Ametryn were detected as representative toxicants of heavy metal ions, phenols and pesticides. The sensitivity of different kinds of microorganism and mixed microbes was evaluated by comparing the inhibition rate of three representative toxicants above. The mixed ratio of multi-species microbes is *E. coli*, *B. subtilis* and *S. cerevisiae* = 2:2:1. As shown in Fig. 1, *B. subtilis* is more sensitive to phenols for its highest 27.29% inhibition rate of 10 mg L^{-1} DCP among all three kinds of microbes; whereas *E. coli* is more suitable for the toxicity assessment of heavy metals and pesticides; *S. cerevisiae* is least sensitive to the test toxicants among all three microorganisms and this may contribute to the complex cell structure of fungus. As contrast to the selective sensitivity of single microbe, the mixed consortium showed broad-spectrum sensitivity to no matter heavy metal ions, phenols and organic pesticides. As we can observed from Fig. 1, the inhibition rate of mixed microbes with ratio of 2:2:1 showed the highest response sensitivity to all the toxicants studied, which implying that the methodology of using multi-species microbes as biosensor signal receptor is feasible to the acute biotoxicity assessment of wastewater samples.

3.2. The optimization of mixed ratio of the multi-species microbes

In order to obtain better detection sensitivity, the mixture proportion of three kinds of microbes was optimized. 10 mg L^{-1} Cu^{2+}, 10 mg L^{-1} DCP and 25 mg L^{-1}Ametryn was used as representative toxicants of heavy metal ions, phenols and pesticides, respectively. Different mixture proportions of *E. coli*, *B. subtilis* and *S. cerevisiae* = 2:2:1, 1:3:1, 3:1:1, 1:1:1 were analyzed, and the optimized ratio was determined by compare the inhibition rate of each mixed proportion. As shown in Fig. 2, among all the mixtures of three microbes, the mixed microbes of *E. coli*, *B. subtilis* and *S. cerevisiae* = 2:2:1 show distinct sensitivity to all the three representative toxicants compared to other mixture of various ratios. The improvement of the sensitivity of mixed strains may ascribe to the interactions (resource competition and competitive exclusion) between different microbial consortium and the susceptibility of each microorganism species to toxicants [27]. Therefore, according to above the discussion, the proportion of *E. coli*, *B. subtilis* and *S. cerevisiae* = 2:2:1 was selected as the optimized ratio and applied in the following experiments.

3.3. The single toxicity tests by multi-species microbial biosensor

To verify the wide applicability of multi-species microbial biosensor to broad spectra toxic chemicals, the biotoxicity of two heavy metal ions (Cu^{2+}, Cd^{2+}), one phenol (DCP) and two pesticides (Ametryn, Acephate) were examined. Chronoamperometry was applied to detect the on-line current change in the monitoring process. As shown in Fig. 3, in the stabilization period, the current is nearly zero, because microbial cells' metabolism activity can hardly be examined without the mediator. After 0.4 mM p-benzoquinone mediator injected into the solution, there is a sharp increase in the current value, which contribute to the amplification effect of the p-benzoquinone mediator. BQ can be reduced to hydroquinone (HQ) during cell respiration, and the resultant HQ can diffuse to the platinum sheet electrode and reoxidized to BQ at the electrode surface. After the second period of current stabilization, the toxicant was added into the system and an apparent current decrease was observed due to the detrimental effect of toxicant. The

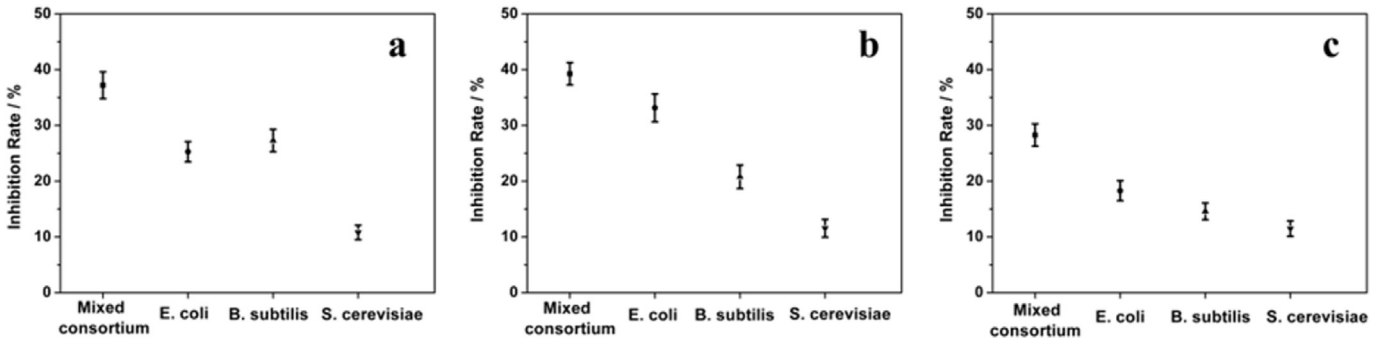


Fig. 1. The inhibition rate of single *E. coli*, *B. subtilis*, *S. cerevisiae* and their mixtures at the proportion of 2:2:1 to 10 mg L^{-1} DCP, 10 mg L^{-1} Cu^{2+} and 25 mg L^{-1}Ametryn. (a) The inhibition rate of 10 mg L^{-1} DCP; (b) The inhibition rate of 10 mg $L^{-1}Cu^{2+}$; (c) The inhibition rate of 25 mg L^{-1}Ametryn.

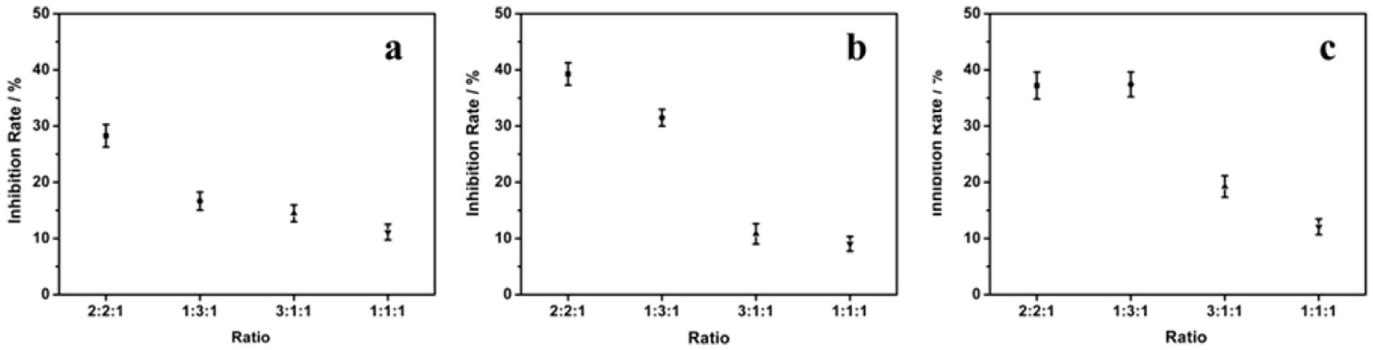


Fig. 2. The inhibition rate of multi-species microbe mixtures at different proportions to 10 mg L^{-1} DCP, 10 mg L^{-1} Cu^{2+} and 25 mg L^{-1}Ametryn. (a) The inhibition rate of 10 mg L^{-1} DCP; (b) The inhibition rate of 10 mg $L^{-1}Cu^{2+}$; (c) The inhibition rate of 25 mg L^{-1}Ametryn.

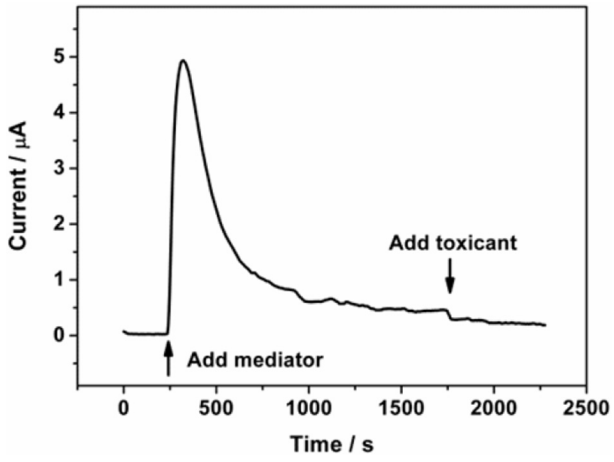


Fig. 3. The chronoamperometry curve of prepared multi-species microbial biosensor in toxicity determination process. The mediator is p-benzoquinone with final concentration of 0.4 mM, the detected toxicant is 20 mg L^{-1} DCP.

inhibition rates were calculated by Eq. (1) in a series of toxicants concentrations. The inhibitory curves of five individual toxicants were shown in Fig. 4. Because of the detrimental effect of the toxicants, the viability of cells decreased dramatically as the toxicants concentration increased. The IC50 values can be deduced from the inhibitory curves, for Cu^{2+}, Cd^{2+}, DCP, Acephate and Ametryn, and their IC50 values were 16.5, 20.5, 17.1, 28.3 and 18.7 mg L^{-1}, respectively. Therefore, the toxic order detected by the multi-species microbial biosensor was ranked as Cu^{2+} > DCP > Ametryn > Cd^{2+} > Acephate according to the IC50. The toxic order is consistent with the results obtained from single-specie microbial biosensors [7,8,11], whereas the sensitivity of prepared biosensor is improved. As shown in Table 1, in the previous reported single microbe biosensors, although the detected IC50 value of a certain toxicant is lower than present method, their detection of broad-spectrum toxicants sensitivity is relatively low. From the comparison, we can conclude that the approach we proposed in this study has a better sensitivity and extended detection spectra, which indicate that above method is a promising way for the risk warning of wastewater acute toxicity.

3.4. Joint toxicity of binary toxicants

Chemical pollutants always exist as mixtures in the environment, and water pollution is also a multi-components problem especially in wastewater acute toxicity detection. To reflect the combined effects of binary toxicants in the aquatic system, five kinds of combination of toxic chemicals were selected and examined in present study and their joint toxicity were analyzed by using the TU model. The combination of binary toxicants include Cu^{2+} + Cd^{2+}, Cu^{2+} + DCP, Cu^{2+} + Acephate, DCP + Acephate and Acephate + Ametryn, which represent the joint toxicity analysis of binary metal ions, metal ion and phenol, metal ion and pesticide, phenol and pesticide and two pesticides, respectively. The IC50 values of the individual toxicants obtained above were used to calculate the IC50 for the mixture, $IC50_{mix}$. The concentration gradients applied in the binary toxicants tests were exactly the same as the individual toxicity experiments whereas the concentrations of each individual toxicants were converted to TUs by dividing the concentrations of individual toxicant IC50 (TU = concentrations/IC50) as shown in Table 2. And the IC50 value of each toxicant was used as 1TU for the subsequent joint toxicity text in the mixture. As for the experiments of combined effects, the joint toxicity was measured by the $\sum TUi$ of binary compounds, as shown in Table 3. The $\sum TUi$ was calculated by Eq. (2), and the inhibitory curves was drawn against $\sum TUi$. From the resulting dose (TU-based)–response relationships, $IC50_{mix}$, sum of toxic unit ($\sum TU_i$) at 50% inhibition for the mixture, were calculated. The combined effect was defined as concentration additive ($IC50_{mix}$ = 1TU), synergistic ($IC50_{mix}$ < 1TU) or antagonistic ($IC50_{mix}$ > 1TU) [22].

As shown in Fig. 5 (a-e), the dose-response curves of five binary mixtures were drawn against TU, and the $IC50_{mix}$ value of each mixture was estimated as toxic unit. From the comparison in Fig. 5 (f), binary combinations of the five kinds of toxicants produced all three types of interactions: less than additive (LA, antagonistic), additive, and greater than additive (GA, synergistic) responses. When Cu^{2+} and Cd^{2+} were added in combination, $IC50_{mix}$ for multi-species was 0.9 indicating a synergistic response of two metal ions to the poisonous effect on the microbes. This may ascribed to the enlargement of cell membrane permeability when two heavy metal ions coexistence in the binary mixture [28], since Cu^{2+} can be readily more bioavailable than other toxicants for its lowest IC50 value compared to others. Antagonistic response to multiple bacteria occurred when Cu^{2+} + Acephate, DCP + Acephate and Acephate + Ametryn mixed together because the $IC50_{mix}$ values were detected to be 1.7, 2.2 and 1.45, respectively, which are significantly greater than 1, indicating that the addition of the second toxic component to the system will decrease the toxicity of the first compound or vice versa. This antagonistic response may be due to the formation of less bioavailable complex to the microorganisms. Binary mixtures of Cu^{2+} + DCP produced additive responses for the joint toxicity test as the $IC50_{mix}$ values for Cu^{2+} + DCP was 1.08 approximately equal to 1. This means that two

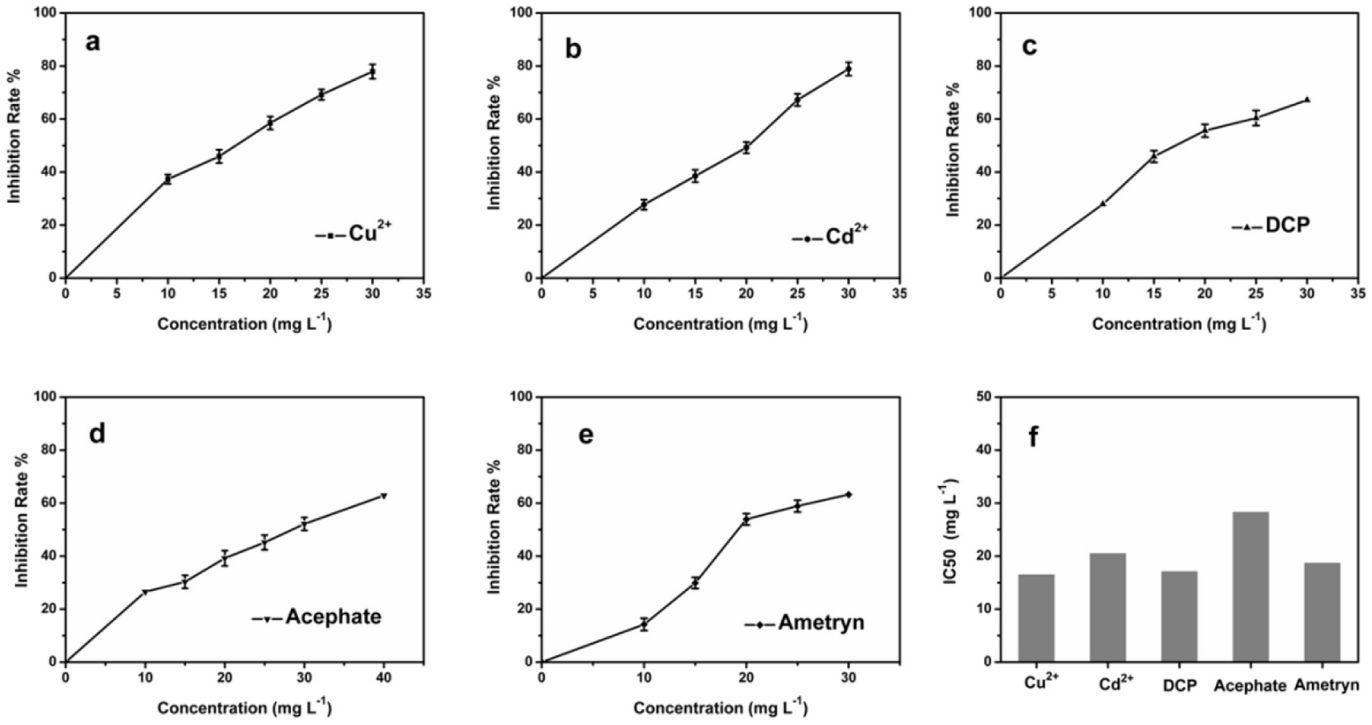


Fig. 4. Inhibitory curves of the toxicants at different concentrations (a) Cu^{2+}, (b) Cd^{2+}, (c) DCP (d) Acephate, (e) Ametryn under the optimized conditions. Data points represent the average of three replicates. (f) IC50 values of five individual toxicants obtained from the inhibitory curves.

Table 1
Comparison of IC50 values by current biosensor with other methods.

Toxicity assays	Toxicants, IC50 (mg L^{-1})					References
	Cu^{2+}	Cd^{2+}	DCP	Ametryn	Acephate	
Amperometry mixed microbes	16.5	20.5	17.1	18.7	28.3	Present study
Amperometry, *E. coli*	–	93.35	8.7	–	–	[29]
Amperometry, *E. coli*	44	79	–	–	–	[8]
Colorimety, *B. subtilis*	5	27.6	–	–	–	[30]
Amperometry, *S. cerevisiae*	–	–	9.83	22	29	[7]
Amperometry, *Psychrobacter. sp*	2.6	47.3	–	–	–	[31]

Table 2
Toxic units of individual toxicants in different concentration gradients.

Concentration (mg L^{-1})	$TU_{Cu^{2+}}$	$TU_{Cd^{2+}}$	TU_{DCP}	$TU_{Acephate}$	$TU_{Ametryn}$
0	0	0	0	0	0
5.0	0.31	0.25	0.29	0.17	0.27
10.0	0.61	0.49	0.58	0.35	0.54
15.0	0.91	0.73	0.88	0.53	0.80
20.0	1.21	0.98	1.17	0.71	1.07
25.0	1.52	1.22	1.46	0.88	1.34
30.0	1.82	1.46	1.75	1.06	1.61

chemicals may not interfere with each other in mixture.

3.5. Analysis of real wastewater samples

Real wastewater is a complicated contaminated sample, which combines of a variety of toxic substances. To evaluate the feasibility of the developed multi-species microbial biosensor, three real wastewater samples were investigated. The chronoamperometry was applied to observe the real-time signal change in the toxicity detection procedure. As shown in Fig. 6, The detected inhibition

Table 3
The sum of TU*i* of binary toxicants.

Concentration (mg L^{-1})	$TU_{Cu^{2+}+Cd^{2+}}$	$TU_{Cu^{2+}+DCP}$	$TU_{Cu^{2+}+Acephate}$	$TU_{DCP+Acephate}$	$TU_{Acephate+Ametryn}$
0	0	0	0	0	0
5.0	0.56	0.60	0.48	0.46	0.44
10.0	1.11	1.19	0.96	0.93	0.89
15.0	1.64	1.79	1.44	1.41	1.33
20.0	2.19	2.38	1.92	1.88	1.78
25.0	2.74	2.98	2.40	2.34	2.22
30.0	3.28	3.57	2.88	2.81	2.67

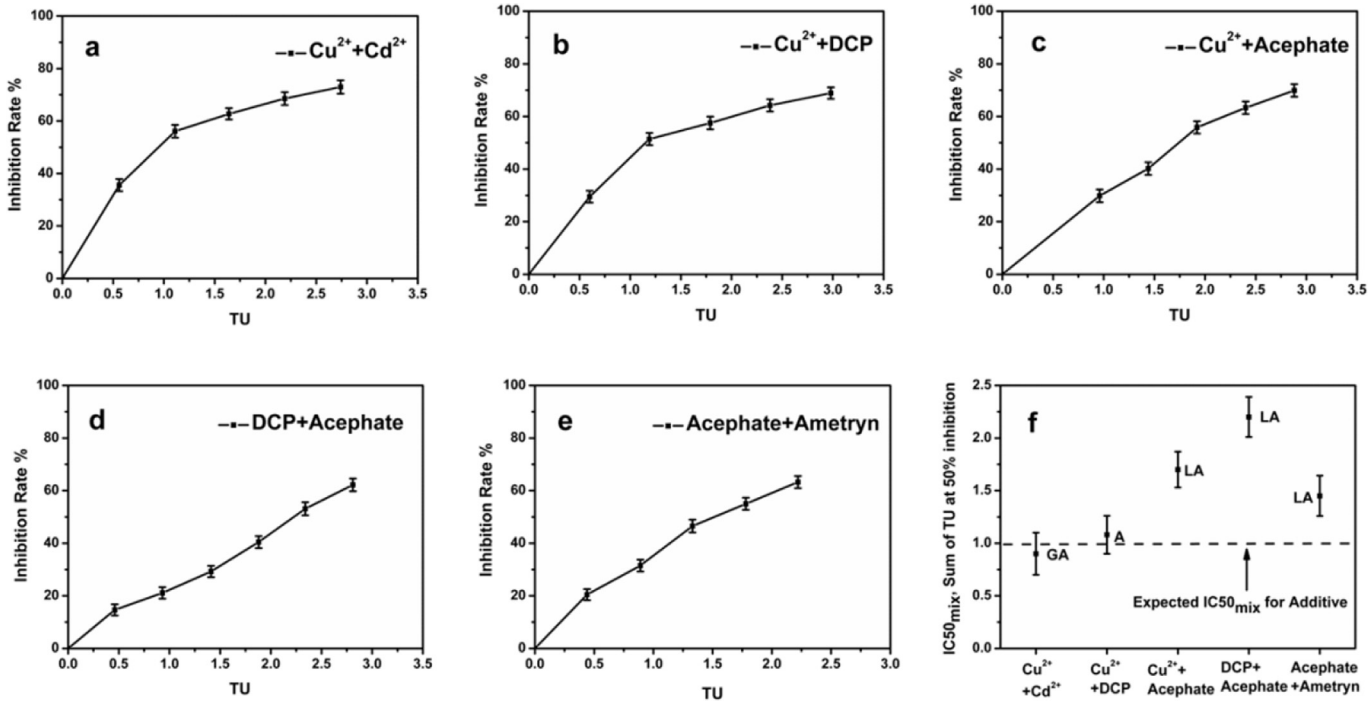


Fig. 5. The dose (TU-based)–response relationship curves of the binary toxicants at different concentrations. (a) Cu^{2+} + Cd^{2+}, (b) Cu^{2+} + DCP, (c) Cu^{2+} + Acephate, (d) DCP + Acephate and (e) Acephate + Ametryn under the optimized conditions. Data points represent the average of three replicates. (f) The half maximal inhibitory concentration of mixture ($IC50_{mix}$) to microbial cells exposed to binary mixtures. $IC50_{mix}$ is estimated as toxic unit (unitless). LA: less than additivity, A: simple additivity, GA: greater than additivity.

rates are 39.83%, 48.15%, 51.77% for landfill wastewater, electroplating wastewater and laboratory wastewater, respectively, which reflect the toxic order of three real wastewater samples is laboratory wastewater > electroplating wastewater > landfill wastewater. The toxicity difference of real samples may contribute to the composition diversity of these samples. As listed in Table 4, the heavy metal ions ingredients of real water samples are measured by ICP-AES and organic toxicants content are deduced from the COD values. The electroplating wastewater have the highest heavy metal ions content whereas the organic toxicants ingredient is relatively low compared to landfill wastewater, which result in the above biotoxicity level. As to laboratory wastewater, either heavy metal ions or organic pollutants content is very low, however, it was estimated to be the most toxic real water sample among others. The acidic pH value may attribute to the above toxic order. In summary,

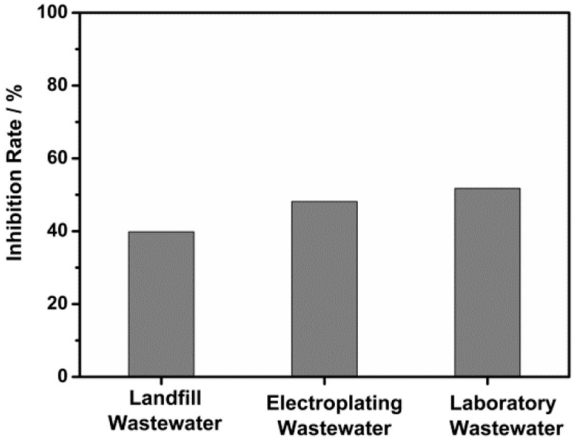


Fig. 6. The inhibition rate of three real wastewater samples.

Table 4
Compositions of real wastewater samples.

Real wastewater samples	Heavy metal ions (mg L^{-1})				COD (mg L^{-1})	pH
	Ni^{2+}	Cu^{2+}	Zn^{2+}	Cr^{2+}		
Landfill wastewater	48	–	–	0.2	19203	6.88
Electroplating wastewater	30–80	30–60	50	–	5760	6.9
Laboratory wastewater	0.7	0.4	–	–	754	4.5

the detected results is coincidence with the predicted toxic order refer from their compositions, which implying that the approach can effectively use to detect the toxicity of real wastewater samples. It cannot only reflect the toxicity of heavy metal ions and organic pollutants but also can indicate the pH abnormal of water samples, which providing an precise early risk warning method of aquatic system.

4. Conclusions

A mediated cell-based electrochemical biosensor with a mixed microbial consortium (*E. coli*, *B. subtilis* and *S. cerevisiae*) was prepared to investigate the single and joint toxicity of five kinds of pollutants, including heavy metal ions, phenol and pesticides. The mixed microbial electrochemical sensor shows improved sensitivity to the five kinds of single toxicants, which means that multi-species microbial biosensor can make up the defect of restricted detection range of single strain biosensors. The joint toxicity tests indicate that TU approach appears to be a good model to estimate the combined effect of toxic substances in water system. Binary combinations of five toxicants produced all three types of interactions, including antagonistic, additive and synergistic responses. This reflects the complexity of water acute toxicity assessment and the necessity of joint toxicity measurements. In conclusion, the biosensors prepared above provide an alternative

and complementary way to water quality detection and a promising approach for wastewater acute toxicity assessment.

Acknowledgments

The authors appreciate the supports of the International Science & Technology Cooperation Program of China (No. 2013DFG50150), the Natural Foundation of Sciences of the People's Republic of China (No. 21175144) and the Key Project of Beijing Natural Science Foundation (No. 2120002).

References

[1] B.I.E. René, P. Schwarzenbach, Kathrin Fenner, Thomas B. Hofstetter, C. Annette Johnson, Urs von Gunten, Bernhard Wehrli, The Challenge of Micropollutants in Aquatic Systems, Science 313 (2006) 1072–1077.
[2] I.D. Green, A. Diaz, M. Tibbett, Factors affecting the concentration in seven-spotted ladybirds (Coccinella septempunctata L.) of Cd and Zn transferred through the food chain, Environ. Pollut. 158 (2010) 135–141.
[3] S. Saltzman, B. Heuer, A Rapid Bioassay for the Detection of Photosynthesis Inhibitors in Water, Pesticide Sci. 16 (1985) 457–462.
[4] A. Tizzard, J. Webber, R. Gooneratne, R. John, J. Hay, N. Pasco, MICREDOX: application for rapid biotoxicity assessment, Anal. Chim. Acta 522 (2004) 197–205.
[5] W.H. Schalie, T.R. Shedd, P.L. Knechtges, M.W. Widder, Using higher organisms in biological early warning systems for real-time toxicity detection, Biosens. Bioelectron. 16 (2001) 457–465.
[6] B. Zhu, Z.F. Wu, J. Li, G.X. Wang, Single and joint action toxicity of heavy metals on early developmental stages of Chinese rare minnow (Gobiocypris rarus), Ecotoxicol. Environ. Saf. 74 (2011) 2193–2202.
[7] J. Qian, J. Li, D. Fang, Y. Yu, J. Zhi, A disposable biofilm-modified amperometric biosensor for the sensitive determination of pesticide biotoxicity in water, RSC Adv. 4 (2014) 55473–55482.
[8] J. Li, Y. Yu, J. Qian, Y. Wang, J. Zhang, J. Zhi, A novel integrated biosensor based on co-immobilizing the mediator and microorganism for water biotoxicity assay, Analyst 139 (2014) 2806–2812.
[9] M.D. Hernando, O. Malato, M. Farre, A.R. Fernandez-Alba, D. Barcelo, Application of ring study: water toxicity determinations by bioluminescence assay with Vibrio fischeri, Talanta 69 (2006) 370–376.
[10] E. Fulladosa, J. Debord, I. Villaescusa, J.C. Bollinger, J.C. Murat, Effect of arsenic compounds on Vibrio fischeri light emission and butyrylcholinesterase activity, Environ. Chem. Lett. 5 (2006) 115–119.
[11] C.-Y. Hsieh, M.-H. Tsai, D.K. Ryan, O.C. Pancorbo, Toxicity of the 13 priority pollutant metals to Vibrio fisheri in the Microtox® chronic toxicity test, Sci. Total Environ. 320 (2004) 37–50.
[12] W. Liu, J. Jin-Gang, G.-Y. Shi, Y. He, Y. Liu, L.-T. Jin, Toxicity assessment of cyanide and tetramethylene disulfotetramine (Tetramine) using luminescent bacteria vibrio-qinghaiensis and PbO2 electrochemical sensor, Chin. J. Chem. 25 (2007) 203–207.
[13] S. Rodriguez-Mozaz, M.J. Lopez de Alda, D. Barcelo, Biosensors as useful tools for environmental analysis and monitoring, Anal. Bioanal. Chem. 386 (2006) 1025–1041.
[14] P. Banerjee, S. Kintzios, B. Prabhakarpandian, Biotoxin detection using cell-based sensors, Toxins 5 (2013) 2366–2383.
[15] S.E. Kintzios, Cell-based biosensors in clinical chemistry, Mini-Reviews Med. Chem. 7 (2007) 1019–1026.
[16] L. Campanella, D. D'Orazio, B.M. Petronio, E. Pietrantonio, Proposal for a metal speciation Study in sediments, Anal. Chim. Acta 309 (1995) 387–393.
[17] J.L. Stauber, N.M. Franklin, M.S. Adams, Applications of flow cytometry to ecotoxicity testing using microalgae, Trends Biotechnol. 20 (2002).
[18] N.M. Franklin, J.L. Stauber, R.P. Lim, Development of multispecies algal bioassays using flow cytometry, Environ. Toxicol. Chem. 23 (2004) 1452–1462.
[19] Y. Yu, F. Kong, M. Wang, L. Qian, X. Shi, Determination of short-term copper toxicity in a multispecies microalgal population using flow cytometry, Ecotoxicol. Environ. Saf. 66 (2007) 49–56.
[20] F. De Laender, K.A. De Schamphelaere, P.A. Vanrolleghem, C.R. Janssen, Comparing ecotoxicological effect concentrations of chemicals established in multi-species vs. single-species toxicity test systems, Ecotoxicol. Environ. Saf. 72 (2009) 310–315.
[21] X. Wang, X. Wang, J. Zhang, Y. Bu, X. Yan, J. Chen, J. Huang, J. Zhao, Direct toxicity assessment of copper (II) ions to activated sludge process using a p-benzoquinone-mediated amperometric biosensor, Sensors Actuators B Chem. 208 (2015) 554–558.
[22] H.G.V.D. Geest, G.D. Greve, M.-E. Boivin, Michiel, H.S. Kraak, C.A.M.V. Gestel, Mixture toxicity of copper and diazinon to larvae of the mayfly (ephoron virgo) judging additivity at different effect levels, Environ. Toxicol. Chem. 19 (2000) 2900–2905.
[23] Y. Wu, C. Lin, L. Yuan, Phototaxis index of Daphnia carinata as an indicator of joint toxicity of copper, cadmium, zinc, nitrogen and phosphorus in aqueous solutions, Ecol. Indic. 8 (2008) 69–74.
[24] J. Ma, J. Chen, How to accurately assay the algal toxicity of pesticides with low water solubility, Environ. Pollut. 136 (2005) 267–273.
[25] T. Backhaus, R. Altenburger, W. Boedeker, M.S. Michael Faust, L.H. Grimme, Predictability of the toxicity of a multiple mixture of dissimilarly acting chemicals to *Vibrio Fischeri*, Environ. Toxicol. Chem. 19 (2000) 2348–2356.
[26] Y.J. An, Y.M. Kim, T.I. Kwon, S.W. Jeong, Combined effect of copper, cadmium, and lead upon Cucumis sativus growth and bioaccumulation, Sci. Total Environ. 326 (2004) 85–93.
[27] M.E. Hibbing, C. Fuqua, M.R. Parsek, S.B. Peterson, Bacterial competition: surviving and thriving in the microbial jungle, Nat. Rev. Microbiol. 8 (2010) 15–25.
[28] A. Gerhardt, Review of impact of heavy metals on stream invertebrates with special emphasis on acid conditions, Water Air Soil Pollut. 66 (1993) 289–314.
[29] D. Yong, L. Liu, D. Yu, S. Dong, Development of a simple method for biotoxicity measurement using ultramicroelectrode array under non-deaerated condition, Anal. Chim. Acta 701 (2011) 164–168.
[30] D. Fang, Y. Yu, L. Wu, Y. Wang, J. Zhang, J. Zhi, Bacillus subtilis-based colorimetric bioassay for acute biotoxicity assessment of heavy metal ions, RSC Adv. 5 (2015) 59472–59479.
[31] X. Wang, M. Liu, X. Wang, Z. Wu, L. Yang, S. Xia, L. Chen, J. Zhao, p-Benzoquinone-mediated amperometric biosensor developed with Psychrobacter sp. for toxicity testing of heavy metals, Biosens. Bioelectron. 41 (2013) 557–562.