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Evaluation of removal efficiency for acute toxicity and genotoxicity on zebrafish in anoxic–oxic process from selected municipal wastewater treatment plants

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HIGHLIGHTS

- Water quality of the effluents from A/O process met the standard to discharge.
- The acute toxicity was completely removed through the A/O process.
- The genotoxicity was not reduced, and even increased during the A/O process.

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ABSTRACT

The anoxic–oxic (A/O) process has been extensively applied for simultaneous removal of organic contaminants and nitrogen in wastewater treatment. However, very little is known about its ability to remove toxic materials. Municipal wastewater contains various kinds of pollutants, some of which have recalcitrant genotoxicity and may cause potential threat to environment, and even can lead to extinction of many species. In this study, we have selected three municipal wastewater treatment plants (WWTPs) employing anoxic–oxic (A/O) process to evaluate their ability to remove acute toxicity and genotoxicity of wastewater. Mortality rate of zebrafish (*Danio rerio*) was used to evaluate acute toxicity, while micronucleus (MN) and comet assays were used to detect genotoxicity. Results showed that in this process the acute toxicity was completely removed as the treatment proceeded along with decrease in chemical oxygen demand (COD) (<50 mg L^{-1}) in the effluent. However, in these treatment processes the genotoxicity was not significantly reduced, but an increase in genotoxicity was observed. Both MN and comet assays showed similar results. The eliminated effluent may pose genotoxic threaten although its COD level has met the Chinese Sewage Discharge Standard. This study suggests that further treatment of the wastewater is required after the A/O process to remove the genotoxicity and minimize the ecotoxicological risk.

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1. Introduction

In many countries, the discharge of municipal wastewater is increased with population growth and economic development, particularly in China. Municipal wastewater contains abundant a mixture of simple to more complex molecules, which are recalcitrant to biodegradation. (Heidrich et al., 2011). Many trace organic pollutants, such as nonylphenol, estrone (E1), estradiol (E2), alkylphenols, bisphenol A (BPA), and phthalates could not be effectively removed by the traditional treatment processes and have been discharged into the water bodies (Anderson and Wild, 1994; Diekmann et al., 2004; Bolong et al., 2009). These substances are potentially hazardous, causing bioaccumulation, genotoxicity, and endocrine disruption, which can be a threat to the health of global environment (Marttinen et al., 2002). Genotoxicity is of special concern, because it may cause adverse reproductive damage to organisms directly or even lead to their extinction (Anderson and Wild, 1994; Diekmann et al., 2004).

Anoxic–oxic (A/O) process is widely used in municipal and industrial wastewater treatment, which is capable of removing suspended solids (SS), nitrogen and biological oxygen demand (BOD). In A/O process, ammonia is converted aerobically to nitrate (nitrification) and then nitrates to nitrogen gas (denitrification) (Cui and Jahng, 2004). However, some substances, which are recalcitrant to be biodegraded, cannot be removed efficiently. The residual toxicants may cause potential ecological hazards like antimicrobial degradation, feminization of organisms, and bioaccumulation of these compounds in soil, plants and animals (Bolong

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et al., 2009; Camacho-Munoz et al., 2010; Fatta-Kassinos et al., 2011).

To date, the evaluation of water quality in wastewater are mainly based on physico-chemical parameters, such as BOD, chemical oxygen demand (COD), SS, total organic carbon (TOC), nitrogen, and phosphorus, which are not appropriate to evaluate the toxicity (Ma et al., 2005). In many countries, some toxic substances such as PCDDs/PCDFs and PCBs have been listed as priority to be controlled. Presently worldwide wastewater discharge standards are not covering all the aspects of toxicity. Thus, there is a lack of knowledge to prove whether the conventional A/O process will actually reduce the toxic effects of wastewater on aquatic organisms. So it is necessary to evaluate the toxicity of the wastewater before its discharge into the environment. At the moment only a few researches have focused on the bioassays for assessing the detoxification efficiency of the municipal wastewater treatment process (Wang et al., 2001, 2003; Ma et al., 2005; Huang et al., 2010). They have employed several techniques to isolate and identify toxins from complex mixtures. Conversely, interactive effects may be reduced as the mixtures were resolved into single components (Desbrow et al., 1998). Therefore, the toxicity evaluation of whole complex mixture is a useful tool to predict the environment risk, which is a comprehensive response to all the contaminants in the wastewater, including synergistic or antagonistic effects (Wang et al., 2003; Petala et al., 2008).

For the evaluation of wastewater toxicity, bioassays have been proved to be valuable tools in aquatic ecotoxicology for detecting the total biological potential risk of a mixture of pollutants (Chen and White, 2004). Different assays are used to evaluate many aspects of the components' activity and obtain a complete overall assessment of the hazards (Feretti et al., 2008). Micronucleus (MN) and comet assays have been extensively used for the evaluation of genotoxicity of single compound and complex mixture. MN assay can measure chromosome fragments, whole chromosomes or chromatids, which lag behind in anaphase. Several studies have shown that the peripheral erythrocytes of fish have a high incidence of micronuclei after exposure to pollutants under field and laboratory condition, which is associated with cancer prediction (Al-Sabti and Metcalfe, 1995; Bolt et al., 2011). The comet assay represents a rapid, inexpensive and sensitive method for measuring genotoxic effects on individual cells (Fairbairn et al., 1995; Lee and Steinert, 2003). It detects the DNA strand lesions and alkali-labile sites (single and double-strand breaks) by measuring the migration of DNA fragments from immobilized nuclear DNA (Singh et al., 1988).

This study focused on evaluating the effectiveness of the A/O process with respect to removal of both acute toxicity and genotoxicity. The acute toxicity was evaluated by the mortality rate of zebrafish, and genotoxicity was tested by MN and comet assays. Three different wastewater treatment plants (WWTPs) represent a complete view of detoxification efficiencies of wastewater from the A/O process, which can better reflect a more realistic exposure scenario.

2. Materials and methods

2.1. Sampling and processing

Three municipal WWTPs (Plants A, B, C) in northeast China were chosen to evaluate the toxicity elimination efficiency. Plant A receives not only municipal wastewater (about 50% of total influent), but also considerable amount of pretreated industrial wastewater such as dye wastewater. Plants B and C receive municipal wastewater. The three plants are performed with the A/O process under similar operating parameters, in which the Hydraulic Retention Time (HRT) of the anoxic tank and oxic tank are about 2.5 h and 5 h, respectively.

The wastewater samples were collected from each unit of municipal WWTPs over a 24 h period according to composite sampling methods (USEPA, 2002), including influent and effluent from aerated grit chamber, anoxic tank, oxic tank and secondary sedimentation tank (Effluent.), respectively. Then the airtight wastewater bottles were stored at 4 °C.

2.2. Chemical analysis of water quality

The samples were precipitated, and the supernatant was collected to determine the water quality parameters like color, pH, DO, COD, NH_4^+-N, TN, TP and water temperature according to Standard Methods for the Examination of Water and Wastewater (APHA, 1998).

2.3. Zebrafish culturing

Zebrafish (wild-type, Tuebingen strain) provided by the Model Animal Research Center of Nanjing University were kept in dechlorinated tap water at a constant temperature of 25 ± 1 °C. They were fed with commercial fish food pellets three times per day.

2.4. Exposure

Ten zerbrafish of both sexes (3–6 months old) were exposed to 3 L water samples for toxicity tests. The zebrafish were not fed for 24 h prior to the experimental period. The dechlorinated tap water without toxicant was served as negative control (NC). The positive controls employed genotoxic compounds like potassium dichromate and 4-nitroquinoline-1-oxide (4-NQO) at different concentrations (Supplemental Figs. 1 and 2).

2.5. Toxicity tests

2.5.1. Acute toxicity assay

An acute-static 96-h toxicity test was performed according to OECD Guideline 203 (OECD, 1992). The wastewater samples were diluted with dechlorinated tap water. Zebrafish (*Danio rerio*) were exposed to wastewater samples at different concentrations (about 1 g fish per liter water). The mortality rate was observed after 96 h of exposure to evaluate the acute toxicity.

2.5.2. Genotoxicity assays

The MN and comet assays were employed to assess the genotoxicity of the wastewater samples. Zebrafish were exposed to wastewater samples at different concentrations for 96 h. The test concentrations for genotoxicity exhibited no significant acute toxicity.

For the MN assay, the zebrafish peripheral blood sample was added to the slides contained fetal bovine serum and dried. Then the blood cells were fixed in methanol for 20 min and dried at room temperature. Afterwards the cells were stained with 10% Giemsa prepared in phosphate buffer solution (PBS, pH 6.8) for 10 min, then washed with PBS, and dried at room temperature before microscope analysis. The MN frequency was determined to evaluate the genotoxicity (Al-Sabti and Metcalfe, 1995).

Comet assay was employed to detect the DNA strand breaks caused by the wastewater. Liver cells of zebrafish were used for the comet assay. The exposed fish was cut open and the liver tissues were collected for preparing the liver cell suspension. The liver tissues were cleaned using PBS, digested by trypsin, and then centrifuged at 100*g* for 10 min. The cell precipitates were suspended in DMEM to obtain the liver cell suspension for preparing the microgels in comet assay. Rough microscope slides were coated

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