2012 International Conference on Geological and Environmental Sciences
IPCBEE vol.36(2012) © (2012)IACSIT Press, Singapoore

Evaluating the Ecological Risk of Nonylphenol Using Comet Assay in Constructed Wetlands

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Abstract. Constructed wetlands are commonly used to remove conventional contaminants due to their low cost and easy maintenance. This study determines the distribution of the endocrine disruptor nonylphenol (NP) and assesses the ecological risk using the genotoxicity test in two selected wetlands. The results show that the NP concentrations ranged from 0.094 to 18.05 μg/L from 35 collected water samples in the Wuluo wetland. For the Old Railroad Bridge wetland, NP was found in approximately 87 % of the water samples with concentrations ranging from undetectable to 0.47 μg/L in 15 collected water samples. The removal efficiency was above 75 % in the system. For ecological assessment, risk quotients were used to evaluate the potential ecological risk of NP using the comet assay. The calculated risk quotients forboth wetlands were greater than one and were up to 40 times higher in the Wuluo constructed wetland than in the Old Railroad Bridge wetland under the worst scenario, indicating that the NP concentrations in both wetland systems cause potential ecological risks to aquatic organisms. Furthermore, the decreasing risk quotient from influent to effluent indicates that alkylphenolic compounds such as NP are removed in these constructed wetlands.

Keywords: Constructed wetlands, Comet assay, Ecological risk, Nonylphenol

1. Introduction

ndustrial wastewater, agricultural wastewater, and non-point source runoff have entered rivers in Taiwan over the last three decades. Wetland systems are used to purify wastewater in order to reduce the loading that enters rivers [1,2]. However, the distribution and removal of xenobiotic compounds such as endocrine disruptors in constructed wetland systems are rarely discussed. Matamoros demonstrated a variety of small-scale domestic sewage treatment systems for removing pharmaceutical and personal care products and found that vertical flow constructed wetlands (VFCWs) are the most reliable and efficient [3]. Conkle found that the removal of pharmaceutically active compounds (PhACs) in a lagoon wetland was greater than 90 %, similar to that obtained in conventional activated sludge wastewater treatment plans [4].

The use of non-ionic surfactants has recently increased. They have entered the environment and have been proven to affect ecological and biological systems. Nonylphenol (NP), a degradation product of alkylphenol polyethoxylate (APE), is an endocrine-disrupting chemical that has estrogenic activities. It has been included in the priority list of substances regulated by the 2000/60/EC Water Framework Directive. The Taiwan Environmental Protection Administration (Taiwan EPA) has also restricted the use of NP as a commercial cleaning product additive due to its resistance to biodegradation and ability to bioaccumulate in biota [1,2]. Therefore, many researchers have reported the occurrence and toxicity of NP in the environment matrices [5]. There are many toxicological tools for evaluating the genotoxicity caused by environmental contaminants, such as chromosome aberration, the micronuclei test, sister chromatid exchanges, the mutation test, unscheduled DNA synthesis, and the comet assay. Among these tests, the comet assay is relatively

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simple and sensitive [6]. Frenzilli et al. analysed the DNA damage of catfish red blood cells exposed to environmental pollutants [7].

The present study investigates the distribution and removal efficiencies of NP in two constructed wetlands. The genotoxicity test and the NP concentration of various processing units are used to determine the ecological risk in the systems.

2. Site description

The water samples were collected from the Wuluo constructed wetland and the Old Railroad Bridge wetland respectively located on the left and right sides of the Kaoping River, which is the second longest river in Taiwan and one of the most important water sources in southern Taiwan. The Wuluo constructed wetland comprises 6 gravel contact filtration/aeration chambers and a 5-cell free-water surface (FWS) flow wetland. The sampling sites were located at the inlet, a small lotus pond, FWS #2, the wetland outlet, and the discharge point to the river. The Old Railroad Bridge wetland comprises a 12-cell FWS wetland and receives different sources of wastewater containing effluent A (paper mill wastewater) and effluent B (domestic wastewater). The sampling sites were at the inlet of effluent A, A1, A2, A3, A4, A5, and A6, and the inlet of effluent B, B1, B2, B3, B4, B5, B6, and B7 (outlet). The water in the A6 unit enters the B7 unit and then flows out of the system.

3. Materials and Methods

3.1. Chemicals and reagents

4-n-nonylphenol (NP) of analytical grade and Dulbecco's Modified Eagle's Medium (DMEM) were purchased from Sigma-Aldrich (Steinheim, Germany). The solvents and chemicals for chemical analysis were purchased from Merck (Darmstadt, Germany). Fetal bovine serum and penicillin/streptomycin were purchased from Gibco (USA) and sodium pyruvate, MEM non-essential amino acids solution, and trypsin were obtained from HyClone (USA). H_2O_2 (30% solution) was purchased from Showa (Japan) and dimethylsulfoxide (DMSO, 99% purity) was obtained from WAKO Pure Chemical Industries (Osaka, Japan). Comet LMAgarose and lysis solution were purchased from Trevigen Inc. (Gaithersburg, USA).

3.2. Sample extraction and analytical procedure

A volume of 1 L of the water sample was passed through 1-μm and 0.45-μm filters to remove suspended materials at a flow rate of 4 mL/min via organic solvent extraction with cartridges (Oasis HLB 6mL, Waters, USA) using a solid-phase extraction system (Varian, USA). The cartridges were eluted by methylene chloride/methanol (50:50), methanol, and de-ionized water to collect the concentrates. The eluates were evaporated and dried to near dryness by a gentle stream of nitrogen and then concentrated (1000x) by acetonitrile/ultra-pure H_2O to a volume of 1 mL as samples for chemical analysis. The NP analysis was carried out using a high-performance liquid chromatography-fluorescence (HPLC-FL) detector (Waters Alliance 2695 Waters, USA). An XTerra® C18 column (4.6 × 250 mm, 5 μm) was employed. The limit of detection of NP was 2 μg/L. The average recoveries for spiked samples for NP were determined to be 88.1～95.7% for quality control purposes.

3.3. Cell culture and maintenance

Hep G2 cells (human hepatocellular liver carcinoma cell line) were purchased from the Bioresource Collection and Research Center (BCRC) and stored in liquid nitrogen until use. The cells were grown at 37 °C in tissue-culture flasks that contained DMEM supplemented with 5% fetal calf serum and 0.4% penicillin/streptomycin, and cultivated in a humidified atmosphere with 5% CO_2.

3.4. Comet assay

The principle of the comet assay, also known as the single-cell gel electrophoresis assay, is based on the ability of denatured DNA fragments to migrate during electrophoresis. Electrophoresis is carried out under alkaline conditions (pH > 12.6) in order to detect DNA strand breaks. The DNA damage experiments were modified from the method described by Singh [8]. In brief, cell suspensions were prepared by washing the cells with phosphate buffer saline (PBS) and treating them with trypsin/EDTA for 5 min at 37 °C. The cell

density was adjusted to 1×105 cells/mL with serum-free medium, and the aliquot of 50 μL was added to 500 μL of 0.5 % molten Low melting point agorose. The cell suspension was rapidly spread onto a pre-coated slide and placed at 4 °C for 10 min while shielded from light. The cover slips were removed and the slides were treated with pre-chilled lysis solution for 1 h at 4 °C. After lysis, the slides were exposed to alkaline electrophoresis buffer (pH>13) and placed in an electrophoresis box filled with tris-borate-EDTA buffer for 10 min (300 mA, 25 mV). The alkali solution was neutralized with Tris buffer. The slides were rinsed with 70 % cold ethanol and dried at room temperature. The DNA was stained with Syber®-Green I (excitation 450–490 nm, emission 520 nm) and observed under a fluorescence microscope (CKS 41-RSL, Olympus, Japan). For slide scoring, 50 individual comets/slide and two slides/concentration were randomly selected and examined using Comet ScoreTM software (TriTek Corp., USA). Three parameters, namely tail DNA%, tail length, and tail moment, were used. Data analysis was performed using GraphPad Prism®4.0 software (GraphPad Software, Inc., San Diego, USA) to determine EC_{50}. The comet assay was validated using hydrogen peroxide (H_2O_2), a known genotoxin.

3.5. Removal efficiencies calculation

The removal efficiencies for NP in wetlands were calculated as $(C_{outflow}-C_{inflow})/C_{inflow} \times 100\%$, where C_{inflow} and $C_{outflow}$ represent the analyte concentrations in the inflow and outflow of the system, respectively. The mean removal efficiencies are reported. When the actual concentrations were below the corresponding method detection limits (MDLs), half of the MDL was used for calculation.

4. Results

4.1. Nonylphenol level in Wuluo constructed wetland

The detection frequencies of NP in the Wuluo constructed wetland were 100 %. The concentrations in the inlet, small lotus pond, and discharge point to the Kuoping river were in the ranges of 0.648 ~ 18.05 μg/L, 0.989 ~ 4.178 μg/L, and 0.094 ~ 0.413 μg/L, respectively. The average removal rate was above 90%. The removal efficiencies from the influent to the effluent were significantly different, as determine using the Kruskal-Wallis test ($p < 0.001$).

4.2. Nonylphenol level in Old Railroad Bridge wetland

The concentrations of NP were in the range of < 0.04 ~ 0.469 μg/L for effluent A. The average detection frequencies for effluents A and B were 86% and 88 %, respectively. The NP concentrations measured in the cells of the right bank of the A system were low, likely due to Alkylphenolic ethoxylates (APnEO) (such as NP_1EO and NP_2EO) not yet having decomposed into NP or NP being removed through decomposition (photochemical processes) or volatilization. The removal efficiency of NP was 79% from the inlet to the outlet in the B system. The concentrations of NP detected from both wetlands along the Kaoping river were higher than the NP concentrations found in Japan (0.052 ~ 0.144 μg/L) [9].

4.3. Genotoxicity of nonylphenol determined using Comet assay

The dose responses of NP were constructed from three comet parameters, namely tail DNA%, tail length, and tail moment, to determine the single-strand, double-strand breaks, as well as the alkali labile sites shown in Fig. 1. The three parameters gave a positive NP concentration response (DNA damage). The lowest EC50 was observed for the percentage of DNA in the comet tail (tail DNA%) following by the tail length and tail moment parameters. The obtained EC_{50} values were 3.996 nM ($r^2 = 0.9263$) for tail DNA%, 9.115 nM ($r^2 = 0.9638$) for tail length, and 29.57 nM ($r^2 = 0.9258$) for tail moment. The EC_{50} values obtained here are lower than those obtained by Choi et al. [10] and proved that the comet assay is capable of detecting DNA damage caused by pollutants at the cellular level.

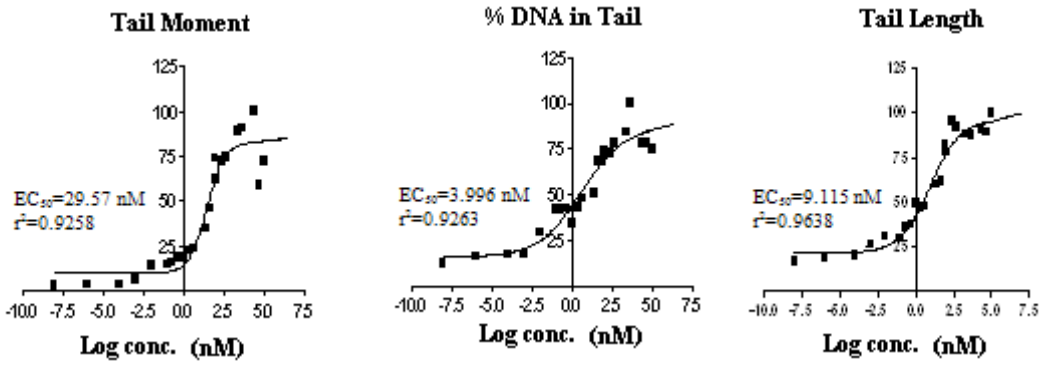

Fig. 1 : Dose response curves of nonylphenol derived from DNA damage assessed by comet assay.

4.4. Evaluation of ecological risks in constructed wetlands

NP is a major environmental concern due to its adverse effects on aquatic organisms. The risk quotients (RQs) were determined as the ratio of NP measured environmental concentration (MEC) to the predicted no-effect concentrations (PNEC) expected to affect the biota based on US EPA's ecological risk assessment framework. The PNEC was obtained from the calculation of the EC_{50} divided by extrapolation factor, which was set to 1000 for the aquatic risk assessment [11]. The confirmation of the potential risks was based on the value being greater or less than one. The RQs calculated from the EC_{50} values obtained from the three parameters show that the ecological risk for the Wuluo constructed wetland is 40 times higher than that for the Old Railroad Bridge wetland. In addition, the decreasing risk quotient from influent to effluent indicates that alkylphenolic compounds such as NP are treated in these constructed wetlands.

5. Acknowledgement

The authors would like to thank the Seventh River Management Office of the Water Resources Agency and National Science Council for financially supporting this research under grant MOEAWRA0980225 and NSC -99-2221-E-020- .

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