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Cytotoxicity and genotoxicity evaluation of urban surface waters using freshwater luminescent bacteria *Vibrio-qinghaiensis* sp.-Q67 and *Vicia faba* root tip

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Abstract

The freshwater luminescent bacteria *Vibrio-qinghaiensis* sp.-Q67 test and the *Vicia faba* root tip test associated with solid-phase extraction were applied for cytotoxicity and genotoxicity assessment of organic substances in three rivers, two lakes and effluent flows from two wastewater treatment plants (WWTPs) in Xi'an, China. Although the most seriously polluted river with high chemical oxygen demand (COD) and total organic carbon (TOC) showed high cytotoxicity (expressed as TII_{50}, the toxicity impact index) and genotoxicity (expressed as RMCN, the relative frequency of micronucleus), no correlative relation was found between the ecotoxicity and organic content of the water samples. However, there was a linear correlative relation between TII_{50} and RMCN for most water samples except that from the Zaohe River, which receives discharge from WWTP and untreated industrial wastewaters. The ecotoxicity of the organic toxicants in the Chanhe River and Zaohe River indicated that cytotoxic and genotoxic effects were related to the pollutant source. The TII_{50} and RMCN were also found to correlate roughly to the dissolved oxygen concentration of the water. Sufficient dissolved oxygen in surface water is thus proved to be an indicator of a healthy water environmental condition.

Key words: cytotoxicity; genotoxicity; surface water; luminescent bacteria; *Vicia faba* root tip

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Introduction

The ecotoxic effect of pollutants on the water environment has become a hot topic of research. These pollutants arise from various sources such as point sources from industrial effluents (Ohe et al., 2004) and treated and/or untreated domestic wastewater discharge (Terzi et al., 2008; Bolong et al., 2009), and non-point sources from agricultural land and urban runoff (Brett et al., 2005; Santhi et al., 2001). In addition to the regulated water quality parameters such as organic content (COD etc.), nutrients (nitrogen and phosphorus), heavy metals and other chemicals, more and more attention is paid to the overall adverse impacts of the pollutants on aquatic ecosystems which can be evaluated as ecotoxicities, including cytotoxicity and genotoxicity (Žegura et al., 2009; Dizer et al., 2002). Due to the limitation of knowledge on the ecotoxic effects of individual chemicals (Chen and White, 2004), biological assays are found to be useful for characterizing the ecotoxicity through chronic and/or acute exposure, without prior information on the chemical components present in the water. The chemicals which have cytotoxic and/or genotoxic effects on the water environment include both inorganic substances and organic substances. Of them, the organic substances are often the main target of ecotoxicity assessment to avoid the difficulties involved in identification and quantification of individual organic substances through physicochemical analysis.

For water cytotoxicity assessment, a marine bacterium *Vibrio fischeri* has been widely used. However, when *V. fischeri* is applied to freshwater samples, a concentration of 2%–3% NaCl must be added to its culture medium, which may change the inherent properties of the freshwater sample. As an alternative approach, a freshwater luminescent bacterium *Vibrio qinghaiensis* sp.-Q67 (denoted as Q67 afterwards) was found to be applicable (Zhu et al., 2009). For water genotoxicity assessment, methods using plants have been widely developed and applied (Villarini et al., 2009). Micronucleus (MCN) assay has been proved to be sensitive and fast for monitoring the genotoxic responses of indigenous aquatic organisms to environmental pollution (Hoshina et al., 2009). To a certain extent, chromosome damage in *Vicia faba* root tip cells exposed to contaminants is considered to reflect the DNA damage in animals.

In environmental waters, due to the coexistence of various substances, the interference from some inorganic substances such as nutrients which are not ecotoxic often

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affects the result of bioassays (Deriabin and Aleshina, 2008). In order to improve the sensitivity of the cytotoxic and/or genotoxic analysis, extraction and concentration of the target organic compounds from the water samples are often prerequisite steps of the operation. The commonly used extraction/concentration methods for bioassay include liquid-liquid extraction, XAD resin adsorption, commercial solid-phase column extraction and the blue rayon/cotton hanging method etc. (Ohe et al., 2004). Of these methods, solid-phase extraction (SPE) can effectively extract organic substances and avoid interference from coexisting inorganic substances (Macova et al., 2010; Smital et al., 2011).

The objective of the present study was to assess the cytotoxicity and genotoxicity of various surface waters, streams and effluent of domestic wastewater treatment plants (WWTPs) in Xi'an, China by a method which combined SPE with bioassays using Q67 for cytotoxicity assessment and *V. faba* root tip for genotoxicity assessment.

1 Materials and methods

1.1 Sample collection and characterization

Water samples were collected from three urban streams, two urban lakes, and the effluents from two WWTPs in Xi'an, China. Table 1 shows the condition of each sampling point. The sample collection was conducted on sunny weekdays when these waters were under normal conditions.

1.2 Water quality analyses

Dissolved oxygen (DO) was measured at each sampling point by using a portable DO meter (SX751, SANXIN, China). After filtration by a 0.45 μm membrane filter, the chemical oxygen demand (COD) was analyzed using the closed reflux, colorimetric method with potassium dichromate as the oxidant (State Envronmental Protection Administration, 2002), and the total organic carbon (TOC) was measured using a TOC analyzer (TOC-VC, Shimadzu, Japan).

1.3 Solid-phase extraction and preparation of testing samples

Each of the samples collected for bioassay was pretreated by SPE for concentrating the organic compounds following the previous work (Ma et al., 2011). The sample was first filtered by a 0.45 μm membrane filter to remove the suspended solids and then SPE was conducted for the extraction of organic matter from the sample. As the target compounds for this study were organic compounds that might be ecotoxic, C18 SPE cartridges (Agilent SampliQ C18, 3 mL, 500 mg) that could extract a broad range of organic compounds were selected. Before the SPE operation, methylene chloride, methanol, and milli-Q water, each with a volume of 7 mL, were successively passed through the cartridge to activate the fillings and keep them under a wetted condition. The sample was then passed through the cartridge at a flow rate of 5 mL/min to extract the organic compounds. Afterwards, 3 mL milli-Q water was passed through to wash out the impurities.

After the above procedures, the cartridge was put in a centrifuge to remove the residual water. It was then eluted with 7 mL methylene chloride. A nitrogen evaporator (Sample Concentrator MD200, Supelco, America) was used to evaporate the eluent at a constant temperature of 40°C. The extracted organic compounds were finally dissolved in 0.5% dimethyl sulphoxide (DMSO) solution for the cytotoxicity and genotoxicity test.

1.4 Bioluminescent bacteria test

For the cytotoxicity test, Q67 luminescent bacteria (purchased from Beijing Hammatsu Photon Techniques Inc., China) were grown in a culture medium that could produce a large quantity of bacteria with fluorescence enzyme (Zheng et al., 1999) up to the logarithmic growth stage, for 10 to 12 hr at (22 ± 1)°C while shaking at 120 r/min.

The acute toxicity test was then conducted using a Modulus™ Single Tube Multimode Reader (Turner Biosystems, America). For each test, four test tubes were prepared, three for parallel samples and one for a blank control. The bacterial suspension of 100 μL was added into each tube, and the same volume of the sample or blank control liquid (0.5% DMSO) was added at 15 sec intervals.

Table 1 Sampling points for the study

Sample No.	Sampling site	Conditions
1	Chanhe River	A stream mainly receiving rural runoff and treated domestic effluent. Sampling location was upstream of the WWTP 2 mentioned below.
2	Zaohe River	A stream receiving urban runoff, treated domestic effluent and untreated industrial wastewater. Sampling location was upstream of the WWTP 1 mentioned below.
3	Weihe River	The largest tributary of the Yellow River flowing through the northern suburb of Xi'an and receiving the flows from all urban streams. Samples were collected downstream from the city.
4	Xingqinghu Lake	A landscape lake in the city.
5	Nanhu Lake	A landscape lake in a newly built tourist district in the southern suburb of the city.
6	Effluent of WWTP 1	From the outlet of a WWTP which discharges its effluent to the lower reach of the Zaohe River.
7	Effluent of WWTP 2	From the outlet of a WWTP which discharges its effluent to the lower reach of the Chanhe River.

After 15 min exposure of the bacteria to the sample at (22 ± 1)°C, the relative light unit (RLU) of Q67 was measured on the Modulus^{TM}, and the acute toxicity of the sample on Q67 was expressed as the inhibition value in percent.

In order to quantitatively compare the ecotoxicity of different water samples, the concentration (times) corresponding to the inhibition value of 50%, namely effective concentration (EC_{50}), was calculated. By definition, the higher the EC_{50} value, the lower the ecotoxic effect. For the convenience of comparison following the common convention, the reciprocal of EC_{50}, namely the toxicity impact index (TII_{50}), was used (Farré et al., 2001).

1.5 *V. faba* root tip test

For genotoxicity testing, the *V. faba* root tip test was conducted (Yi and Si, 2007). Dry seeds of *V. faba* stored at 4°C were soaked in milli-Q water for 24 hr. Then the seeds were allowed to germinate between two layers of moist cotton at (23 ± 1)°C in the dark. When the newly emerged roots were of 1.00–2.00 cm in length, they were used in the test.

Growing roots were treated for 12 hr with concentrated organic matter in water samples after SPE. The 0.5% DMSO and 10 mg/L methyl methanesulphonate (MMS) were used as negative and positive controls respectively. The roots were maintained in milli-Q water for a 24 hr recovery period. All experimental groups were kept in an incubator at (23 ± 1)°C. Afterwards, the roots were fixed in a mixture of methanol-acetic acid (3:1, *V/V*) at 4°C overnight. The fixative was replaced by 70% alcohol for long-term storage. For slide preparation and microscopic examination, the root tips were rinsed in milli-Q water and dissociated in 1 mol/L HCl at 60°C for 12 to 15 min. After staining with Schiff's reagent, 1 mm of the mitotic zone from a well-stained root tip was immersed in a drop of milli-Q water on a clean slide and flattened under a cover glass.

The analysis results are expressed as the relative frequency of micronucleus (RMCN) by observing the number of micronucleus in 1000 cells of each *V. faba* root tip cells above the blank control, and the mitotic index (MI) which was defined as the number of dividing cells per 100 cells. All analyses were carried out with a 400× magnification optical light microscope (ECLIPSE 90i, Nikon Instruments, Japan). Replicates of five root tips were analyzed for each treatment.

2 Results and discussion

2.1 Comparison of organic content and toxicity parameters

Table 2 summarizes the results of COD, TOC, and DO analyses and toxicity tests. From the organic contents in terms of COD or TOC, it can be seen that sample No. 2, i.e., the Zaohe River, is the most polluted surface water (COD as high as 117.57 mg/L, TOC as 31.80 mg/L and DO as low as 0.4 mg/L) because it receives urban runoff and direct discharge of untreated industrial wastewaters. As expected, this polluted stream showed the highest toxicity (TII_{50} = 6.73%, RMCN = 33.68‰) among all samples. Sample No. 1, i.e., the Chanhe River, which was not of high organic content (COD as 15.01 mg/L and TOC as 4.83 mg/L) but of low dissolved oxygen concentration (DO as 0.8 mg/L), also showed high genotoxicity (RMCN = 29.57‰) but lower cytotoxicity (TII_{50} = 0.84%). The difference in the pollutant sources of these two streams shown in Table 1 may be the main reason. Unknown organic chemicals from industries may contribute to the high cytotoxicity of the Zaohe River, while rural runoff, which measureably contains certain agricultural originated organic substances that may be toxic even at a low concentration, may contribute to the high genotoxicity of the Chanhe River. Compared with urban streams, the two urban lakes showed very low toxicity values (TII_{50} = 0.65% and 0.43%, RMCN = 2.56‰ and 1.79‰), though their COD or TOC values were not the lowest. Of the rivers monitored, as the Weihe River is the main waterway of the local basin which receives inflow from all side streams and has a large flow rate, its toxicity values were not high (TII_{50} = 0.81%, RMCN = 7.74‰) in comparison with its organic content (COD = 53.80 mg/L, TOC = 10.52 mg/L).

The secondary effluents from the two WWTPs well meet the national discharge standard of pollutants for municipal wastewater treatment plants in term of COD (GB 18918-2002). However, their genotoxicity values (RMCN = 11.98‰ and 14.58‰) are higher than that of some streams and lakes of similar or higher COD values (such as samples No. 3, No. 4 and No. 5). This may be due to the mutagenicity of certain residual organic substances in the WWTP effluent (Claxton et al., 1998).

Direct Q67 and *V. faba* root tip tests without SPE were also tried for these water samples but useful data could not be obtained due to the strong stimulating effect of the coexisting inorganic substances.

2.2 Relation between cytotoxicity and genotoxicity of water samples

Figure 1 compares the TII_{50} obtained from the Q67 test with RMCN obtained from the *V. faba* root tip micronucleus test. It can be seen that for many of the samples, higher TII_{50} occurred simultaneously with higher RMCN such as for samples No. 1, No. 3, No. 4, and No. 5, indicating that most of the organics which are cytotoxic may also be

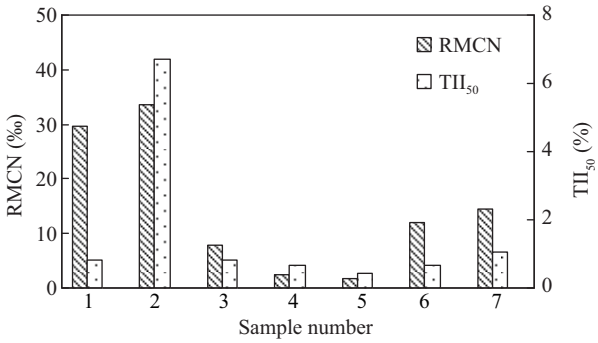

Fig. 1 Comparison of bioluminescent bacteria test (expressed as TII_{50}) and *V. faba* root tip test (expressed as RMCN).

Table 2 Results of chemical and ecotoxicity analyses of water samples

Sample number	DO (mg/L)	COD (mg/L)	TOC (mg/L)	TII_{50} (%)	RMCN (‰)	MI reduction rate (%)
1	0.8	15.01	4.83	0.84	29.57	22.36
2	0.4	117.57	31.80	6.73	33.68	25.41
3	2.1	53.80	10.52	0.81	7.74	9.81
4	3.5	14.11	10.91	0.65	2.56	7.36
5	4.6	22.17	7.05	0.43	1.79	5.23
6	2.2	27.94	14.66	0.68	11.98	13.67
7	2.3	13.99	5.69	1.06	14.58	15.64
Negative control	–	–	–	–	0	0
Positive control	–	–	–	–	36	24.43

"–" mean that was not detected. DO: dissolved oxygen; COD: chemical oxygen demand; TOC: total organic carbon; TII_{50}: toxicity impact index; RMCN: relative frequency of micronucleus; MI: mitotic index.

genotoxic (Osano et al., 2002; Baun et al., 2004). By plotting the TII_{50}-RMCN relation on logarithmic coordinates, Fig. 2a is obtained to show a linear regressive relation of all the data except for sample No. 2, which is an exceptional case as discussed above.

The RMCN also well correlates to the mitotic index (MI) reduction rate as shown in Fig. 2b. As the MI reflects the frequency of cell division, the MI reduction rate is related to mutagens leading to chromosome abnormalities (Duquesnoy et al., 2010).

2.3 Relation between ecotoxicity and dissolved oxygen content

As shown in Fig. 3, the water ecotoxicity, either as TII_{50} or RMCN, roughly shows a trend of decreasing with increasing DO concentration of the water. It is widely recognized that DO is an important indicator of the healthy state of a water body and in many countries DO concentration is regulated according to the function of the surface water for various uses, such as the Environmental Quality Standards for Surface Water in China (GB 3838-2002) which specifies DO > 5 mg/L for rivers, lakes and reservoirs suitable as source water for domestic water supply. Figure 3 can thus provide support to this kind of regulation from the viewpoint of ecotoxicity control. Some researchers pointed out that toxic substances could often be generated from the decomposition of organic matter under anaerobic conditions (Lesteur et al., 2010), and a well-aerated condition can bring about substantial reduction of water ecotoxicity (Zhao et al., 2006). In this sense, the very low dissolved oxygen (DO = 0.8 mg/L) in sample No. 1 can be taken as an indication of the possible existence of ecotoxic substances in the Chanhe River though its total organic contents were not at low levels (COD = 15.01

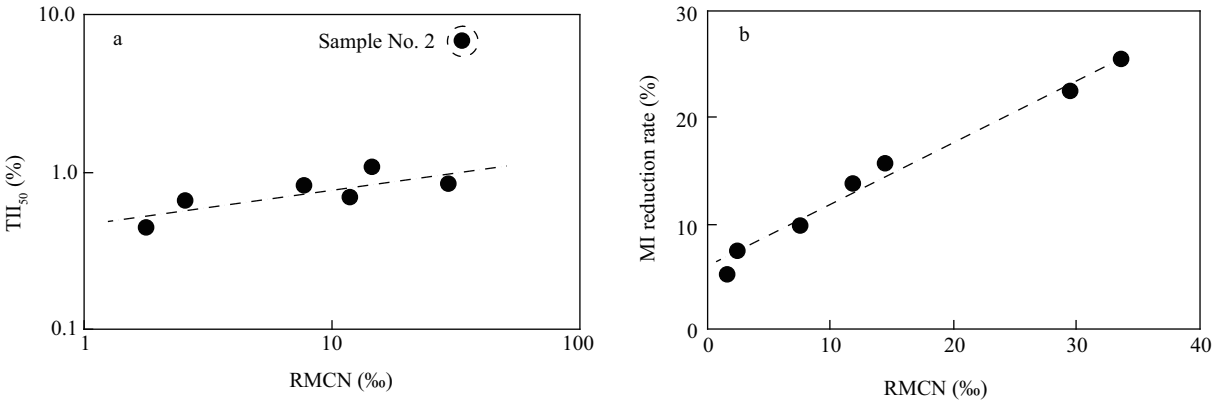

Fig. 2 Relationship between RMCN and TII_{50} (a), and between RMCN and MI reduction rate (b).

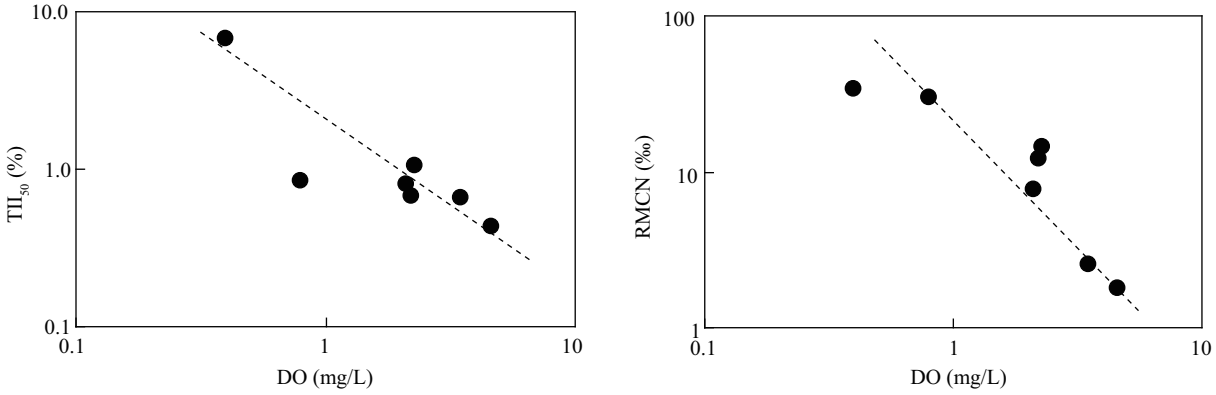

Fig. 3 Relationship between water ecotoxicity and dissolved oxygen.

mg/L, TOC = 4.83 mg/L). By ecotoxicity analyses, it was found that the water was genotoxic from its RMCN as high as 29.57‰ and MI reduction rate as high as 22.36%.

3 Conclusions

By bioassays using the Q67 test and *V. faba* root tip test associated with SPE, the cytotoxicity and genotoxicity of organic compounds in surface waters and streams of secondary effluents were evaluated. By SPE operation, a wide range of organic substances could be enriched from water samples, which enabled an assessment of their overall toxic effects without detailed investigation of individual organic toxicants. Although high organic contents, such as expressed by COD or TOC, are often associated with high cytotoxicity and genotoxicity, as with the case of sample No. 2 from the seriously polluted Zaohe River in this study, there is principally no clear correlation between them. The cytotoxicity expressed by TII_{50} and genotoxicity expressed by RMCN may have a correlative relationship in most cases but organic toxicants may show differences in these two kinds of toxicities according their sources. Correlative relationships were also found between DO concentration and cytotoxicity or genotoxicity. This provides a strong proof that sufficient dissolved oxygen in surface water is an indicator of its healthy environmental condition.

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