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Cell-based biosensor for measurement of phenol and nitrophenols toxicity

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ABSTRACT

A cost-effective whole cell biosensor based on electrochemical technique to detect toxicities of phenol and nitrophenols has been developed. This method relied on the inhibition effect for respiratory chain activity of microorganism by toxicant, which was measured by chronoamperometry using mediator (ferricyanide). The current signals produced by suspended microorganisms and reoxidation of ferrocyanide were transformed to inhibiting efficiency directly, and 50% inhibiting concentration (IC_{50}) was chosen as the quantitative standard of toxicity. The test microorganisms used here consist of three bacilli (*Escherichia coli*, *Enterobacter cloacae* and *Alcaligenes faecalis*), two pseudomonas (*Pseudomonas fluorescens* and *Pseucomonas putida*) and one fungus (*Trichosporon cutaneum*). 3,5-Dichlorophenol (DCP) was taken as the reference toxicant. The results showed that the microorganisms which belong to the same bacterial family had similar trends of inhibitions on respiratory activity and similar IC50 values. By comparing the IC_{50} values, *P. fluorescens* was the most sensitive one to DCP toxicity, its IC_{50} was estimated to be 4.2 mg/L. pH 7.0 and together with the standard glucose–glutamic acid (GGA) as an exogenous material were taken for optimum conditions in this study. Here, *P. fluorescens* as model test microorganism was employed to assess toxicities of phenol and nitrophenols under the optimum conditions. IC_{50} values of 291.4 mg/L for phenol, 64.1 mg/L for 2-NP, 71.4 mg/L for 3-NP and 14.0 mg/L for 4-NP were determined at 60 min, respectively. Comparison with the results of published data has confirmed that this cell biosensor is a sensitive and rapid alternative to toxicity screening of chemicals.

1. Introduction

Since chemical abuse became a global problem, many scientists have focused on the development of technologies to enforce environmental monitoring and homeland security [1]. New technique not only requires the sensitive response to chemicals but also needs to reflect the effects that the chemicals may have on human health. Conventional chemical-based methods can only quantify concentrations of all pollutants, are not suitable for the current status of environmental security and chemicals screening. Consequently, bioassay has been introduced for reflecting the toxic bioavailability to biota, particularly when complex chemical cocktails are involved.

There have been intense activities into bio-based assays in recent years and a wide range of organisms such as plants, invertebrates and fish are involved [2]. Unfortunately, many of these tests are time consuming, expensive and inhuman. Furthermore, necessary specialized equipment and trained personnel also limited the techniques promotion. Hence, there is a great challenge for effective technique that provides rapid, simple, reliable and low-cost measurements to complement traditional chemical and biological methods.

The whole cell sensors based on microorganisms are very easy to handle and have much shorter generation times. Microtox based on the bioluminescence fading, in the presence of toxic materials, is the most widely used method for toxicity assessment. However, some disadvantages and particular characteristics must be mentioned. Luminescence tests depending on optical detection are restricted by cell populations strictly, and are not suitable for samples of high turbidity, which would cause the fluorescence scattering. Furthermore, the luminous bacterial must work in 3% saline solution in order to maintain osmotic pressure of bacteria. This would decrease the solubility of some organic chemicals.

In order to remedy the defects of luminescent test, microorganisms based sensors combined with electrochemical measurement have been developed [3–6]. Direct toxicity assessment (DTA) developed by Lincoln Technology is considered to be a superior and rapid toxicity assessment for chemicals screening and water monitoring. MICREDOX is characterized by high levels of microorganisms and mediator in order to facilitate the reaction. Mediators can 'shuttle' electrons produced within the cell during respiration to an electrode, and can be reduced by certain microorganisms [7]. Injury sustained by a cell in response to a toxicant changes the capacity of some central function of cellular metabolism, compared to unaffected cells. These toxic effects can be easily measured as a

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Table 1
Detailed information of the microorganisms in experiments.

Microorganism name	*Escherichia coli*	*Enterobacter cloacae*	*Alcaligenes faecalis*	*Pseudomonas fluorescens*	*Pseudomonas putida*	*Trichosporon cutaneum*
Shortened form	*E. coli*	*E. cloacae*	*A. faecalis*	*P. fluorescens*	*P. putida*	*T. cutaneum*
Culture medium	LB	Beef extract peptone medium	Beef extract peptone medium	Beef extract peptone medium	Beef extract peptone medium	YEPD
Incubation temperature (°C)	37	30	30	30	30	30
Incubation time (h)	10	12	12	12	12	40

deviation away from the respiration signal produced by healthy cells. The ratio of the electrochemical signals, recorded in the presence and absence of toxin, provided an index of inhibition. 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 [8–10]. Especially ultramicroelectrode array (UMEA) used, effectively amplifying the signal from the total limiting currents of multi individuals one to 3,5-dichlorophenol (DCP) [11]. The ability to monitor the changes in the cellular 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.

To date, MICREDOX has been demonstrated using three microorganisms (*E. coli*, *Bacillus subtilis* and *Pseudomonas putida)* for toxicity assessment of DCP. Pasco et al. [12] also used MICREDOX to study differences in the inhibitory properties of various chlorinated phenol compounds, using *E. coli* and *Klebsiella oxytoca* as test microorganisms. Recently, Liu et al. [11] adopt *E. coli* to detect many toxicants, including arsenic and several heavy metals. However, the microorganisms have been tested were still limited and were not analyzed clearly to the responses of toxicants.

In this work, DCP was chosen as the reference toxicant, the effect of DCP on various kinds of microorganisms were investigated in detail. The results showed that the microorganisms which belong to the same bacterial family showed similar trends of inhibitions on respiratory activity and similar IC_{50} values. By comparing the IC_{50} values, *Pseudomonas fluorescens* (*P. fluorescens*) was the most sensitive one responsed to DCP toxicity. Accordingly, we took *P. fluorescens* as a model microorganism to investigate the toxicities of other toxicants such as phenol and nitrophenols under the optimum conditions (solution pH 7.0 and together with the standard glucose–glutamic acid (GGA)).

2. Experimental

2.1. Chemicals and solution

DCP was purchased from Aldrich. Phenol was obtained from Tianjin Guangfu Fine Chemical Research Institute. 2-NP was taken from Xihui Chemical Factory (Chaoyang, Beijing). 3-NP was purchased from Sinopharm Chemical Reagent Co., Ltd. 2-NP and 4-NP were of chemical purity, and other chemicals used in this study were of analytical reagent grade. Stock solutions were prepared in deionized water and adjusted to 800 mg/L using liquid chromatography (Waters 600, Waters, USA).

The standard GGA solution (150 mg/L glucose and 150 mg/L glutamic acid) was prepared according to APHA standard methods [13]. The Luria Bertani (LB, 10.0 g/L tryptone, 5.0 g/L yeast extract, and 10.0 g/L NaCl, pH 7.0), YEPD medium (beef extract 3.0 g/L, yeast extract 3.0 g/L, tryptone 3.0 g/L, glucose 10.0 g/L, pH 5.5) and Beef Extract Peptone medium (beef extract 3.0 g/L, tryptone 10.0 g/L, NaCl 5.0 g/L, pH 7.0) broth were adjusted to the desired pH with 2 mol/L HCl or 2 mol/L NaOH and sterilized in high-pressure steam at 120 °C for 20 min. $K_3[Fe(CN)_6]$ was freshly prepared before use.

2.2. Microorganisms preparation

All microorganisms were obtained from China General Microbiological Culture Collection Center (CGMCC) and maintained on nutrient agar plates at 4 °C. The suitable medium and the incubation temperature were confirmed by guide of CGMCC. *E. coli* was grown aerobically in a shaker bath (220 rpm) for 10 h at 37 °C, *E. cloacae*, *A. faecalis*, *P. fluorescens* and *P. putida* were grown aerobically in a shaker bath (220 rpm) for 12 h at 30 °C. *T. cutaneum* was grown aerobically in a shaker bath (220 rpm) for 40 h at 30 °C. Table 1 showed detailed information of the microorganisms in experiments. Cells were harvested by centrifugation at 6000 rpm for 10 min at room temperature, then washed twice with phosphate buffer solution (PBS) and resuspended in PBS. The concentration of cells was adjusted to an absorbance value of 24, measured at 600 nm (OD_{600}) using a Cary 500 Scan UV-vis-NIR Spectrophotometer. The bacterial suspension was used for the further 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 appropriate temperature in a water bath. 10 mL sample mixture for incubation containing 45 mM potassium ferricyanide, standard GGA solution, toxicant at the appropriate level and cells suspension with the ultimate absorbance value of ~6.0 were incubated anaerobically using oxygen-free nitrogen at temperature appropriate to individual microorganism for 60 min. Control incubations contained PBS in place of the toxicant; positive and negative controls refer to the presence and absence of GGA substrate in toxic samples, 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.

Chronoamperometry at microelectrode array was used to measure the quantity of reduced mediator produced during the microbial incubation. 9 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 were adopted. The working electrode was polished using slurry of 0.05 μm α alumina before use.

Amperometric detection was performed at 450 mV, and the pulse width was of 10 s. For each toxicant concentration, the limiting current can be converted to equivalent inhibitory percentage values following Eq. (1) [20]:

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

where $i_{\lim(\text{toxicant})}$ is the output of limiting current at an appropriate concentration of toxicant and standard GGA solution; $i_{\lim(t-\text{control})}$ is the output of limiting current at an appropriate concentration of

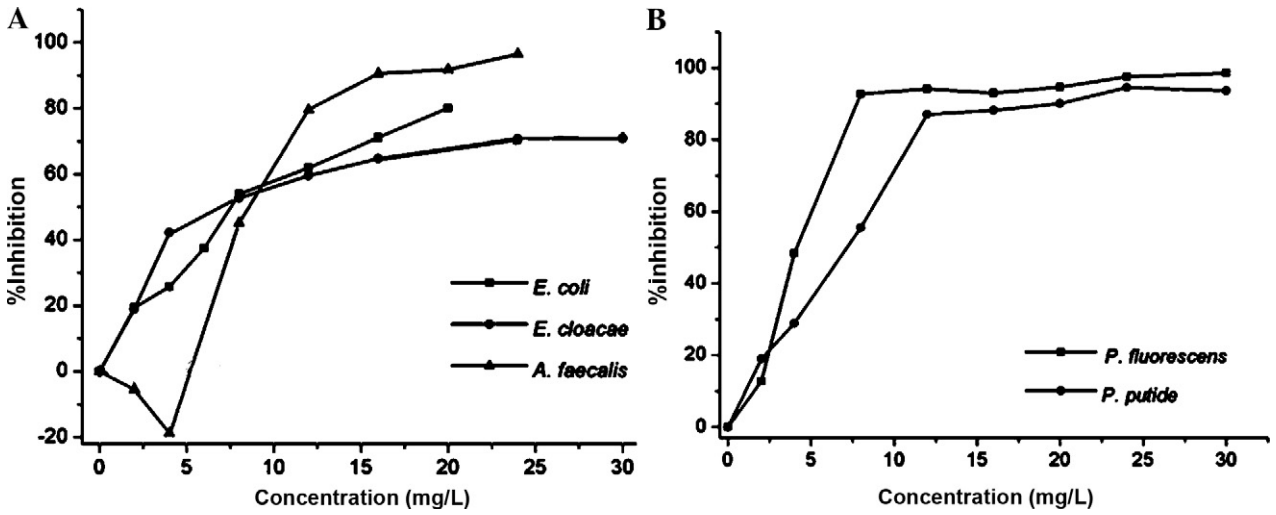

Fig. 1. Inhibitory curves of bacterial respiration at different concentrations of DCP. (A) Three bacilli were *E. coli*, *E. cloacae* and *A. faecalis*, respectively. (B) Two pseudomonades were *P. fluorescens and P. putide*, respectively.

toxicant without GGA solution; $i_{\lim(p-\text{control})}$ is the output of positive control current; $i_{\lim(n-\text{control})}$ is the output of negative control current.

2.4. Screening the most sensitive microorganism

DCP was chosen as the reference toxicant for sensitivity comparison of 6 strains of microorganism, because it has been widely studied in previous assays of toxicity. The OECD 209 Activated Sludge Respiration Inhibition Test (ASRIT) [14] also advised the use of DCP as a reference toxicant. In this work, the toxicity of DCP was examined at least with five different concentrations for each microorganism. According to the limiting currents, the inhibitory percentages at each toxicant concentration were calculated using Eq. (1). Then the IC_{50} values of each microorganism were obtained by inhibitory curves directly. The smallest value of IC_{50} indicated that it was the most sensitive microorganism among all the detected objects.

2.5. The effects of exogenous materials

The standard GGA solution was used as substrate to investigate the effect of exogenous materials on *P. fluorescens*. We took PBS in place of GGA in positive controls. DCP was used as the only carbon and nitrogen source. Solutions were incubated anaerobically at 30 °C for 60 min and centrifuged at 10,000 rpm for 10 min.

2.6. The effects of pH

Replicate samples were prepared according to Section 2.3 exception of 2.5 mL buffer solutions replacing the toxic sample. All suspensions were incubated anaerobically at 30 °C for 60 min. To terminate the microbial reaction, samples were removed and centrifuged at 10,000 rpm for 10 min. The supernatant solutions were maintained without oxygen and then taken for analysis.

3. Results and discussion

3.1. Screening the most sensitive microorganism to DCP

Fig. 1 showed inhibitory curves of five bacteria at different concentrations of DCP. Inhibitory curves of three bacilli respiration (*E. coli*, *E. cloacae* and *A. faecalis*) at different concentrations of DCP were recorded as Fig. 1A. The toxicities of DCP on *E. coli* and *E. cloacae* had the similar trends of inhibition on respiratory activity, while small difference was observed between 10.0 and 20.0 mg/L at 60 min. DCP showed a negative inhibition on *A. faecalis* when its concentrations were less than 4.0 mg/L. With the concentration increasing, the influences of DCP on *A. faecalis* enhanced rapidly even exceeded the responses of other bacilli. Accordingly, IC_{50} values of 7.6 mg/L for *E. coli*, 7.5 mg/L for *E. cloacae*, 8.6 mg/L for and *A. faecalis* were determined under 60 min incubation. Obviously, *A. faecalis* was the lowest sensitive one among the three detected microorganisms, whereas the lower IC50 values for *E. cloacae* and *E. coli* indicating the more sensitive response to DCP toxicity.

Fig. 1B showed inhibitory curves of two pseudomonades respiration (*P. fluorescens and P. putide*) at different concentrations of DCP, respectively. *P. fluorescens* and *P. putide* also showed the similar trends of inhibition because they belong to the same bacterial family. IC_{50} values were 4.2 mg/L for *P. fluorescens* and 7.2 mg/L for *P. putide*, respectively.

As shown in Fig. 2, the inhibitory curve of one fungus (*T. cutaneum*) was obtained at different concentrations of DCP. Compared to five bacteria tested, *T. cutaneum* had a relatively small response to DCP. Also of note, when DCP was less than 4.0 mg/L, it has small promoting effect to respiration. The inhibition just reached to 50% as DCP in 32.1 mg/L. Then, there is no clear difference inhibition between 40.0 and 80.0 mg/L DCP at 60 min.

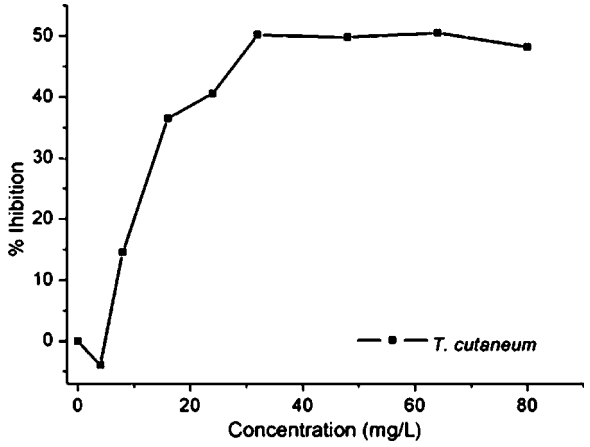

Fig. 2. Inhibitory curves of one fungus (*T. cutaneum)* at different concentrations of DCP.

Table 2
Comparison of IC_{50} values by Electrochemical method to other DTA assays.

Toxicants	DTA assay (IC_{50} mg/L)			
	Microtox *V. fischeri*	ToxAlert *V. fischeri*	Electrochemical method *P. fluorescens*	ASRIT (activated sludge)
Phenol	21.1 [17]	7.99 [19]	291.4	783.0 [20]
2-NP	34.9 [18]	–	64.1	–
3-NP	–	43.05 [19]	71.4	–
4-NP	13.6 [21]	25.51 [19]	14.0	57.0 [22]

By comparing the IC_{50} values, the microorganisms we used have different IC50 values because the different microorganisms have different sensitivities to the toxicants. *E. coli*, *E. cloacae*, *A. faecalis* and *B. subtilis*, *P. fluorescens* and *P. putide* have similar IC_{50} values, respectively. Especially, it should be noted that *E. coli* and *E. cloacae*, *P. fluorescens* and *P. putide* have the similar trends of inhibitions on respiratory activity, which are ascribed to the same bacterial family. *E. coli*, *E. cloacae* and *A. faecalis* belong to bacilli, *P. fluorescens* and *P. putide* belong to pseudomonades, respectively. DCP showed a negative inhibition on *A. faecalis* when its concentration was less than 4.0 mg/L. In contrast, *E. coli* and *E. cloacae* did not show the same phenomenon. Thus, we speculated that some microorganisms could degrade DCP as a substrate, leading to promote the respiration of microorganism. On the other hand, when the concentration of DCP was higher than 4.0 mg/L, the inhibiting effect of DCP on *A. faecalis* was larger than that of degradation, resulting in the inhibition became dominate. Similar with *A. faecalis*, *T. cutaneum* also has a negative inhibition at low concentration of DCP. Based on discussion above, owing to the nature of microorganism, the same toxicant has distinct effects on different microorganism individuals. The microorganisms belong to the same family have the similar trends of inhibitions on respiratory activity and IC_{50} values.

P. fluorescens is the most sensitive microorganism in this study. Compared with EC_{50} values reported before, it is only higher than that of Microtox [15] and inhibition of nitrification [16] with 2.9 mg/L and 0.51 mg/L, respectively. So we choose *P. fluorescens* as a model microorganism in following experiments.

3.2. Influence of standard GGA solution

Fig. 3 showed inhibitory curves of *P. fluorescens* at different concentrations of DCP with GGA (●) and without GGA (■), respectively. In the presence of GGA, *P. fluorescens* respiration declined quickly to more than 85% when the concentration of DCP changed from 2.0 mg/L to 8.0 mg/L. In the absence of GGA, its inhibitory curve gradually reached 85% when DCP raised up to 20.3 mg/L. This result indicated that the effects of DCP became more pronounced when GGA was as an exogenous material added.

3.3. Influence of pH

As shown in Fig. 4, the effects of pH values (2.2–10.0) on *P. fluorescens* after 1 h exposure to 45 mM ferricyanide and GGA standard solutions were depicted. Limiting current increased slowly at pH 2.2–5.0. There was a significant increase of the limiting current at pH 5.0–7.0. In contrast, the limiting current was decreased when pH over 7.0 up to 10.0. This result was mainly due to the changes in chemical speciation of ferricyanide in the alkaline solution. Hence, pH 7.0 was used as one of the optimum condition for all further studies.

3.4. Toxicities of phenol and nitrophenols to P. fluorescens

The important chemical pollutants, such as phenol and nitrophenols, were investigated under optimum conditions in present study. The results obtained were compared with that of published data by other methods. According to Table 2, the Microtox and ToxAlert assays were more sensitive than of ASRIT because of different microorganisms used. While this sensor combining with electrochemical method for toxicity detection displayed good sensitivities to phenol and nitrophenols. Their IC_{50} values of phenol, 2-NP, 3-NP and 4-NP were 291.4 mg/L, 64.1 mg/L, 71.4 mg/L and 14.0 mg/L, respectively. Our result for 4-NP (using *P. fluorescens*) was similar as Microtox (using *V. fischeri*), but it was more sensitive than that of ToxAlert and ASRIT. However, the resulting sensitivity to phenol was 10 times lower than that of Microtox. The reasons were discussed as following: (1) the different bacteria have different sensitivities to phenol, the sensitivity of *V. fischeri* to phenol is much higher than *P. fluorescens*. (2) The nature of the chemicals, this phenomenon was found in the use of higher organisms to detect phenol. (3) In electrochemistry test, there are some factors, such as upon addition of PBS, ferricyanide and nitrogen, can affect current signal. (4) It was reported that the toxicity depended greatly on the pH of the medium [23]. The pH value adopted in our experi-

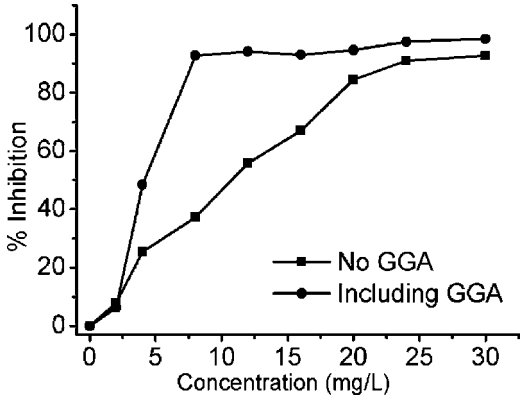

Fig. 3. Inhibitory curves of *P. fluorescens* at different concentrations of DCP under GGA and no GGA conditions, respectively.

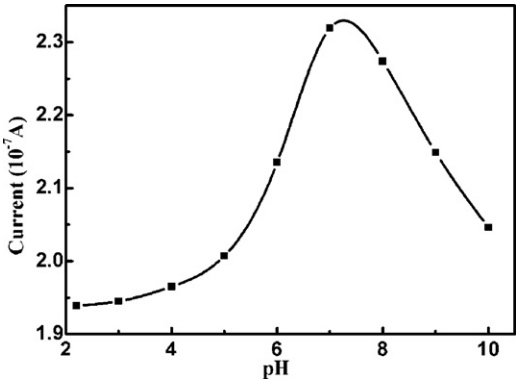

Fig. 4. Dose–response curve for the influences of pH. Changes of limiting currents after 60 min exposure of *P. fluorescens* incubated with 45 mM ferricyanide at different pH. Each point plotted was the average of two samples.

ment could affect the obtained sensitivity to some extent, whereas Microtox and Toxalert assays were not be strictly defined.

4. Conclusions

A simple, rapid and reliable whole cell biosensor combining with electrochemical method has been developed successfully for the detection of various toxic substances. In optimized assays, *P. fluorescens* showed the most sensitivity to DCP, and its IC_{50} for DCP was 4.2 mg/L at 60 min incubation. Additionally, sample solutions with pH 7.0 and GGA added could improve the sensitivity to *P. fluorescens* for DCP. Phenol and nitrophenols as the serious environmental pollutants and toxic chemicals were examined using this electrochemical method under optimum conditions. IC_{50} values of 291.4 mg/L for phenol, 64.1 mg/L for 2-NP, 71.4 mg/L for 3-NP and 14.0 mg/L for 4-NP were determined by using *P. fluorescens* as model microorganism, respectively. These results obtained were comparable to bioluminescent method and ASRIT, although different microorganisms were used. The improvement is needed in further investigation. It is known that different species of microorganisms have their own metabolic response to toxicant, so single microorganism can hardly reflect the true and comprehensive values for the toxicant in real samples. To remedy this defect, the mixed microorganisms should be adopted. In general, the electrochemical method based on whole cell described is a sensitive, rapid, reproducible and inexpensive alternative with no ethical argument for toxicity screening of chemicals and environmental water monitoring.

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References

[1] G. Yeates, V. Orchard, T. Speir, J. Hunt, M. Hermans, Biol. Fertil. Soils 18 (1994) 200.
[2] N. Pasco, K. Baronian, C. Jeffries, J. Hay, Appl. Microbiol. Biotechnol. 53 (2000) 613.
[3] N. Pasco, J. Hay, J. Webber, Biomarkers 6 (2001) 83.
[4] P. Ertl, E. Robello, F. Battaglini, S.R. Mikkelsen, Anal. Chem. 72 (2000) 4957.
[5] J. Zhao, Z. Yang, M. Wang, Y. Lu, Z. Yang., J. Agric. Food Chem. 52 (2004) 7246.
[6] J. Zhao, M. Wang, Z. Yang, Q. Gong, Y. Lu., Biotechnol. Lett. 27 (2005) 207.
[7] A. Tizzard, J. Webber, R. Gooneratne, R. John, J. Hay, N. Pasco, Anal. Chim. Acta 522 (2004) 197.
[8] K.R. Rogers, M. Mascini, 2001, http://www.epa.gov/heasdweb/edrb/biochem/intro.htm.
[9] K. Morris, K. Catterall, H. Zhao, N. Pasco, R. John, Anal. Chim. Acta 442 (2001) 129.
[10] G. Evtugyn, H. Budnikov, E. Nikolskaya, Talanta 46 (1998) 465.
[11] C. Liu, T. Sun, X. Xu, S. Dong, Anal. Chim. Acta 641 (2009) 59.
[12] N. Pasco, R. Goonerate, R. Daniel, A. Cznoller, A. Scott, Int. J. Environ. Anal. Chem. 88 (2008) 1063.
[13] Standard Method for the Examination of Water and Wastewater, American Public Health Association: American, 1997.
[14] L. dos Santos, L. Defrenne, A. Krebs-Brown, Anal. Chim. Acta 456 (2002) 41.
[15] B. Dutka, K. Kwan, D. Liu, B.D. Eds. Toxicity Screening Procedures Using Bacterial Systems, Marcel Dekker, New York, 1984.
[16] K. Gernaey, L. Verschuere, L. Luyten, W. Verstraete, Water Environ. Res. 69 (1997) 1163.
[17] M. Elnabarawy, R. Robideau, S. Beach, Toxicity Assess. 3 (1988) 361.
[18] R. Speece, Drexel University, Pittsburgh, PA, private communication, 16 (1987) 2259.
[19] M. Farré, D. Barceló, TrAC Trends Anal. Chem. 22 (2003) 299.
[20] G. Klecka, L. Landi, K. Bodner, Chemosphere 14 (1985) 1239.
[21] L. Somasundaram, J. Coats, K. Racke, H. Stahr, Toxicology 44 (1990) 254.
[22] L. Reynolds, J. Blok, A. Demorsier, P. Gerike, H. Wellens, W. Bontinck, Chemosphere 16 (1987) 2259.
[23] G. Nalecz-Jawecki, J. Sawicki, Chemosphere 52 (2003) 249.