

Genotoxicity and cytotoxicity reduction of the polluted urban river after ecological restoration: a field-scale study of Jialu River in northern China

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Received: 17 February 2016 / Accepted: 28 December 2016 / Published online: 13 January 2017
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Abstract To further treat the reclaimed municipal wastewater and rehabilitate the aquatic ecosystem of polluted urban rivers, an 18.5-km field-scale ecological restoration project was constructed along Jialu River, a polluted urban river which receives only reclaimed municipal wastewater from Zhengzhou City without natural upland water dilution. This study investigated the potential efficiency of water quality improvement, as well as genotoxicity and cytotoxicity reduction along the ecological restoration project of this polluted urban river. Results showed that the chemical oxygen demand (COD) and ammonia nitrogen (NH₃-N) of the reclaimed municipal effluent were reduced by more than 45 and 70%, respectively, meeting the Chinese surface water environmental quality standard level IV, while the total phosphorus and metal concentrations had no significant reduction along the restoration project, and Pb concentrations in all river water samples exceeded permissible limit in drinking water set by WHO (2006) and China (GB5749-2006). The in vitro SOS/umu

assay showed 4-nitroquinoline-1-oxide equivalent (4-NQO-EQ) values of reclaimed municipal wastewater of $0.69 \pm 0.05 \mu\text{g/L}$ in April and $0.68 \pm 0.06 \mu\text{g/L}$ in December, respectively, indicating the presence of genotoxic compounds. The results of 8-hydroxy-2'-deoxyguanosine (8-OHdG) and hepatic cell apoptosis in zebrafish after a chronic long-term (21 days) in vivo exposure also demonstrated that the reclaimed municipal wastewater caused significant DNA oxidative damage and cytotoxicity. After the ecological purification of 18.5-km field-scale restoration project, the genotoxicity assessed by in vitro assay was negligible, while the DNA oxidative damage and cytotoxicity in exposed fish were still significantly elevated. The mechanisms of DNA oxidative damage and cytotoxicity caused by the reclaimed municipal wastewater need further study.

Keywords Urban river · Reclaimed wastewater · Ecological restoration · Genotoxicity · Cytotoxicity · 8-OHdG

Responsible editor: Markus Hecker

Electronic supplementary material The online version of this article (doi:10.1007/s11356-016-8352-z) contains supplementary material, which is available to authorized users.

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Introduction

Along with accelerating and rapidly growing urbanization and industrialization of China, demand and usage of accessible runoff from urban rivers both by municipalities and industries are increasing. In recent years, more than 3×10^7 t of reclaimed wastewater from municipal wastewater treatment plants (MWWPs) were discharged into urban rivers per year (Yi et al. 2011). Many urban freshwater bodies are receiving these wastewaters, and downstream aquatic ecosystems have experienced considerable degradation. In order to further improve the water quality of reclaimed municipal wastewater and rehabilitate the aquatic ecosystems of polluted urban rivers, the advanced treatment of reclaimed municipal wastewater using ecological technology has

gained attention in China, as the treatment is less costly and requires less space and time compared to other advanced technologies. Sheng et al. (2012) reported that chemical oxygen demand (COD) was reduced from 250 to 50 mg/L, and ammonia nitrogen (NH₃-N) was reduced from 27 to 4 mg/L along a 23-km river remediation project in the Shijin River, North China. In another separate field-scale experiment, COD concentrations were reduced by 70% in an ecologically restored urban river in Guangzhou City, South China (Sheng et al. 2012, 2013). Although the COD of municipal wastewater was significantly reduced in the Shijin River, there were still a number of contaminants left that cannot be removed (Sheng et al. 2012, 2013).

Despite the fact that most of the synthetic chemical contaminants present in municipal wastewater are at very low and fluctuating concentrations, reclaimed wastewater from MWWPs can still pose genotoxicity and cytotoxicity to organisms (Kaur et al. 2014; Wang et al. 2007, 2013; Wu et al. 2010). While it has been shown that advanced treatment reduces the genotoxicity and cytotoxicity of reclaimed wastewater in an ecologically restored urban river, a basic understanding of this process is still lacking and its extent (Schwarzenbach et al. 2006). A large number of micropollutants that cause genotoxicity are present in the reclaimed municipal wastewater; however, they typically occur at low concentrations or at levels below the detection limit, which makes it difficult to estimate the genotoxicity by chemical analytical detection methods. In recent decades, several bioassays, including the micronucleus test, the SOS/umu test, and the Ames test, have been successfully used to assess the genotoxicity of wastewater (Mathur et al. 2007). The SOS/umu test is frequently used as a short-term, high-throughput *in vitro* genotoxicity assay for detecting DNA-damaging agents (Xu et al. 2014). Using the SOS/umu test, Magdeburg et al. (2014) found that some pollutants in MWP effluents could induce genotoxicity. Margot et al. (2013) also found that the metabolites of some pollutants in municipal wastewater could lead to genotoxic effects by using SOS/umu test. In recent years, *in vivo* genotoxicity assays gradually gained attention, as it can provide more information about the actual ecological risk (Roos and Kaina 2006; Valavanidis et al. 2009). In animal tissues and cells, DNA is the main target for environmental genotoxins, and a predominant and stable form of an oxidative product of DNA, 8-hydroxy-2'-deoxyguanosine (8-OHdG), has been widely used as a biomarker for oxidative stress and DNA mutagenesis risks in animals (Esperanza et al. 2015). Flow cytometry (FCM) is another effective way to investigate necrosis apoptosis and the genotoxicity. Using FCM, Xian et al. (2011) found that cells exposed to 10 mg/L NH₃-N for 24 h showed a significant increase in apoptosis and genotoxicity (Xian et al. 2011). Cytotoxicity and genotoxicity of prooxidant herbicides to the microalga *Chlamydomonas* was also demonstrated by using the FCM (Esperanza et al.

2015). There are many studies that investigated the relationship of DNA damage induced apoptosis and genotoxicity of pure toxic substances such as tributyltin and certain polycyclic aromatic hydrocarbons with *in vitro* bioassays (CHAKRABARTI 2003; Li et al. 2015).

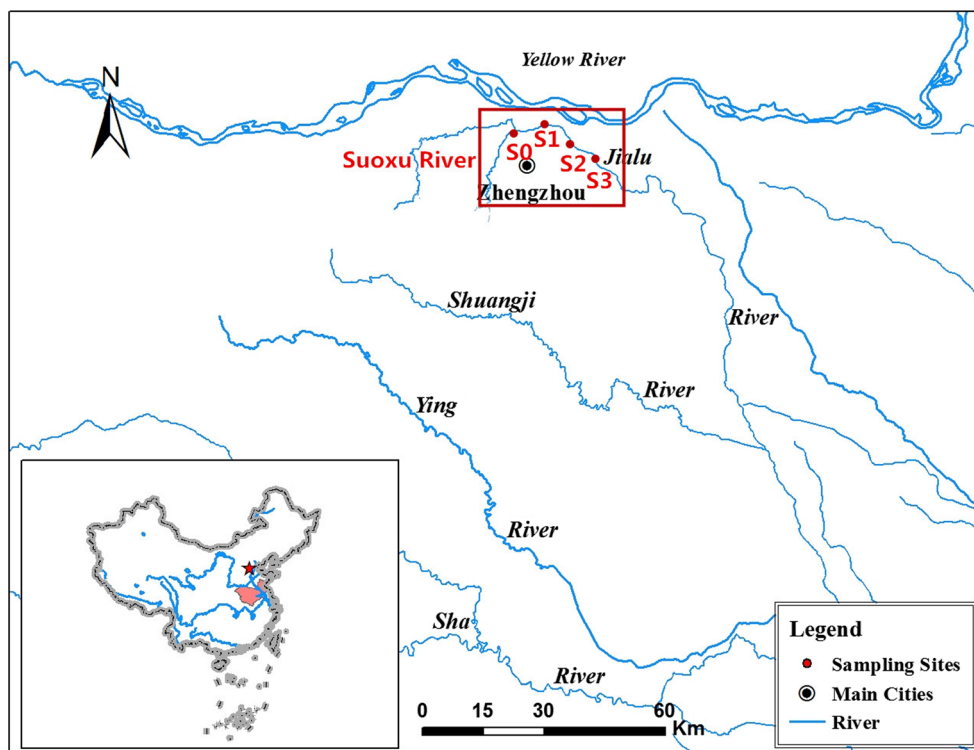
Jialu River, which is a polluted urban river flowing through Zhenzhou City and represents a tributary of the Huaihe River basin in North China, is estimated to receive about 200 kt of reclaimed wastewater from Zhengzhou City, without any natural upland water dilution. The COD and NH₃-N concentrations in Jialu River did not reach the level V value of Chinese surface water environmental quality standard (GB 3838-2002), which may lead to serious ecological risk. To further treat the reclaimed municipal wastewater and rehabilitate the aquatic ecosystem of the Jialu River, an 18.5-km field-scale ecological restoration project was implemented between 2009 and 2011. The river channel was newly reconstructed, and the channel was made wider (120–170 m) than before (30–60 m). Diverse species of aquatic plants, such as *Acorus calamus* L., *Cyperus imbricatus* Retz., *Ceratophyllum demersum* L., and *Scirpus tabernaemontani* Vahl, were planted along the restoration site in the Jialu River. Solar aerators at the Xiangyunsi section and rubber dams were used to add dissolved oxygen (DO) to river water (Supplementary materials). This study aimed to investigate the potential efficiency of the reclaimed municipal wastewater along the 18.5-km field-scale ecological restoration project to improve water quality, and to reduce genotoxicity and cytotoxicity of conventionally treated wastewater.

Materials and methods

A field-scale ecological restoration project along the Jialu River

The study was conducted in a segment of the Jialu River in northern China (Fig. 1). Specifically, the drainage systems designed to treat wastewater and help restore the habitats were in between site S0 (Shijiahe section, the first rubber dam, 34° 52' 13.5" N, 113° 34' 02.1" E) and site S3 (Xiangyunsi section, 18.5 km to S0, 34° 52' 09.1" N, 113° 43' 30.3" E) with a total length of 18.5 km. There are two more sites along the channel were selected for this study, which are site S1 (the second rubber dam, 7.7 km to S0, 34° 52' 12.2" N, 113° 36' 04.2" E) and site S2 (the third rubber dam, 11.7 km to S0, 34° 52' 12.2" N, 113° 36' 04.2" E) (Fig. 1). The average river flow upstream of the sewage treatment works was 216.5–328.9 L/s, and nearly 100% of river flow was reclaimed municipal

Fig. 1 Sampling sites located in upper reaches of Jialu River



wastewater from Zhengzhou City, without dilution by natural runoff.

Water sample collection

Water samples were collected every day at 0.5 m below the water surface between December 10 and 18 (air -2 – 5 °C, water 2 – 3 °C) of 2013 and between April 1 and 8 (air 4 – 9 °C, water 5 – 6 °C) of 2014 from sites S0 to S3. There was no rainfall on any of the sampling days. The hydraulic retention times (HRTs), average length of time the municipal wastewater remains in the 18.5 km stretch, between December 10 and 18, 2013, were 40.7 h (S0), 42.6 h (S1), 44.8 h (S2), and 40.2 h (S3), respectively. The HRTs between April 1 and 8, 2014, were 40.2 h (S0), 43.5 h (S1), 45.2 h (S2), and 39.6 h (S3), respectively.

Conventional water quality measurement

Water samples were first filtered through a 0.45 μm GFF filter (Xinya Purify Apparatus Company, Shanghai, China) to remove suspended particles. The same samples were also used to measure routine water quality parameters including chemical oxygen demand (COD), $\text{NH}_3\text{-N}$, total phosphorus (TP), and DO, which were determined using a DR2800 instrument (HACH, USA). Concentrations of metals and other elements, including Na, Ca, Mg, K, Ba, Fe, Al, Cu, Li, Mn, Ni, and Pb, were measured by inductive coupled plasma-atomic emission spectrometry (ICP-AES, J-A1100 Jarrel-Ash, USA). DO was

determined using dissolved oxygen analyzer (Eutech DO700, ThermoFisher, USA). Water samples used for use in the *in vivo* toxicity assay were stored in glass bottles at 4 °C before being used for fish exposure within 48 h.

Genotoxicity by SOS/umu assay

After being filtered, 1 L water samples were transferred to C_{18} cartridges using a solid-phase extraction apparatus at a flow rate of 10 mL/min (Zhenxiang Commerce and Trade Co., Ltd., China). Ten milliliters of both acetone and dichloromethane were pumped into each cartridge at 2.0 mL/min for elution. Then, eluates were mixed in a scaled centrifuge tube, and air-dried under a slow nitrogen flow. The residues were then dissolved in 1 mL dimethyl sulfoxide (DMSO). The concentrated stock was used to prepare exposure solutions for *in vitro* genotoxicity assay.

The genotoxicity of water samples was evaluated following the SOS/umu method based on *Salmonella typhimurium* TA1535/pSK1002 (Yan et al. 2014). Briefly, bacterial cultures grown overnight were diluted ten times with fresh TGA medium (KeyGEN Biotech, Nanjing, China) and incubated at 37 °C for 1.5 h with shaking (150 rpm). The test was performed on a microplate with the mixture consisting of 20 μL of $10 \times$ TGA (KeyGEN Biotech, Nanjing, China), 70 μL of bacterial culture, and 180 μL concentrated stock (180 μL DMSO used as control sample). The microplate was then incubated at 35 °C for 2 h with shaking (150 rpm). Then, the incubated mixture was diluted

ten times with fresh TGA medium and was further incubated for 2 h. The absorbance at 595 nm was measured, and then, 20 μL O-nitrophenyl β -D-galactopyranoside (6 mg/mL solution) were added to the mixture and incubated for 20 min. After that, 50 μL Na_2CO_3 (1 mol/L

solution) were added to terminate the reaction. Then, the absorbance was measured at 550 and 415 nm to calculate the genotoxicity. Toxicity tests were performed in triplicate. The activity of all samples was calculated by the following formula (Eq. 1):

$$\text{Activity}(\text{unit}) = 1000 \times [(A_{420_{\text{sample}}} - A_{420_{\text{control}}}) - 1.75 \times (A_{550_{\text{sample}}} - A_{550_{\text{control}}})] / [20 \times 0.18 \times (A_{595_{\text{sample}}} - A_{595_{\text{control}}})] \quad (\text{Eq. 1})$$

4-Nitroquinoline-1-oxide (4-NQO), of which eight concentration gradients from 0.1 to 25 $\mu\text{g/L}$ were set, was used as reference for the dose-response curve. The genotoxicity effect of water sample was then expressed as the equivalent concentration of 4-NQO (4-NQO-EQ, $\mu\text{g/L}$).

8-OHdG measured in zebrafish

Zebrafish (*Danio rerio*), which were obtained from the Institute of Hydrobiology of Chinese Academy of Sciences (Wuhan, China), were used for a 21-day exposure chronic toxicity test. Each individual used weighed between 0.25 and 0.3 g. Fish were first acclimatized in recirculating water for 3 weeks (25 ± 0.5 °C, 12: 12-h light/dark cycles). Fish were then exposed to water samples for 4 days to test their acute toxicity, and survival rate was monitored. Bioassays were conducted for 21 days chronic toxicity effects, while the control was exposed to MilliQ water with inorganic salt ($\text{CaCl}_2 = 2$ mmol/L, $\text{MgSO}_4 = 0.5$ mol/L, $\text{NaHCO}_3 = 0.5$ mol/L, $\text{KCl} = 0.05$ mol/L) to maintain the osmotic pressure balance in fish cells (GB/T 21808-2008). A total of 60 zebrafish were used for each treatment of water samples.

After 21-day exposure, each fish was weighed and the liver was collected. Hepatic tissue samples were then homogenized with PBS (0.1 g liver with 1 ml PBS, pH = 7.4) by 2 mL cold liver grinder that was prestored at -20 °C in a freezer. The supernatant was then transferred into clean tubes and was used for *in vivo* genotoxicity assay. Total protein content was measured by the Bradford assay using serum albumin as standard (Bradford 1976; Sun et al. 2016). The concentration of 8-OHdG in zebrafish, which indicates hepatic oxidative DNA damage, was monitored using a zebrafish 8-OHdG enzyme-linked immunosorbent assay (ELISA) kit (Nanjing Jiancheng Bioeng Inst., China). Competitive ELISA for 8-OHdG was performed according to manufacturer's protocol. Each experiment was performed in triplicate. The average concentration of 8-OHdG per microgram of DNA for each group was then calculated. The detection limits for 8-OHdG analysis using ELISA was 0.1 ng/L.

Cytotoxicity measured by flow cytometry

After 21-day exposure, ten zebrafish were randomly selected from each treatment group and their livers were aseptically isolated, and resuspended in cold 10 mM PBS buffer (137 mM NaCl, 2 mM KCl, 0.1% NaN_3 , pH = 7.2), and the blood vessels were carefully removed. Tissues were then digested by 0.25% pancreatin at 37 °C for 5 min, and 40 μL of trypsin-neutralized solution (KeyGen Biotech, Nanjing, China) was used to terminate the reaction. Then, approximately $1 \times 10^5 \sim 5 \times 10^5$ cells were collected by centrifugation at 2000 rpm for 5 min and washed by PBS twice. Cells were then stained with by 5 μL Annexin V (Annexin V-FITC Apoptosis Kit, KeyGEN Biotech, Nanjing, China) and 5 μL propidium iodide (PI) in 500 μL binding buffer and incubated for 5 min. Cells stained by both Annexin V and PI are necrotic cells, while those stained only by Annexin V represent apoptotic cells, cells neither stained by Annexin V nor PI are normal. Apoptosis and necrosis counts were then determined using FCM (BD Bioscience, USA).

Statistical analysis

Graphs were generated using GraphPad Prism 5.0 software (San Diego, CA, USA). All data were expressed as mean \pm standard deviation (SD). Tests for normality and homogeneity of variances indicated that all the data were normally distributed and had homogeneous variance in SPSS 13.0. Then, the data were analyzed statistically using Dunnett's multiple *t* test in SPSS 13.0. Statistical significance was set at a level of *p* ($p \leq 0.05$).

Results and discussion

Conventional water quality improvement along the ecological restoration project

A summary of the conventional water quality determined in water samples collected along 18.5-km ecologically restored urban river is presented in Table 1. Results showed that conventional water quality was improved

Table 1 Data and standards of conventional water quality (mg/L)

Time	Sites	COD	NH ₃ -N	TP	DO	Level
April	S0	81 ± 2	4.7 ± 1.1	0.15 ± 0.02	3.14 ± 0.17	Worse than level V
	S1	54 ± 1	2.1 ± 0.2	0.14 ± 0.03	4.72 ± 0.45	Worse than level V
	S2	39 ± 2	1.4 ± 0.3	0.15 ± 0.02	5.23 ± 0.28	IV
	S3	39 ± 3	1.1 ± 0.1	0.13 ± 0.01	5.86 ± 0.31	IV
December	S0	62 ± 3	4.4 ± 2.0	0.29 ± 0.04	2.16 ± 0.04	Worse than level V
	S1	50 ± 4	2.6 ± 0.6	0.27 ± 0.01	4.06 ± 0.12	Worse than level V
	S2	39 ± 2	1.3 ± 0.2	0.19 ± 0.03	4.89 ± 0.24	IV
	S3	38 ± 1	1.4 ± 0.1	0.24 ± 0.02	5.92 ± 0.18	IV
Chinese surface water standard level (GB3838-2002)		<20	<1.0	<0.2	<5	III
		<30	<1.5	<0.3	<3	IV
		<40	<2.0	<0.4	<2	V

Values are denoted as mean ± SD (n = 3)

significantly through the restoration of 18.5-km field-scale ecological restoration project. The COD concentrations at S0 site samples were significantly higher than the level V values of Chinese surface water environmental quality standard (GB3838-2002, 2002). And the COD concentrations were significantly reduced from 81 ± 2 mg/L (in April) and 62 ± 3 mg/L (in December) at site S0 to 54 ± 1 mg/L (in April) and 50 ± 4 mg/L (in December) at site S1, respectively. At site S3, COD levels decreased to 39 ± 3 and 38 ± 1 mg/L in April and in December, respectively, hence meeting the IV level of Chinese surface water environmental quality standard (i.e., 30 ~ 40 mg/L). NH₃-N levels were also significantly reduced along the ecological restoration project, dropping from 4.7 ± 1.1 mg/L (April) and 4.2 ± 2.0 mg/L (December) at site S0 to 1.1 ± 0.1 mg/L (April) and 1.8 ± 0.1 mg/L (December) at site S3, which were better than the IV (1.5 mg/L) and V (2.0 mg/L) threshold levels of Chinese surface water standard (GB3838–2002). It was reported that NH₃-N could induce production of reactive oxygen species at concentrations greater than 1 mg/L that could potentially damage DNA (Jiang et al. 2012; Xian et al. 2011). In this study, the NH₃-N levels at S3 site were greater than 1 mg/L, which may indicate that the water posed the risk of DNA damaged to aquatic lives. In short, the COD and NH₃-N concentrations were reduced by more than 45 and 70%, respectively. However, the TP in river samples collected from all four sampling sites did not change significantly (Table 1).

There are only a few reports on urban river remediation and restoration of effluents of municipal wastewater, and most of which are still based on laboratory work on constructed wetlands (Cao et al. 2012; Wu et al. 2011). An urban river after ecological restoration in Guangzhou (South China) was observed to naturally remove more than 70% COD from MWP effluents. Sheng et al. (2013) also reported that a field-scale experiment in an urban river in northern China removed 80%

COD and decreased its levels to 50 mg/L at the end of the river. At the same time, it also reduced NH₃-N from nearly 30 to 4 mg/L (Sheng et al. 2012, 2013). These two field-scale experiments demonstrated that COD and NH₃-N in polluted urban rivers could be removed effectively by ecological restoration projects. DO is essential for the oxidation of pollutants (Wang et al. 2015), and thus helpful in ecosystem restoration in a river (Ning et al. 2014). In this study, the DO concentrations continuously and significantly increased along the ecological restoration project in Jialu River (Table 1). The cost of the Jialu River restoration was about 0.018 Yuan/t, which was mainly labor costs such as cleaning of the rubber dams, sampling and control, and site inspection. It requires less cost compared to other advanced treatment processes, such as ozonation, active carbon adsorption, and MBR (Rousseau et al. 2008).

Concentrations of most heavy metal elements such as Cu, Fe, Ni, and Pb had partly or slightly reduction through 18.5-km field-scale ecological restoration project, while the Mg, K, Na, Si, and Mn concentrations were not significantly changed (Table 2). Pb, Cr, Ni, Fe, and Cu also known to cause genotoxicity by damaging DNA or inhibiting fidelity of DNA synthesis (Kaur et al. 2014; Esperanza et al. 2015). The Pb concentrations in Jialu River exceeded permissible limit in drinking water set by WHO (2006) and China (GB5749-2006, 2006) at 0.01 mg/L. The permissible limit in drinking water set by WHO (2006) and China (GB5749-2006, 2006) of Ni, Fe, and Cu were 0.02, 0.3, and 2 mg/L, respectively (Kaur et al. 2014). The concentration of Ni at S3 was 0.011 ± 0.001 mg/L in April and 0.010 ± 0.001 mg/L in December; the concentration of Fe at S3 was 0.07 ± 0.01 mg/L in April and 0.08 ± 0.01 mg/L in December; the concentration of Cu at S3 was 0.011 ± 0.001 mg/L in April and 0.007 ± 0.001 mg/L in December. Although some metals were partly removed, the residual heavy metals, such as Pb, may still cause genotoxicity.

Table 2 Metal concentrations in water samples (mg/L)

Time	Sites	Na	Ca	Mg	K	Ba	Fe	Al	Cu	Li	Mn	Ni	Pb
April	S0	106 ± 7	52.8 ± 1.6	31.8 ± 1.2	5.29 ± 0.38	0.11 ± 0.01	0.10 ± 0.01	0.059 ± 0.002	0.014 ± 0.001	0.024 ± 0.002	0.004 ± 0.001	0.017 ± 0.001	0.092 ± 0.007
	S1	117 ± 6	51.6 ± 2.2	34.6 ± 0.5	7.39 ± 0.41	0.11 ± 0.02	0.13 ± 0.01	0.048 ± 0.004	0.008 ± 0.001	0.026 ± 0.001	0.003 ± 0.001	0.011 ± 0.001	0.044 ± 0.003
	S2	110 ± 2	45.7 ± 0.9	33.6 ± 1.0	6.63 ± 0.22	0.11 ± 0.01	0.07 ± 0.01	0.045 ± 0.009	0.012 ± 0.002	0.025 ± 0.001	0.002 ± 0.001	0.012 ± 0.001	0.052 ± 0.001
	S3	111 ± 5	34.5 ± 0.5	33.0 ± 0.8	5.38 ± 0.30	0.11 ± 0.00	0.07 ± 0.01	0.070 ± 0.011	0.011 ± 0.001	0.024 ± 0.001	0.003 ± 0.001	0.011 ± 0.001	0.045 ± 0.002
December	S0	101 ± 2	50.9 ± 2.2	27.9 ± 0.2	4.33 ± 0.17	0.09 ± 0.00	0.14 ± 0.01	0.051 ± 0.008	0.009 ± 0.001	0.017 ± 0.001	0.003 ± 0.001	0.013 ± 0.001	0.107 ± 0.011
	S1	109 ± 4	44.7 ± 1.9	24.7 ± 0.6	4.21 ± 0.32	0.09 ± 0.01	0.09 ± 0.01	0.047 ± 0.006	0.008 ± 0.001	0.015 ± 0.001	0.003 ± 0.001	0.012 ± 0.001	0.055 ± 0.004
	S2	114 ± 6	39.2 ± 2.3	26.3 ± 0.9	4.56 ± 0.29	0.09 ± 0.01	0.12 ± 0.02	0.042 ± 0.008	0.009 ± 0.001	0.014 ± 0.001	0.002 ± 0.001	0.012 ± 0.001	0.060 ± 0.003
	S3	96 ± 4	41.0 ± 1.9	26.1 ± 0.4	4.62 ± 0.16	0.09 ± 0.00	0.08 ± 0.01	0.063 ± 0.010	0.007 ± 0.001	0.015 ± 0.001	0.003 ± 0.001	0.010 ± 0.001	0.057 ± 0.002
Permissible limit in drinking water by WHO													
						0.3		2			0.02	0.01	

Values are denoted as mean ± SD (n = 3)

Genotoxicity by SOS/umu assay in vitro

4-NQO-EQ values of site S0 samples were $0.69 \pm 0.05 \mu\text{g/L}$ in April and $0.68 \pm 0.06 \mu\text{g/L}$ in December, and slightly decreased to 0.50 ± 0.03 and $0.49 \pm 0.04 \mu\text{g/L}$ at site S1, and further decreased to 0.27 ± 0.02 and $0.28 \pm 0.01 \mu\text{g/L}$ at site S3, respectively (Fig. 2). The predicted no-effect concentration of 4-NQO was $0.64 \mu\text{g/L}$ 4-NQO, which was based on 12 no observed effect concentration data points of genotoxicity (carcinogenesis) obtained from a number of studies with fish, mice and cell lines following exposure to 4-NQO (Xu et al. 2014). In cases where 4-NQO equivalents exceeded $0.64 \mu\text{g/L}$, as the case at site S0, the water samples were likely to pose genotoxic risk to aquatic life. The 4-NQO-EQs of site S3 samples in both April and in December ranged from 0.25 to $0.30 \mu\text{g/L}$ and were less than the PNEC value, which indicated that the genotoxicities of site S3 samples in Jialu River were reduced to be negligible after the purification of 18.5-km field-scale ecological restoration project.

DNA damage in zebrafish after 21-day exposure

8-OHdG levels in zebrafish exposed to site S0 water were $134.6 \pm 8.0 \text{ ng/L}$ in April and $132.2 \pm 11.4 \text{ ng/L}$ in December after 21 days of exposure, which were significantly greater than those of the control group. This revealed that exposure to the S0 water samples resulted in significant oxidative damage to DNA in zebrafish, indicating that the river water was genotoxic. These results were congruent with the results of the SOS/umu assay. Along the ecological restoration project stretch of the Jialu River, 8-OHdG levels in zebrafish were significantly decreased for water samples from sites S1 and S2, and that of site S3 further decreased to $63.6 \pm 2.8 \text{ ng/L}$ in April and $68.4 \pm 3.4 \text{ ng/L}$ in December (Fig. 3).

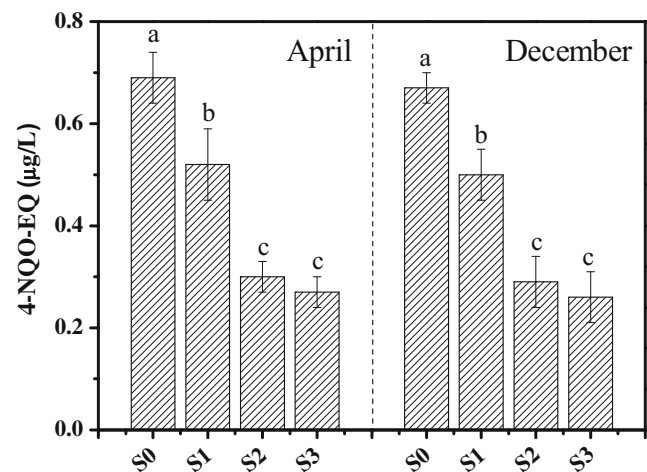


Fig. 2 The 4-NQO-EQ values of SOS/umu assay. Values are denoted as mean ± SD (n = 3), and the different superscript letters indicate significant differences ($p < 0.05$)

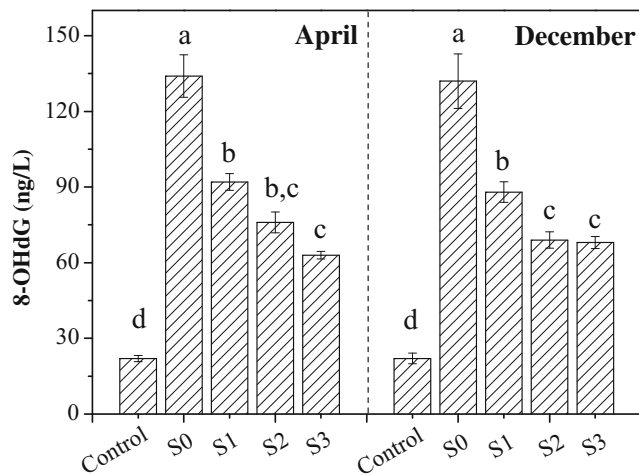


Fig. 3 The 8-OHdG levels in zebrafish after 21-day exposure. Values are denoted as mean ± SD (n = 3), and the different superscript letters indicate significant differences (p < 0.05)

Compared to the control group, 8-OHdG levels at site S3 showed a significantly greater genotoxicity than the control sample during both sampling seasons, which suggests that long-term exposure to effluent-dominated river water, even after treatment through the ecological restoration project, may still cause DNA damage in zebrafish. These results were different from those of the in vitro (SOS/mum) assay. These differences could be due to the following possibilities. Firstly, in SOS/mum in vitro assay experiments, some volatile organic pollutants could be lost during the nitrogen-drying process; in addition, NH₃-N and metal ions were not captured by the enriching process using C₁₈ cartridges (Magdeburg et al. 2014). It is then possible that the in vitro test undertaken in this study might not have been able to evaluate the toxic effects of NH₃-N or heavy metals in water, while many of these pollutants can cause genotoxicity to zebrafish (Kaur et al. 2014; Xian et al. 2011). Secondly, *S. typhimurium* and zebrafish have very different metabolic capacity, as well as are characterized by different structural properties that can affect the uptake of chemicals. For example, the bacterial cell wall can prevent some micropollutants entering into the cells, while fish cells have no cell wall (Petit et al. 1995). Meanwhile, some chemicals could be metabolically activated in zebrafish, but not in bacteria. For example, many polycyclic and heterocyclic aromatic hydrocarbons, such as 3,4-diol-DB[a,j]A, had represented more genotoxicity in eukaryote cells rather than in *S. typhimurium*, as these chemical may be metabolic activated by cytochrome P450 enzymes (CYPs) which were only expressed in eukaryote cells (Xu et al. 2005). Lastly, zebrafish may bioaccumulate many pollutants in river water during 21-day exposure, which would result in greater exposure than in the short-term SOS/mum assay.

Cytotoxicity assay using FCM

Chakrabarti (2003) and Li et al. (2015) reported that DNA damage may lead to cytotoxicity, which can be induced by toxic pollutants such as tributyltin and polycyclic aromatic hydrocarbons (PAHs) (CHAKRABARTI 2003; Li et al. 2015). Excessive mitochondrial injury and oxidized DNA products such as 8-OHdG may directly cause apoptosis and necrosis in cells (Kultz 2005). Two cellular strategies have evolved for coping with DNA damage. First, DNA damage is repaired (Kultz 2005). Second, cells that contained DNA damage due to exposure to toxic substance are removed from the tissue mainly by apoptosis or necrosis. Nonrepaired DNA damage often has harmful consequences that manifest as gene mutations (or called genotoxicity) (Kultz 2005).

FCM has been shown to provide an objective, reproducible, and sensitive characterization of the cytotoxicity level in a large number of cells. However, very few studies have investigated the link between the percentage of necrosis and apoptosis and the genotoxicity level in natural water body with zebrafish bioassay. Zebrafish exposed for 21 days, were observed to have significantly upregulated concentrations of 8-OHdG (Fig. 3), which may indicate genotoxic effects of contaminants in the water samples tested. To better confirm the genotoxic effects in zebrafish, the FCM-based detection of necrosis and apoptosis were analyzed in hepatic cells. Results showed that the apoptosis percentages of hepatic cells of fish exposed to S0 water were 20.9 ± 1.1% in April and 18.9 ± 1.1% in December, and the necrosis percentages were 7.9 ± 0.3% in April and 6.7 ± 0.5% in December, which all were significantly greater than that of control group (Table 3). This indicated that the reclaimed municipal wastewater poses obvious cytotoxicity to zebrafish after 21 days of exposure. Although apoptosis and necrosis percentages of

Table 3 The percentage of necrosis and apoptosis in hepatic cells

Time	Sites	Normal cells (%)	Apoptosis (%)	Necrosis (%)
April	Control	96.3 ± 3.1 ^a	2.3 ± 0.2 ^a	1.6 ± 0.1 ^a
	S0	71.3 ± 4.9 ^c	20.9 ± 1.1 ^c	7.9 ± 0.3 ^c
	S1	80.6 ± 6.2 ^b	14.0 ± 1.7 ^c	5.8 ± 1.0 ^c
	S2	88.5 ± 3.4 ^{a,b}	8.5 ± 0.9 ^b	3.0 ± 0.2 ^b
	S3	93.0 ± 5.2 ^{a,b}	4.2 ± 0.6 ^b	2.4 ± 0.1 ^b
December	Control	95.4 ± 2.3 ^a	2.6 ± 0.3 ^a	2.1 ± 0.1 ^a
	S0	74.2 ± 3.6 ^c	18.9 ± 1.1 ^c	6.7 ± 0.5 ^{b,c}
	S1	83.3 ± 5.8 ^b	11.7 ± 0.8 ^c	4.8 ± 0.6 ^b
	S2	91.6 ± 3.4 ^{a,b}	6.4 ± 0.8 ^b	2.6 ± 0.1 ^{a,b}
	S3	92.7 ± 4.0 ^{a,b}	4.7 ± 0.5 ^b	2.2 ± 0.2 ^a

Values are denoted as mean ± SD (n = 3); the different superscript letters indicate significant differences (p < 0.05)

hepatic cells of fish exposed to site S1 and S2 water were significantly decreased compared to S0 water, they were still significantly greater than that of the control group, indicating the river water still posed cytotoxic risks to zebrafish. This is in agreement with the results of 8-OHdG in zebrafish. The apoptosis and necrosis percentages of fish exposed to S3 water had further decreased to 4.2 ± 0.6 and $2.4 \pm 0.1\%$ in April and 4.7 ± 0.5 and $2.2 \pm 0.2\%$ in December, respectively. The percentages of apoptosis at S3 during both sampling seasons were also significantly greater than those of the control, which indicates that the water still had genotoxic properties and caused DNA damage and DNA damage-induced apoptosis. Considering this, we hypothesized that the river water still posed cytotoxic and genotoxic risks to zebrafish.

For $\text{NH}_3\text{-N}$ could interfere with energy metabolism through impairment of the tricarboxylic acid cycle in the mitochondria and cause oxidative damage to cells, it may lead to multiple deleterious effects on aquatic lives (Chew and Ip 2014). Notably, many fishes may suffer from oxidative stress exposed to 1 mg/L $\text{NH}_3\text{-N}$ within 2 weeks, and this concentration of ammonia was not regarded as high environmental ammonia (Chew and Ip 2014; Jiang et al. 2012; Xian et al. 2011). In this study, the $\text{NH}_3\text{-N}$ concentrations in all water samples were more than 1 mg/L, indicating that all water samples might pose oxidative stress to zebrafish. As one of the most toxic heavy metals, prolonged Pb exposure could increase the toxicity effects in fish because Pb could be accumulated in fish from contaminated water (Alves and Wood 2006). Alsop et al. (2016) had examined the possible toxicity effects of Pb in juvenile rainbow trout and found that the 0–97 $\mu\text{g/L}$ Pb could be accumulated and would cause damage to the gill, liver, and kidney (Alsop et al. 2016). Jurczuk et al. (2006) had also found that Pb could enhance the concentration of malondialdehyde and reduce the glutathione concentrations, which might be the main mechanisms of the peroxidative action by Pb (Jurczuk et al. 2006). The Pb concentrations in S3 water samples were 57 $\mu\text{g/L}$ in December and 45 $\mu\text{g/L}$ in April, indicating the S3 might pose oxidative stress to zebrafish.

Another possibility of genotoxicity in S3 is that the micropollutants causing genotoxicity present in the municipal wastewater may not be totally removed in the ecological restoration project. There is no direct evidence from literature that the ecological restoration project would remove the genotoxic organics, such as polycyclic aromatic hydrocarbons, some pharmaceuticals and so on. However, some literature had reported that the polycyclic aromatic hydrocarbons cannot be effectively removed in wetlands, and the water in wetlands may still pose genotoxicity and carcinogenesis risks (Li et al. 2014; Zhang et al. 2011). The genotoxic organics in the ecological restoration project will be studied further.

Conclusions

This study showed that COD and $\text{NH}_3\text{-N}$ in reclaimed municipal wastewater could be effectively removed, and DO could be continuously and significantly increased along 18.5-km field-scale ecological restoration project in Jialu River. The results of SOS/umu, FCM, and 8-OHdG assay demonstrated that the reclaimed municipal wastewater could induce genotoxicity, obvious DNA oxidation, and cytotoxicity, which may correlate with the presence of $\text{NH}_3\text{-N}$, exceedingly high concentrations of Pb and other pollutants. After the ecological purification of the 18.5-km field-scale restoration project, the genotoxicity assessed by in vitro assay was negligible, while the DNA oxidative damage and cytotoxicity level were still significant in exposed fish. The mechanisms of DNA oxidative damage and cytotoxicity caused by the reclaimed municipal wastewater need further study.

Acknowledgements We gratefully acknowledge generous support provided by NSFC (41203062 and 51438008), Jiangsu Nature Science Fund (BK20151378), China's National Key Project of Science and Technology (2012ZX07204-001; 2015ZX07204-007), and the Fundamental Research Funds for the Central Universities (090514380001).

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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