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# Reverse osmosis pretreatment method for toxicity assessment of domestic wastewater using *Vibrio qinghaiensis* sp.-Q67

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ABSTRACT

Luminescent bacterial test is a fast and sensitive method for acute toxicity assessment of water and wastewater. In this study, an improved toxicity testing method was developed using the freshwater luminescent bacteria *Vibrio qinghaiensis* sp.-Q67 that involved pretreatment of water samples with reverse osmosis (RO) to eliminate the interferences caused by nutrients in concentrated samples and to improve the reliability and sensitivity of the analysis. Because water samples contain low concentrations of several target toxic substances, rapid acute toxicity testing method that is commonly employed does not achieve enough sensitivity. The proposed RO pretreatment could effectively enrich organic and inorganic substances in water samples to enable a more effective and sensitive toxicity evaluation. The kinetic characteristics of toxicity of raw sewage and secondary effluent were evaluated based on the relative luminescence unit (RLU) curves and time–concentration-effect surfaces. It was observed that when the exposure time was prolonged to 8-h or longer, the bacteria reached the logarithmic growth stage. Hence, the stimulating effects of the coexisting ions (such as $Na^+$, $K^+$, $NO_3^-$) in the concentrated samples could be well eliminated. A 10-h exposure time in proposed Q67 test was found to quantitatively evaluate the toxicity of the organic and inorganic pollutants in the RO-concentrated samples.



## 1. Introduction

Ecotoxicity is an index to reflect the impact of pollutants on environment and human health (Bundschuh and Schulz, 2011). In the formulation of laws and regulations, ecotoxicity control is one of the effective approaches from the viewpoint of aquatic environment protection (Mendonca et al., 2009), especially when a water body is receiving effluent discharge from wastewater treatment plants (Bundschuh et al., 2011). In many countries ecotoxicity monitoring has already been adopted routinely for wastewater management (Hernando et al., 2005; Carafa et al., 2011).

Compared to chemical analysis targeting individual pollutants, ecotoxicity is a measure of the integrated toxic effects of all the pollutants on aquatic fauna and flora. The toxicity is usually tested by bioassays using chosen aquatic organisms that are sensitive to toxic substances. If the detection limit of the bioassay is sufficiently low or the concentration of the toxic substances in the testing sample is sufficiently high, it is preferable to use the original water sample for the toxicity tests to avoid any change in the chemical composition. However, in many cases the concentration of the toxic substances in the test sample is very low and the sensitivity of toxicity test may be much affected by the coexisting nutrient salts that often have strong stimulating effects on the bioassay (Ore et al., 2007; Macken et al., 2008). Therefore, sample pretreatment is often required for isolating and/or concentrating the target toxic substances and eliminating the interference.

When organic substances are the targets of ecotoxicity test, liquid–liquid extraction (Pérez et al., 2009), resin adsorption (Reginatto et al., 2009) and solid-phase extraction (SPE) (Dagnino et al., 2010; Smital et al., 2011) are the common pretreatment methods for effective extraction of organic substances and elimination of all inorganic interferences. If heavy metals become the target substances, passive sampling can effectively concentrate the metals ions by utilizing diffusive gradient in thin-films (Roig et al., 2011). However, the ecotoxicity test should virtually be an integrated evaluation of the ecotoxic effects of all possible toxic substances in the water sample. Any loss of either inorganic or organic toxic substances following these methods will affect the reliability of the test. Therefore, it needs to find a way to

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concentrate a low-concentration sample without much change in its chemical composition.

For the ecotoxicity test, the bioassay using luminescent bacteria has been recognized as a useful method for its short testing time, good sensitivity and reproducibility (Mantis et al., 2005; Parvez et al., 2006; Ye et al., 2011). The mechanism of the luminescence emission is that luciferase catalyzes oxidation reaction of the reduced state flavin mononucleotide ($FMNH_2$) to the oxidized state (FMN) accompanying light release, which is closely related to the bacterial metabolism (Girotti et al., 2008). Any factors that may affect the bacterial metabolism will have effect on the luminescence emission. When the luminescent bacteria are exposed to toxic substances, the metabolism of luminescent bacterium will be disturbed so that the light emission is weakened. By measuring the luminescence before and after a certain exposure time, the toxicity can be quantitatively evaluated using a bioluminescence inhibition value (Zhou et al., 2010). In a water sample, some inorganic matter such as nutrient salts may promote the respiration of the luminescent bacteria, and stimulate light emission (Rosal et al., 2010). Thus, to assure the reliability of toxicity tests, the method for decreasing the stimulating effect from these interference substances is also an essential study.

Due to the relatively short generation cycle of bacteria, the exposure time for the bioassay using luminescent bacteria is often set as shorter as 15 min and the toxicity is taken as the acute toxicity (ISO 11348, 2008). However, the time-dependent toxicity of herbicides, pentachlorophenol and chloramphenicol etc. in the chronic toxicity assay has also been identified using luminescent bacteria (Froehner et al., 2002; Zhu et al., 2009). Therefore, the time-dependent toxicity assay using luminescent bacteria may need further investigation especially for practical water samples for which little information is available.

In this study, the main objective was to develop a pretreatment procedure in order to improve the sensitivity of the luminescent bacterial assay for toxicity assessment. A new scheme of bioassay was thus proposed with prolonged exposure of the luminescent bacteria *Vibrio qinghaiensis* sp.-Q67 (Q67) to the substances in water samples from a domestic wastewater treatment plant (WWTP) concentrated by reverse osmosis (RO). The kinetic characteristics of toxicity of raw sewage and secondary effluent were also analyzed.

## 2. Materials and methods

### 2.1. Sample collection and preparation

Raw sewage and secondary effluent samples were collected from a WWTP in Xi'an, China where an oxidation ditch process was applied. The sampling was conducted when the WWTP was operated under normal weather and operational conditions. The collected samples were firstly filtered by a 0.45 μm mixed cellulose ester membrane mounted on a stainless steel single filter holder ($\Phi$ 150 mm) to remove the suspended solids. The membrane was boiled in Milli-Q water before use and the filtrate of the first batch was discarded.

A series of inorganic salt solutions were also prepared for a preliminary experiment to compare the recoveries of different cationic and anionic ions by RO concentration. The target cationic ions were $Na^+$, $Ca^{2+}$ and $Al^{3+}$, and anionic ions were $Cl^-$, $NO_3^-$ and $SO_4^{2-}$. For this purpose, NaCl, $CaCl_2$, $AlCl_3$, $NaNO_3$ and $Na_2SO_4$ salts of analytic grade were used and solutions were prepared by dissolving each of these salts into deionized water.

### 2.2. Sample concentration

For concentrating organic and inorganic substances from water samples, a RO device (MSM-2008, Mosu Co. Shanghai, China) was used. The 0.45 μm filtered sample was pumped into the RO device where the permeated solution was discharged and the concentrate was sent back to the sample reservoir, thus forming a circulating flow for continuous concentrating the sample until it reached the prescribed concentration. The final concentrate was collected for subsequent time-dependent toxicity test. After each batch of concentrating operation, the RO membrane module was firstly washed with 0.1–0.2 percent NaOH solution and then with purified water. The final concentration fold of the concentrated sample was calibrated by measuring the total organic carbon (TOC) concentration before and after RO concentration using Shimadzu TOC-VC, Japan. The ionic concentration of the sample was estimated by conductivity measurement using HQ30d (HACH, USA). The concentrated samples were stored at 4 °C for later use.

To concentrate only the organic substances from the water samples, SPE was conducted following the authors' previous work (Ma et al., 2011).

### 2.3. Q67 toxicity test

For the toxicity test, the Q67 luminescent bacteria purchased from Beijing Hammatsu Photon Techniques Inc. was grown in a culture medium that could produce larger quantity of bacteria with fluorescence enzyme (Ma et al., 2012). The bacteria were inoculated from the stock culture medium maintained at 4 °C to a 50 ml liquid medium and grown up to the logarithmic growth stage after 10 to 12 h under 22 ± 1 °C while shaking at 120 rpm.

The toxicity test was conducted in two ways. One was the acute toxicity test with exposure time as 15 min following the authors' previous work (Ma et al., 2011). Another was the toxicity test with exposure time prolonged up to 12 h, and operation procedures as below.

The grown-up Q67 bacteria from the liquid medium were used as the inoculum for the time-dependent bioassay performed on Centrol IApc LB962 Microplate Luminometer (Berthold Technologies Company, Germany). The initial RLU was controlled in the range of $3\times10^6$–$4\times10^6$. For each concentrated water sample, ten testing solutions with gradient diluting ratios were prepared and each solution was tested in three parallels plus one blank control which were placed in the wells of the 96-well Microplate (Corning, American). In each of the wells, the volume ratio of the inoculum, two-fold concentrated medium and the testing solution (or blank) was 1:4:5, and the final test volume was 200 μl. All the marginal wells were filled with 200 μl Milli-Q water to avoid marginal effect on the accuracy of the measurement. The microplate was incubated at 22 °C for 12 h with RLU measurement in every 30 min. Eq. (1) was used for calculating the integral inhibition value (Zhu et al., 2009).

$$Inhibition_m\ (\text{percent}) = \left(1-\frac{\sum_{i=0}^{m-1}(Lt_{i+1}+Lt_i)}{\sum_{i=0}^{m-1}(LCt_{i+1}+LCt_i)}\right)\times 100 \quad (1)$$

where $Inhibition_m$ is the integral inhibition value at time $t_m$ (percent), $Lt_i$ is the average RLU of the parallel testing solutions at time $t_i$, and $LCt_i$ is the average RLU of the controls at time $t_i$.

Eq. (1) is suitable for evaluating the response of exposure time on light inhibition in the time-dependent ecotoxicity test. The time–concentration-response (integral inhibition value) surface diagrams were designed to show the three-dimensional (3D) experience. Contrast to integral inhibition, for the acute toxicity text the exposure time was fixed as 15 min and the inhibition value could be calculated as Eq. (2)

$$I(\text{percent}) = \frac{R_0-R}{R_0}\times 100 \quad (2)$$

where $I$ is the inhibition value (percent), $R$ and $R_0$ are the average RLU values of the parallel samples and the controls, respectively, after 15 min exposure.

To obtain the growth curve of the Q67 bacteria, the optical density of bacteria suspension was measured using a spectrophotometer (UV-1650PC, Shimadzu, Japan) at a wavelength of 600 nm.

## 3. Results and discussion

### 3.1. Recovery of inorganic and organic substances by RO-concentrating

To evaluate how inorganic and organic substances could be recovered by RO-concentrating, a preliminary experiment was conducted using solutions of known concentrations of NaCl, $CaCl_2$ and $AlCl_3$ for comparing the recoveries of different cationic ions, and that of NaCl, $NaNO_3$ and $Na_2SO_4$ to compare the recoveries of different anionic ions. The secondary effluent collected from the WWTP was also used for comparing the recoveries of ions and organic substances from practical samples. Each of these samples was firstly pumped into the RO device where the permeated and concentrated solutions were separately collected. The concentrated solution was then sent to the RO device for the next round of concentrating operation. Similar operation was repeated four rounds. In each round, the volumes, conductivity, and/or TOC concentration of the permeated solutions were measured to