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Genotoxic and Neurotoxic Potential in Marine Fishes Exposed to Sewage Effluent from a Wastewater Treatment Plant

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Abstract

Concentrations of industrial, agricultural and natural chemicals have been increasing in secondary effluents without their combined sub-lethal effects having been elucidated. In this study, two assays (the comet and acetylcholinesterase assays) were combined to evaluate the genotoxic and neurotoxic effects of effluent from the Noksan wastewater treatment plant (WWTP) on two local marine fish species (flounder and sea eel). The fish were exposed to WWTP secondary effluent that had been diluted with filtered seawater to final concentrations of 1%, 10% and 50%. Analysis of fish samples collected 3 and 5 days after exposure showed that DNA damage occurred in flounder exposed to 50% effluent and in sea eels exposed to 10% or 50% effluent. Furthermore, it was found that acetylcholinesterase (EC:3.1.1.7, AChE) activity decreased in both species when exposed to 10% effluent, indicating the presence of large amounts of genotoxic and neurotoxic chemicals in the effluent. Our results indicate that the comet and AChE assays are promising tools for biomonitoring of secondary effluents.

Keywords: Effluent, Comet assay, Acetylcholinesterase assay, Flounder, Sea eel

Secondary effluents are a major source of toxic pollutants in aquatic environments, and the toxicity and complexity of chemicals in industrial wastewater are increasing. Secondary effluents contain a variety of synthetic materials, and chemicals including heavy metals, polycyclic aromatic hydrocarbons (PAHs), pesticides, health-related medicines, and industrial byproducts[1,2]. The presence and adverse effects of industrial secondary effluents in aquatic ecosystems is a significant threat to marine organisms, as these ecosystems are often a final reservoir for the accumulation of natural and synthetic pollutants[3,4]. Field studies of secondary effluents from wastewater treatment plants (WWTPs) have reported genotoxicity and neurotoxicity in fishes and invertebrates downstream from WWTPs[5-9]. Numerous WWTPs have been built in Korea to process increasing quantities of industrial and domestic wastewater, and many secondary effluents are discharged into receiving waters including rivers and the sea. Although knowledge of the toxicity of WWTP secondary effluents is fundamental to development of discharge controls and ecological risk assessments, few studies have been conducted on the adverse effects of these effluents on marine organisms and ecosystems[10]. Moreover, despite substantial reports of the potential toxicity of secondary effluents from industrial and municipal WWTPs, field studies have been extremely limited for many practical reasons[6,11,12], and *in vivo* laboratory bioassays have usually been used as an alternative approach for evaluating the toxicity of secondary effluents to aquatic organisms[10].

Biomarkers are indicators for evaluating the biological significance of environmental contaminants[13]. Biomarkers have been used as a promising ecotoxicological tool to evaluate toxicity, and their application is amenable to assessment of the effects on biological systems of untreated pollutants in secondary effluents[14,15]. Molecular and biochemical responses can enable early diagnosis of environmental pollution before perturbation of the whole community occurs[16,17], and biochemical and molecular biomarkers are robust tools for identifying and monitoring environmental risks to aquatic species. The comet assay has been widely used in toxicological studies assessing geno-

toxic potential in fresh water and sea water[18,19]. In this test, genomic DNA of cells in an agarose suspension is denatured under highly alkaline conditions and electrophoresed. The DNA fragments migrate to the positive pole, forming a long fuzzy "comet" tail. The tail length is a measure of the degree of damage to the DNA in the sample. The replication machinery will be nonfunctional on the damaged and mutated DNA strand, resulting in incomplete transcription and abnormal metabolites in the living organism[20]. Acetylcholinesterase (EC:3.1.1.7, AChE) is an enzyme involved in the hydrolysis of excessive acetylcholine in the body, and has been used as an enzyme biomarker for chemical pollutants including organic agrichemicals and carbamates[21,22]. AChE activity has been mainly studied in fish and bivalves. There have been many recent reports concerning the inhibitory effect of pesticides, insecticides and some of heavy metals on AChE activity[23-25].

Flounder (*Pleuronichthys cornutus*) and sea eels (*Conger myriaster*) are widely distributed bottom-dwelling species in South Korean coastal waters, and are of high economic value. They are easy to catch and maintain in the laboratory, and are considered good indicators of pollution[26-28]. In this study, short-term *in vivo* tests of the toxicity of secondary effluents on these marine species were conducted. The main objective was to evaluate the genotoxic and neurotoxic potential of secondary effluents on fish using environmental toxicology methods, and to assess the economic impact of effluents on coastal fisheries. Flounder and sea eels were exposed to a range of secondary effluent concentrations for fixed periods, at which time DNA damage and AChE activity were assessed as indicators of the genetic and neurological effects of the effluent on the test fish.

The inflow wastewater and secondary effluent of the Noksan WWTP, Korea (Figure 1), were collected. The test fish were exposed to three concentrations of secondary effluent (1%, 10% and 50%) in seawater in a toxicity test conducted over 5 days. Negative controls (seawater without added secondary effluent) and positive controls (10% inflow wastewater in seawater) were included. The temperature, pH and salinity of the treatment solutions remained almost unchanged during all experiments (Table 1). The salinity in the 50% effluent treatment was 10‰ lower than in the other treatments, but was adequate for fish survival during the experiment. The subsequent toxicity tests involved monitoring DNA damage and measuring AChE enzyme activity.

Genotoxicity of Secondary Effluent

The genotoxic potential of the secondary effluent was evaluated in the comet assay, which provided an indication of the degree of single strand breakage of DNA from the blood of the test fish. The degree of DNA tailing was classified as zero, low, medium, high or complete (values of 0, 1, 2, 3 and 4, respectively; Figure 2). The values were reported as percentages relative to the positive control[29-31]. The results showed that DNA damage increased with increasing concentration of the effluent, and that sensitivity to DNA damage varied between the fish tested (Figure 3). There was a significant increase in the comet tail for positive control fish relative to those comprising the negative

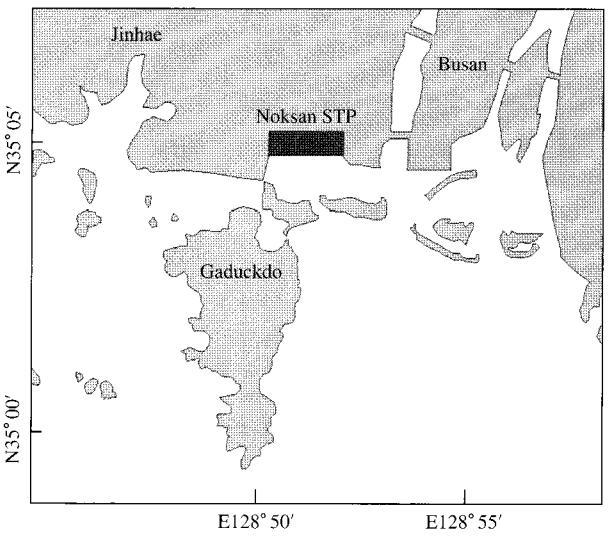

Figure 1. Map of sampling stations. The Noksan wastewater treatment plant is located in South Korea, and the treated secondary effluent is discharged into the coastal area.

Table 1. Temperature, pH and salinity in the culture vessels during experiments involving various percentages of secondary effluent mixed with filtered sea water. The positive control (PC) was 10% inflow wastewater in filtered seawater. The data are the means of two independent experiments.

	Temperature (°C)					pH					Salinity (psu)				
	0%	1%	10%	50%	PC	0%	1%	10%	50%	PC	0%	1%	10%	50%	PC
0 day	17.1	16.3	16.4	16.9	17.0	7.86	7.80	8.00	7.87	7.86	31.2	30.9	29.5	19.3	29.0
3 day	16.5	16.8	16.7	17.2	17.5	7.87	7.84	8.12	7.85	7.85	31.1	30.2	29.3	19.2	29.1
5 day	17.1	16.3	16.4	16.9	17.2	7.87	7.84	8.12	7.85	7.85	31.1	30.2	29.3	19.2	29.1

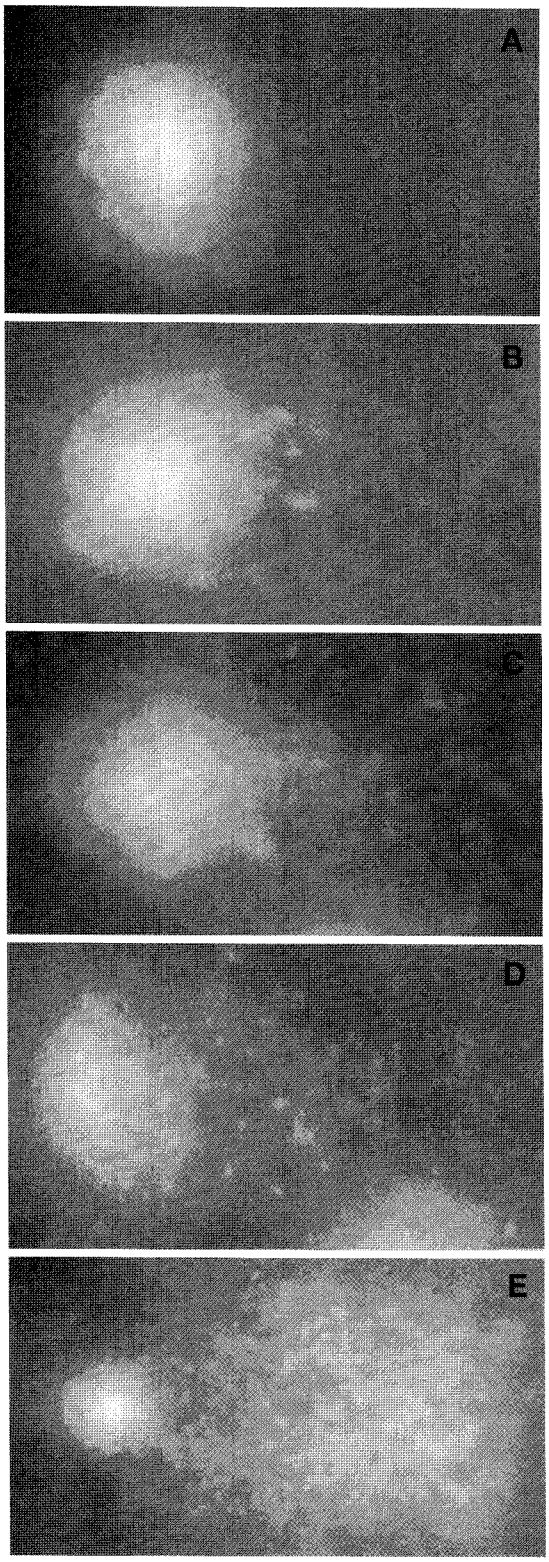

Figure 2. Gel electrophoresis of fish blood cells (× 1,000). A high degree of DNA damage, with a greatly reduced nucleoid core and significant migration of DNA fragments from the core, is evident. A, zero tailing; B, low tailing; C, medium tailing; D, high tailing; E, complete tailing.

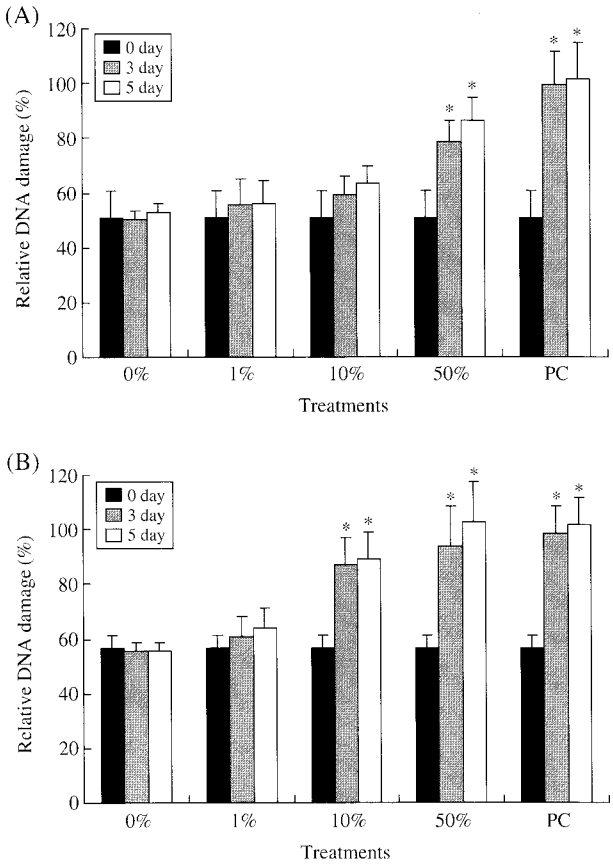

Figure 3. Damage to DNA from blood cells isolated from flounder (A) and sea eels (B) exposed to secondary effluent for 3 and 5 days. Data are means ± SDs for duplicate experiments (n=5). Data were analyzed by ANOVA and Duncan's multiple-range test. Asterisks donate means significantly different from the 0% effluent group at day 3 ($P < 0.05$).

control, indicating that the inflow wastewater contained genotoxic chemicals. Furthermore, significant DNA damage was found for flounder and sea eel exposed to both 50% and 10% secondary effluent ($P < 0.05$), but there was no difference between these treatments in the degree of DNA damage at 3 and 5 days after exposure. For sea eels exposed to 50% effluent, the effect was similar to that observed in the positive control. These results suggest a high correlation between DNA damage and the concentration of the secondary effluent.

Neurotoxicity of Secondary Effluent

AChE activity was markedly inhibited in the positive control (10% inflow wastewater), and similar results were obtained for the 50% effluent treatment for both fish species (Figure 4). Significant inhibition of AChE activity was also induced in both fish species by 10% effluent ($P < 0.05$), and there was no differ-

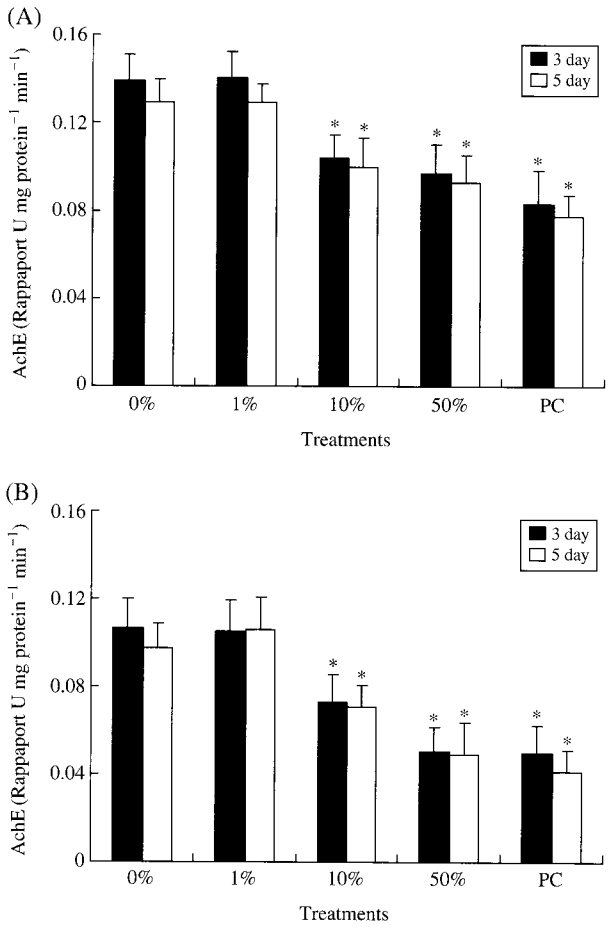

Figure 4. *In vivo* acetylcholinesterase activity for flounder (A) and sea eels (B) exposed to secondary effluent for 3 and 5 days. Data are means ± SDs for duplicate experiments (n= 5). Data were analyzed by ANOVA and Duncan's multiple-range test. Asterisks donate means significantly different from the 0% effluent group at day 3 ($P<0.05$).

ence in the degree of DNA damage 3 and 5 days after exposure to the effluent. These results show that there was a clear correlation between effluent concentration and the studied biochemical parameters for both species, and provide strong evidence for the presence of substantial amounts of genotoxic and neurotoxic chemicals in the secondary effluent as well as in the inflow wastewater.

Discussion

Secondary effluent is an important source of entry into the marine ecosystem for thousands of chemicals, some of which are toxic[32-34]. The detection and quantification of toxic chemicals in secondary effluent is a critical step in wastewater management. However, water quality policy has been mainly based on chemical-specific approaches, which have limitations associated with analysis sensitivity for the various chemicals of interest. In addition, a limited number of toxic chemicals is identified for control through secondary effluent regulations and laws, leaving others in secondary effluent undetected and unregulated. As secondary effluents from agricultural and municipal sources are a potential health and environmental hazard[35], sensitive analyses for the detection of chemical-induced toxicity and other biological impacts originating from secondary effluents of wastewater treatment plants are required. Moreover, the interactions of chemicals in the secondary effluent (synergism, antagonism, addition) can only be detected through whole-effluent assessment using effect-specific parameters. Thus, development of whole-effluent approaches to toxicity testing will potentially provide an effective tool for characterizing and regulating secondary effluents. Bioassays involving living species should constitute a sensitive indicator system for detecting individual and/or combined effects of known and/or unknown toxic chemicals in secondary effluents[36,37].

In conclusion, the combined assessment of DNA damage and AChE activity provided a rapid and comprehensive evaluation of the adverse effects on flounder and sea eel of inflow water and secondary effluent from a WWTP. The results showed that the effluent from the WWTP contained genotoxic and neurotoxic chemicals with the potential for adverse effects on marine fish and the marine ecosystem, as well as for human populations in coastal areas. Our results indicate that the comet and AChE assays are a promising biomonitoring tool for evaluation of secondary effluents and in development of water quality policies.

Materials & Methods

Water Sampling from the WWWP

The inflow wastewater and secondary effluent of the Noksan WWTP, Korea, were collected and stored at 4°C until used in experiments.

Test Animals

Live specimens of flounder (*Pleuronichthys cornutus*; 12-15 cm) and sea eel (*Conger myriaster*; 25-40 cm) were purchased at a market close to the Noksan WWTP. They were transported to the laboratory and acclimated for 5 days to the experimental tank environment (16 h : 8 h light:dark photoperiod; temperature =20 ± 2°C). The fish were not fed during the experiments.

Experimental Conditions and Exposure Strategy

The acclimated fish were exposed for 0, 3 and 5 days to each of three concentrations (1%, 10% and 50%) of the secondary effluent in filtered seawater (n=5 per treatment). The effects of exposure of the test fish to the inflow wastewater and the secondary effluent was also monitored. Filtered seawater without the addition of secondary effluent was used as a negative control, and seawater containing 10% inflow wastewater was used as the positive control. Experiments were conducted in 20 L tanks (50 × 30 × 30 cm) with constant aeration at a temperature of 20-22°C.

Comet Assay

Whole blood of flounder was collected by tail vein puncture using heparinized syringes, and diluted 1 : 1,000 with phosphate-buffered saline (PBS). The blood cells were washed twice with PBS and processed in a slightly modified comet assay[38-40]. A glass slide was covered evenly with 1% normal agarose and cooled. A mixture of 5 μL of sample with 100 μL of 0.65% LM agarose was loaded onto the slide and covered with a coverslip. The coverslip was removed after hardening the gel on ice, and the slide was placed in pre-cooled lysis buffer at 4°C for at least 2 h. The slide was then washed twice for 5 min in distilled water in Coplin jars, to remove salts. The electrophoresis chamber was filled with electrophoresis unwinding buffer, and after 15 min the slide was placed in the chamber and electrophoresis was conducted at 25 V (300 mA) for 20 min. The slide was removed and placed in distilled water for 5 min, then transferred to a Coplin jar containing 0.4 M Tris buffer (pH 7.5) to neutralize the sample. The slide was finally transferred to pre-cooled EtOH (4°C) for 5 min and dried at room temperature. EtBr (50 μL) was added to the sample, which was covered with a coverslip to enable microscope observations (× 1,000) of DNA single strand breakage.

Acetylcholinesterase Activity

Fish tissues for assessment of acetylcholinesterase activity were collected, washed twice in 0.01 M phosphate buffer (pH 7.5), and then homogenized in buffer (10 : 1 volume : tissue weight). The homogenate was centrifuged at 3,500 rpm for 20 min and the supernatant was used for measurement of enzyme activity. All solutions and equipment used in the AChE enzyme assay were stored at −80°C before use. The assay was performed by measuring the concentration of acetic acid produced from cholinesterase-mediated hydrolysis of acetylcholine using m-nitrophenol as an indicator[41]. The assay involved addition of 0.75 mL of distilled water, 0.05 mL of pre-heated (25°C) acetylcholine chloride solution, and 0.05 mL of tissue homogenate supernatant to a test tube, and incubation of the solution at 25°C for 30 min. The acetic acid produced by enzymatic hydrolysis of acetylcholine was assayed by spectrophotometric measurement (420 nm) of the decolorization of m-nitrophenol (yellow), associated with a decline in the solution pH with increasing concentration of acetic acid. Protein was quantified by measurement of the absorbance at 590 nm in the Bradford protein assay.

Data Analyses

All data are presented as means ± SDs. The data were statistically analyzed using a non-parametric ANOVA followed by Dunn's pair-wise multiple comparison test. Differences were considered to be statistically significant at $P < 0.05$.

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