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In situ impact assessment of wastewater effluents by integrating multi-level biomarker responses in the pale chub (*Zacco platypus*)

Woo-Keun Kim [a], Jinho Jung [b,*]

[a] System Toxicology Research Center, Korea Institute of Toxicology, Daejeon 34114, Republic of Korea
[b] Division of Environmental Science and Ecological Engineering, Korea University, Seoul 02841, Republic of Korea

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ABSTRACT

The integration of biomarker responses ranging from the molecular to the individual level is of great interest for measuring the toxic effects of hazardous chemicals or effluent mixtures on aquatic organisms. This study evaluated the effects of wastewater treatment plant (WWTP) effluents on the freshwater pale chub *Zacco platypus* by using multi-level biomarker responses at molecular [mRNA expression of catalase (CAT), superoxide dismutase (SOD), glutathione *S*-transferase (GST), and metallothionein (MT)], biochemical (enzyme activities of CAT, SOD, GST, and concentration of MT), and physiological [condition factor (CF) and liver somatic index (LSI)] levels. The mRNA expression levels of GST and MT in *Z. platypus* from a site downstream of a WWTP significantly increased by 2.2- and 4.5-fold ($p < 0.05$) when compared with those from an upstream site. However, the enzyme activities of CAT, SOD, and GST in fish from the downstream site significantly decreased by 43%, 98%, and 13%, respectively ($p < 0.05$), except for an increase in MT concentration (41%). In addition, a significant increase in LSI (46%) was observed in *Z. platypus* from the downstream site ($p < 0.05$). Concentrations of Cu, Zn, Cd, and Pb in the liver of *Z. platypus* were higher (530%, 353%, 800%, and 2,200%, respectively) in fish from a downstream site than in fish from an upstream location, and several multi-level biomarker responses were significantly correlated with the accumulated metals in *Z. platypus* ($p < 0.05$). Integrated biomarker responses at molecular, biochemical, and physiological levels (multi-level IBR) were much higher (about 4-fold) at the downstream site than at the upstream site. This study suggests that the multi-level IBR approach is very useful for quantifying *in situ* adverse effects of WWTP effluents.

1. Introduction

Wastewater treatment plant (WWTP) effluents, consisting of complex mixtures of chemicals, represent a significant source of water pollution (Metcalfe et al., 2010). For instance, hazardous chemicals such as metals, polycyclic aromatic hydrocarbons (PAHs), pesticides, alkyl phenols, and perfluoroalkyl substances have been found in WWTP effluents in Korea (Choi and Park, 2010; Kim et al., 2014; Lee et al., 2015b). Although chemical analyses can measure many of these compounds qualitatively and quantitatively, it is not possible to quantify all of the hazardous chemicals in affected water bodies (Cazenave et al., 2014). Furthermore, chemical analyses alone do not reveal the impact of chemical pollution on the aquatic environment because of the potential synergistic/antagonistic effects among chemical pollutants (Kerambrun et al., 2011). In addition, chemical analyses per se, without toxicological studies, do not reflect the pollutant effects in the organisms. In this context, alternative monitoring methods using biomarkers have been developed to provide a reliable assessment of environmental quality (van der Oost et al., 2003). In particular, the use of a battery of complementary biomarkers is recommended to gain an understanding of how an organism responds to exposure to hazardous chemicals (Cazenave et al., 2014; Lavado et al., 2006; Li et al., 2011).

Biomarker responses ranging from the molecular to the individual level have been widely used to measure the toxic effects of effluent mixtures (Houde et al., 2014; Triebskorn et al., 2001; Yeom et al., 2007) and hazardous chemicals (Kim et al., 2010, 2013). In particular, the integration of biomarker responses (IBR) index was found to be useful for comparing several toxicants, including perfluorinated organic compounds, heavy metals, and PAHs (Kim et al., 2010, 2013; Kim et al., 2014a). However, these studies primarily focused on pure chemicals in the laboratory, suggesting the need for the *in situ* application of this technique in a natural aquatic environment. The *in situ* biomarker approaches

* Corresponding author.
E-mail address: jjung@korea.ac.kr (J. Jung).

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from the biochemical level to the community level have been used to evaluate the impact of environmental stressors (Triebskorn et al., 2001; Yeom et al., 2007). In particular, Serafim et al. (2013) demonstrated that the IBR approach was successful for assessing seasonal and spatial variation in environmental contamination in Portuguese estuaries. However, biological responses at the individual or higher levels were not well related to the concentrations of contaminants in the field, mainly due to the natural variability in an organism's ability to adapt to environmental changes (van der Oost et al., 2003).

This study was conducted to evaluate the adverse effects of WWTP effluents on the pale chub (*Zacco platypus*) in the Miho Stream by integrating *in situ* multi-level biomarker responses in *Z. platypus*. The biomarker responses were quantified at the molecular level [mRNA expression of catalase (*CAT*), superoxide dismutase (*SOD*), glutathione *S*-transferase (*GST*), and metallothionein (*MT*)], biochemical level (enzymatic activities of CAT, SOD, GST, and MT concentration) and physiological level [condition factor (CF) and liver somatic index (LSI)]. The Miho Stream is a small stream in central Korea that is characterized as moderately to heavily polluted (Yeom et al., 2007). *Z. platypus* is a sentinel species in the Miho Stream and is widespread in eastern Asian countries, including Korea, eastern China, Japan, and Taiwan (Kim et al., 2013).

2. Materials and methods

2.1. Sampling sites and sample collection

Field surveys were conducted in August 2011 at two selected sites in the Miho Stream, which is located in the Chungchungbuk-do province of Korea (Fig. 1). The upstream site is a natural stream with little contamination. The downstream site is located near wastewater treatment plants (WWTP) that receive industrial wastes from about 200 companies in the industrial complex area. The industrial complex accommodates metal (55%), petrochemical (12%), textile (5%), food (4%), and paper (4%) industries and others (14%) (http://cjsandan.net/).

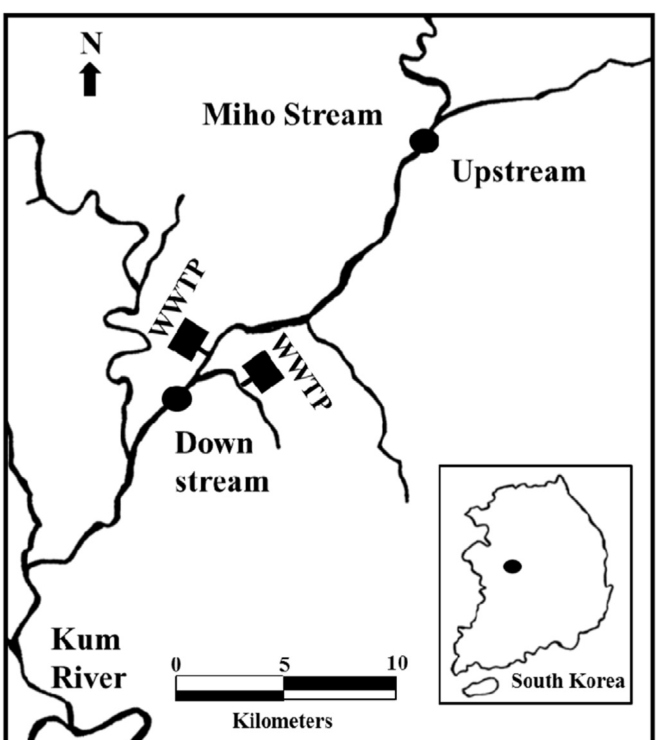

Fig. 1. Sampling sites (upstream and downstream) in the Miho Stream and location of two wastewater treatment plants.

Fish (*Z. platypus*) samples were collected from various habitats, including riffles, runs, and pools, in the upstream direction using casting nets (5×5 mm mesh) and hand nets (4×4 mm mesh). The distance and sampling time at each site for the catch per unit efforts (CPUE) were 200 m and 60 min, respectively. After collecting the fish, we selected over 30 individuals that were > 10 cm in total length from each sampling site, then transferred the fish to the laboratory within 1 h. Groups of 10 individuals were contained in a 20 L plastic bag with aeration and placed in an ice box to prevent warming and physiological stress. Fish samples were maintained at a water temperature of 21.2 ± 0.2 °C, pH of 7.5 ± 0.1, and dissolved oxygen level of 7.0 ± 0.4 mg/L. In the laboratory, all the fish were anesthetized in 150 mg/L of tricaine methanesulfonate (MS-222; Sigma-Aldrich, St. Louis, MO, USA), following the guidelines of the Korea Institute of Toxicology for Animal Care and Use Committee. The liver of each fish was collected and stored at -80 °C for further analysis.

2.2. Water quality parameters and chemical analyses

Information on the water quality of the sampling areas between August 2010 and August 2011 was obtained from the water quality monitoring network of the Ministry of Environment, Korea (MEK; http://water.nier.go.kr/weis). The parameters investigated were dissolved oxygen (DO), pH, temperature, biological oxygen demand (BOD), chemical oxygen demand (COD), suspended solids (SS), total nitrogen (TN), total phosphorus (TP), ammonia nitrogen (NH_3–N), and nitrate nitrogen (NO_3–N).

Chemical analyses of surface water from the upstream and downstream sites were conducted. The concentrations of two PAHs (benzo[*a*]pyrene and benzo[*k*]fluoranthene), identified as priority pollutants by the U.S. Environmental Protection Agency (EPA), were measured using high-performance liquid chromatography (HPLC) following previously published methods (Kim et al., 2008; Kim et al., 2013). The perfluoroalkyl substances (PFAS)–perfluorooctanoic acid (PFOA) and perfluorooctane sulfonate (PFOS) and alkyl phenols–bisphenol A and nonylphenol were measured using combined liquid chromatography–mass-mass spectrometry (LC/MS/MS) (Kim et al., 2010). In surface water, Cu, Cd, Cr, Pb, Hg, and Zn were determined using a Perkin-Elmer inductively-coupled plasma mass spectrometer (ICP-MS, ELAN DRC II, Perkin Elmer, Norwalk, CT, USA).

For metal analysis of the liver of *Z. platypus*, liver samples of approximately 0.5 g ($n = 6$) per replicate were digested with 5 mL HNO_3 at 120–150 °C for at least 2 h following previously published methods (Alam et al., 2002). At the same time, digestion blanks were also prepared. All digestates were diluted to 25 mL with 1 N HNO_3, and were filtered through a 0.45 µm nitrocellulose membrane. All samples were analyzed in triplicate for Cu, Cd, Cr, Pb, Hg, and Zn by the ICP-MS. For all of the chemical analyses, quality assurance and quality control were monitored by processing blank and reference standard materials. The concentrations were always within the 95% confidence intervals of certified values. The limit of detection for all chemicals was 0.1 µg/L.

2.3. Determination of molecular, biochemical and physiological biomarkers

Quantitative PCR (Q-PCR) was conducted to measure the relative mRNA expression levels of *CAT*, *SOD*, *GST*, and *MT*. All mRNA was isolated from the liver samples of *Z. platypus* individuals ($n = 6$) using the Qiagen Total RNA Isolation kit (Hilden, Germany).