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Acute toxicity evaluation of explosive wastewater by bacterial bioluminescence assays using a freshwater luminescent bacterium, *Vibrio qinghaiensis* sp. Nov.

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ABSTRACT

The compositions of explosive wastewater generated from TNT (2,4,6-trinitrotoluene) purification stage were characterized by using UV–vis spectroscopy, Fourier transform infrared spectroscopy (FTIR), high performance liquid chromatograph (HPLC) and gas chromatograph/mass spectroscopy (GC/MS). The acute toxicity was evaluated by bacterium bioluminescence assay using a freshwater luminescent bacterium (*Vibrio qinghaiensis* sp. Nov.) and a marine luminescent bacterium (*Photobacterium phosphoreum*). The results showed that the wastewater's biodegradability was poor due to the high amount of chemical oxygen demand (COD). The main organic components were dinitrotoluene sulfonates (DNTS) with small amount of TNT, dinitrotoluene (DNT), mononitrotoluene (MNT) and other derivatives of nitrobenzene. It was highly toxic to luminescent bacteria *P. phosphoreum* and *V. qinghaiensis* sp. Nov. After reaction time of 15 min, the relative concentration of toxic pollutants (expressed as reciprocal of dilution ratio of wastewater) at 50% of luminescence inhibition ratio was 5.32×10^{-4} for *P. phosphoreu*, while that was 4.34×10^{-4} for *V. qinghaiensis*. *V. qinghaiensis* is more sensitive and suitable for evaluating the wastewater's acute toxicity than *P. phosphoreum*. After adsorption by resin, the acute toxicity can be greatly reduced, which is helpful for further treatment by biological methods.

1. Introduction

TNT (2,4,6-trinitrotoluene) is a kind of high explosive widely used in bombs, shells, grenades, demolition explosives and propellant compositions [1–7]. During the purification stage of TNT production, sodium sulfite is introduced to remove asymmetric TNT. Large amounts of wastewater containing dinitrotoluene sulfonate (DNTS), TNT, dinitrotoluene (DNT), mononitrotoluene (MNT) and other nitrobenzene derivatives are generated, which is highly toxic to peoples' health and surrounding environment. So it is essential to assess the acute toxicity of explosive wastewater before its discharge.

Toxicity measurements are often conducted to evaluate wastewater's toxicity by using micro-organisms [8,9], invertebrate [10,11], small animals [12,13], fish [14] and plants [15]. However, these methods are time-consuming and the obtained results are not satisfied due to its poor reproducibility and repeatability.

The luminescent bacteria test is a rapid and cost-effective method used to evaluate the acute toxicity of wastewater by measuring the reduction of light output of luminescent bacteria [16,17], where luminescent marine bacteria are often used, such as *Photobacterium phosphoreum* [18,19] and *Vibrio fischeri* [20–22]. However, high concentrations of sodium chloride (2–3%) are needed to supplement to the testing solution, which may change the inherent properties of freshwater samples. *Vibrio qinghaiensis* sp. Nov. is a kind of freshwater luminescent bacterium separated from body surfaces of *Gymnocypris przewalskii* (a fish species living in Qinghai Lake, China) [23]. Ma et al. [24] evaluated the toxicity of polluted river water samples using *V. qinghaiensis* and *V. fischeri*. The results showed that *V. qinghaiensis* was effective and reliable as well as the conventional *V. fischeri*, which was more suitable for freshwater bioassay due to its wide pH tolerance.

The objective of the present work was to evaluate the acute toxicity of explosive wastewater generated from TNT purification stage by using this freshwater luminescent bacterium. *P. phosphoreum* was also used for comparison. In addition, the change of its toxicity was also assessed after adsorption treatment by two kinds of macro-porous resins.

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2. Materials and methods

2.1. Materials

The explosive wastewater was obtained from Dongfang Chemical Corporation, Hubei Province, China. It has an intense red color with the density of 1.101 g mL^{-1}. The luminescent bacteria *V. qinghaiensis* and *P. phosphoreum* were provided by Beijing HAMAMATSU Photon Techniques Inc.

2.2. Water quality detection

The chemical oxygen demand (COD) was determined by using potassium dichromate oxidation method. The biochemical oxygen demand after 5 days (BOD_5) was determined according to Chinese standards GB 7488-87. The chemical properties of wastewater samples were detected by using UV–vis spectroscopy, FTIR spectroscopy, HPLC analysis and GC/MS analysis.

The UV–vis absorption of wastewater sample was determined by using a UV1800 spectrophotometer (Shimadzu, Japan) with deionized water as reference. The FTIR spectrum of the sample was conducted by using a NICOLET spectrometer (Thermo Scientific, USA) with 64 scans and a resolution of 4 cm^{-1}.

An Agilent 1100 liquid chromatograph (Agilent Corporation, USA) was used to determine the concentrations of DNTS with the detection wavelength of 230 nm, where an Agilent SB-Aq column with inner diameter of 4.6 mm and length of 250 mm was adopted as the separation system.

A gas chromatograph coupled with a mass spectrometer (GC6890/MSD5973N, Agilent Corporation, USA) was also used to analyze wastewater samples. The mass spectra obtained were utilized to identify the components according to NIST 05 mass spectral library database.

2.3. Acute toxicity test

The acute toxicity test of explosive wastewater sample was conducted according to ASTM D5660-96 (2009) (Standard Test Method for Assessing the Microbial Detoxification of Chemically Contaminated Water and Soil Using a Toxicity Test with a Luminescent Marine Bacterium) by using a BHP9511 water quality toxicity analyzer (Beijing HAMAMATSU, China). Two kinds of luminescent bacteria were used, *P. phosphoreum* and *V. qinghaiensis* sp. Nov. A 0.05 mL bacterial suspension and 2 mL water sample with different dilution ratios were thoroughly mixed in a test tube and the relative light intensity was recorded after different reaction times. The luminescence inhibition ratio LIR (%) was calculated according to the equation:

$$\text{LIR}\quad(\%) = \frac{RLI_{ref} - RLI_s}{RLI_{ref}} \times 100,$$

where *RLI* is the relative light intensity of the luminescence emitted from luminescent bacteria. The indices *ref* and *s* represent the reference and sample, respectively.

2.4. Acute toxicity evaluation of wastewater after adsorption treatment

The static adsorption test was used to investigate the feasibility of adsorption resin produced by Xian Putian Biological Technology Co., Ltd. (Shanxi, China) for reducing the acute toxicity of explosive wastewater. Due to its high concentrations of toxic pollutants, the wastewater was diluted 10 times for later use. The pre-weighed amount of resin (6.0 g) was put into a 250 mL flask containing 50 mL wastewater samples. The flasks were sealed and shaken at 30 °C in a constant temperature oscillator (Taicang Laboratory Equipment Factory, Jiangsu Province, China). When the adsorption process reached equilibrium, the solution was withdrawn and filtered, and then it was used for acute toxicity test.

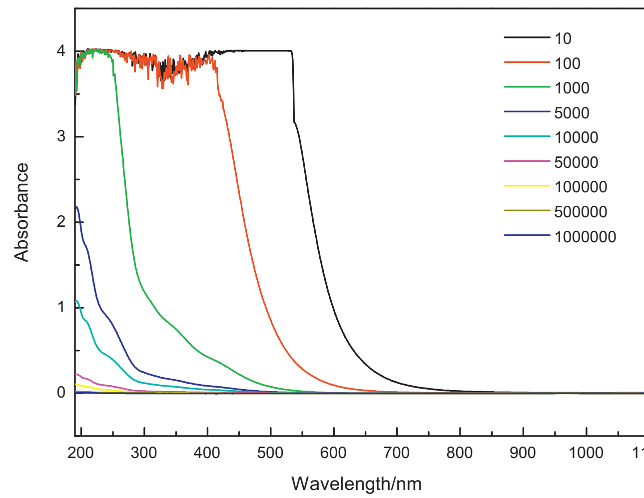

Fig. 1. Absorption spectra of wastewater samples at different dilution ratios.

3. Results and discussion

3.1. Water quality analysis

The explosive wastewater quality was evaluated by determining COD and BOD_5. The COD is as high as 100,800 mg L^{-1}, while BOD_5 is 28,200 mg L^{-1}. BOD_5/COD is only 0.28, meaning that it is difficult to treat the wastewater directly by conventional biological method.

Fig. 1 shows the absorption spectra of wastewater samples at different dilution ratios in the ultraviolet and visible region. Strong absorption in ultraviolet region (A_{200} = 1.90) can still be observed when it was diluted 5000 times, indicating a large concentration of aromatic compounds [25].

FTIR was used to identify the organic functional groups in wastewater sample. Because water absorbs strongly in the infrared region, the wastewater FTIR spectrum was obtained by subtracting infrared spectrum of water. Nine characteristic peaks were observed (Fig. 2). The peak at 1606 cm^{-1} is characteristic of vibration of benzene ring. The peak at 1544 cm^{-1} is assigned to

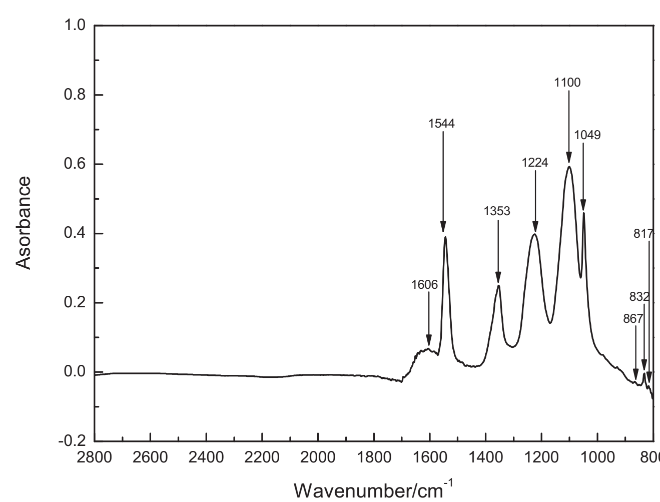

Fig. 2. FTIR spectrum of explosive wastewater sample.

asymmetric stretching vibration of nitro group, while the peak at 1353 cm^{-1} is assigned to symmetric stretching vibration of nitro group. The peaks at 1224 cm^{-1} and 1049 cm^{-1} are assigned to asymmetric stretching vibration and symmetric stretching vibration of sulfonate, respectively. The peak at 1100 cm^{-1} is assigned to asymmetric stretching vibration of O=S=O. In addition, there are two weak peaks at the positions of 867 cm^{-1} and 817 cm^{-1}, which are assigned to out-of-plane bending wagging of methyne in 1,2,4,5-tetra substituted benzene ring and 1,2,3,4-tetra substituted benzene ring, respectively. The peak at 832 cm^{-1} is assigned to stretching vibration of C–N band. From the above analysis, it can be suggested that the main organic components of the wastewater are 2,4-dinitrotoluene-3-sulfonate (2,4-DNT-3-SO_3^-) and 2,4-dinitrotoluene-5-sulfonate (2,4-DNT-5-SO_3^-). The concentrations of 2,4-DNT-3-SO_3^- and 2,4-DNT-5-SO_3^- were determined quantitatively by HPLC [26], which were 19.69×10^3 and 31.03×10^3 mg L^{-1}, respectively.

In our study, GC/MS analysis was used to determine micro amounts of organic molecules that cannot be detected by FTIR analysis. A 100 mL wastewater sample was extracted with 5 mL dichloromethane 3 times under acidic, neutral and basic conditions. The extracts were combined, dried under nitrogen, and the residue was dissolved in 1 mL of dichloromethane. Over 20 peaks can be detected from gas chromatogram, indicating the complexity of organic components in wastewater. The strongest peak appeared at 67.127 min is assigned to 1,3,5-trinitrobenzene (TNB). Other organic components include 2-methyl-3,5-dinitrophenol (2-CH_3-3, 5-DNP), TNT, 3,5-dinitro-*p*-toluidine, 2,6-dinitrotoluene (2,6-DNT), 2,5-dinitrotoluene (2,5-DNT), 2,4-dinitrotoluene (2,4-DNT), 2-nitro-toluene, 3-methyl-2-nitrophenol, 5-methyl-2-nitrophenol, 4-nitro-toluene and 3-methyl-6-nitrobenzoic acid.

From the above results, it can be concluded that the composition of the wastewater is complex with high amount of COD, containing toxic pollutants such as DNTS, TNT, DNT, MNT and other nitrobenzene derivatives.

3.2. Acute toxicity test

Luminescent bacterium emits luminescence in clear water substance. Toxic pollutants in wastewater can destroy its metabolic process and inhibit the luminescence. The inhibition extent is correlated with the whole amount of the toxic pollutants, which can be used to evaluate the wastewater's toxicity. Because the composition of toxic pollutants in the explosive wastewater is complex, the reciprocal of dilution ratio is used to express the relative concentration of toxic pollutants (RCTP) in our study.

Fig. 3 illustrates the dependence of luminescence inhibition ratio on RCTP after 15 min of reaction. For *P. phosphoreum*, LIR increased with increasing RCTP. When RCTP changed from 0.0001 to 0.01, LIR increased from 1.9% to 97.6%. The luminescence was completely inhibited when RCTP increased further. For *V. qinghaiensis*, LIR changed in a similar way as that for *P. phosphoreum*. It increased from 1.3% to 99.9% in the RCTP range of 0.0001–0.01 and then leveled off in the following concentration. It can be noted that for *V. qinghaiensis*, the LIR increased more quickly in the range of 0.0001–0.01 than that for *P. phosphoreum*. For example, when the RCTP was 0.001, LIR was 66.0% for *P. phosphoreum*, while it was 77.4% for *V. qinghaiensis*. This suggests that *V. qinghaiensis* is more sensitive to the toxicity of the wastewater than *P. phosphoreum*.

Additionally, the effect of reaction time on RCTP at 50% LIR was presented in Fig. 4. It can be observed that RCTP at 50% LIR decreased with increasing reaction time. For *P. phosphoreum*, RCTP at 50% LIR was 9.78×10^{-4}, 6.83×10^{-4} and 5.32×10^{-4} at 5, 10 and 15 min reaction times, respectively. For *V. qinghaiensis*, the corresponding RCTP at 50% LIR was 5.13×10^{-4}, 4.60×10^{-4} and 4.34×10^{-4}. It looks like exposure time varying from 5 to 15 min was not important to the response of *V. qinghaiensis*. From the above results, it can be concluded that the wastewater is highly toxic to marine bacterium *P. phosphoreum* and freshwater bacterium *V. qinghaiensis*. *V. qinghaiensis* is more sensitive and suitable for evaluating the acute toxicity of the explosive wastewater than *P. phosphoreum*. So in our later study, *V. qinghaiensis* was used for evaluating the change of wastewater's acute toxicity.

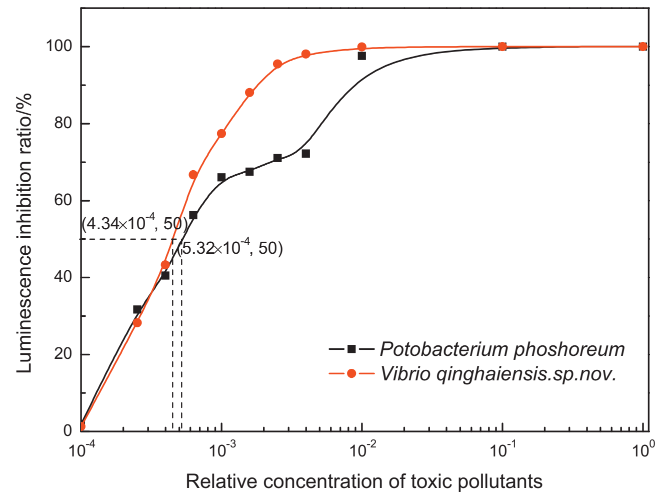

Fig. 3. Dependence of luminescence inhibition ratio on relative concentration of toxic pollutants for *Photobacterium phosphoreum* and *Vibrio qinghaiensis* sp. Nov.

3.3. Change of wastewater's acute toxicity after adsorption

Because of the high amounts of DNTS and other nitrobenzene derivatives in the explosive wastewater, it was diluted 10 times before it was treated by adsorption resin. Fig. 5 illustrates the dependence of luminescence inhibition ratio on relative concentration of toxic pollutants in wastewater sample before and after adsorption by two kinds of resins RS 50A and RS 50B. The LIR% increased with increasing RCTP. The unprocessed water sample can cause 99.99% of LIR, while the water samples processed by RS 50A and RS 50B can cause 62.68% and 32.34% of LIR. It can be clearly seen that the curve of LIR%–RCTP for water sample after adsorption is below that for unprocessed water sample, meaning that the acute toxicity of wastewater sample decreased after adsorption. For water samples before and after adsorption by RS 50A and RS 50B,

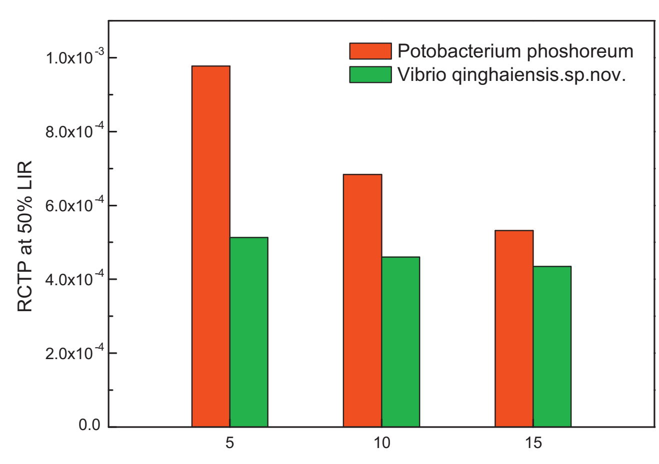

Fig. 4. Effect of reaction time on RCTP at 50% luminescence inhibition ratio for *Photobacterium phosphoreum* and *Vibrio qinghaiensis* sp. Nov.

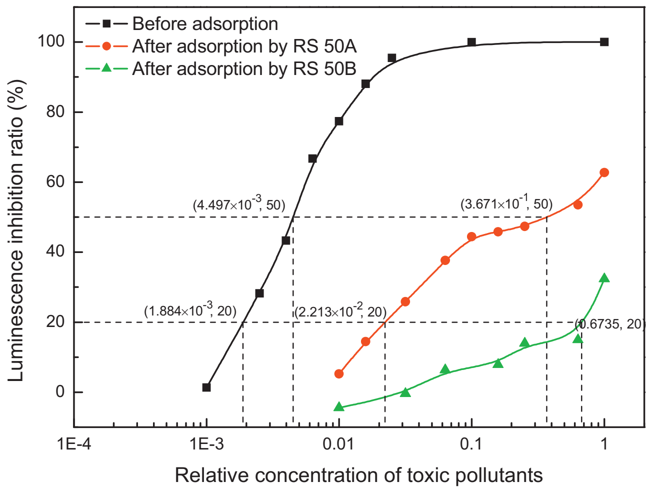

Fig. 5. Dependence of luminescence inhibition ratio on relative concentration of toxic pollutants before and after adsorption.

the RCTP at 20% LIR is 1.884×10^{-3}, 2.213×10^{-2} and 6.735×10^{-1}, respectively, which suggested that the acute toxicity of wastewater sample reduced 91.49% and 99.72% after adsorption by RS 50A and RS 50B, compared with that of unprocessed water sample. It can also be concluded that RS 50B can reduce the wastewater's acute toxicity more effectively than RS 50A.

4. Conclusions

The biodegradability of explosive wastewater generated from TNT purification stage is poor due to its high amount of COD. The main organic components are DNTS with small amount of TNT, DNT, MNT and other derivatives of nitrobenzene. It presents high acute toxicity to luminescent bacteria *P. phosphoreum* and *V. qinghaiensis*. Compared with *P. phosphoreum*, *V. qinghaiensis* is more sensitive and suitable for evaluating the wastewater's acute toxicity. RS 50B can reduce its acute toxicity more effectively than RS 50A. For the explosive wastewater, the acute toxicity can be greatly reduced by adsorption method, which is helpful for further treatment by biological methods.

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