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Research paper

Comparative assessment of the adverse outcome of wastewater effluents by integrating oxidative stress and histopathological alterations in endemic fish

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HIGHLIGHTS

- The IBR study identified the adverse effect of effluent discharge on fish clearly.
- The cool water species was the most impacted by thermal hot spring effluent.
- Pathological alterations gave more consistent IBR index than oxidative stress.

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ABSTRACT

This study evaluated the adverse effect of wastewater effluents on three fish species, *Carassius auratus*, *Zacco platypus*, and *Zacco koreanus*, collected in the Eungcheon, Mihocheon, and Busocheon streams, respectively. Fish gills, liver, and kidneys from the mixing zone (MZ) and sites upstream (US) and downstream (DS) of the MZ were analyzed for oxidative stress responses and histology. Catalase and glutathione *S*-transferase activity was significantly higher at MZ and DS than from US ($p < 0.05$), indicating induction of antioxidant defense mechanisms. Additionally, degree of tissue changes (DTC) indicated highest histopathological alteration in MZ, followed by DS and US. Integrated biomarker response (IBR) for oxidative stress and histopathological alterations showed higher values consistently for *Z. koreanus* than other two species. Water temperature, EC, and TN levels seemed to be responsible for the observed biomarker responses. These findings indicate that thermal hot spring effluent discharged into Busocheon steam induced the most significant impact on the cool water species (*Z. koreanus*). Overall, this study suggests that the IBR index is a very useful tool for monitoring *in situ* adverse effects of wastewater effluents on fish, particularly for histopathological alterations representing prolonged impact.

1. Introduction

The complex mix of effluents discharged into the aquatic environment from industrial and domestic wastewater treatment plants (WWTPs) contains high levels of pollutants, including polycyclic aromatic hydrocarbons (PAHs), solvents, heavy metals, pharmaceuticals, and flame-retardants, posing a constant threat to aquatic ecosystems [1]. However, inventory-based chemical monitoring provides very little information regarding the biological significance of this [2]. Much emphasis has recently been placed on assessing causal relationships between contaminant exposure and biological impacts [3]. Wastewater effluents promote physiological effects [4], immune responses [5], oxidative stress, and estrogenic effects [5,6] in fish caged or caught in contaminated waterbodies. Therefore, aquatic organisms can be used to evaluate the ecological impacts of effluents in receiving waterbodies. Fish are indicators of environmental stress, providing a definite biological end-point of xenobiotic exposure [7].

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Oxidative stress, *i.e.*, the production of reactive oxygen species (ROS), is the most obvious effect of effluents on fish [7]. Generally produced during metabolism, ROS are advantageous under natural conditions [8], although they might cause damage, including lipid peroxidation (LPO). Oxidative stress also induces antioxidant enzymes, such as catalase (CAT), and glutathione S-transferases (GST). Oxidative stress biomarkers can therefore provide early-stage diagnostic tools by detecting sublethal effects of wastewater effluents before other effects appear at higher levels of biological organization [9]. Histological biomarkers are also relevant at intermediate levels of biological organization, providing powerful tools in detecting and characterizing biological end-points of xenobiotic exposure, by integrating the cumulative effects of biochemical and physiological alterations, both in the laboratory [10,11], and in field studies [3,12]. Moreover, histopathological alterations in organs are generally easier to identify than malfunctions [13], warning of potential damage to animal health [14].

No single biomarker can fully support a diagnosis based on the effects of effluent exposure on aquatic organisms, so a battery of complementary biomarkers is recommended [2]. The integrated biomarker response (IBR) approach is useful in toxicological studies for comparing the specific adverse effects of xenobiotics, regardless of considerable variability in biochemical biomarker sets, contamination profiles, or test species [10,11]. Moreover, the IBR index can be a quantitative tool to assess the integrative responses of multiple biomarkers to effluent mixtures and contaminated streams [3,9]. In particular, Serafim et al. [15] demonstrate IBR's success in assessing seasonal and spatial variations of environmental contamination in Portuguese estuaries. However, most IBR studies use molecular (mRNA expression) or biochemical (antioxidant enzymes) biomarkers, though histopathological alterations are related more directly to adverse effects on aquatic organisms.

The present study was aimed to investigate the adverse outcome of domestic, industrial, and hot spring effluents on fish inhabiting in contaminated streams by integrating oxidative stress and histopathological alterations in the gills, liver, and kidneys of fish. The freshwater crucian carp (*Carassius auratus*), the pale chub (*Zacco platypus*), and Korean chub (*Zacco koreanus*) in the Eungcheon, Mihocheon, and Busocheon streams, respectively, were selected due to their site-specific dominance. *Z. platypus* and *Z. koreanus* are omnivores while *C. auratus* is insectivore in nature, and they have been identified as sentinels in biological monitoring programs.

2. Experimental

2.1. Fish sampling

The study sites were three streams in Korea: the Eungcheon, Mihocheon, and Busocheon. All three receive effluents: the Eungcheon from the Geumwang sewage treatment plant (6000 m^3/day) in Gaghoe-ri, Chungcheongbuk-do; the Mihocheon from the Gwanghaewon WWTP (8000 m^3/day) in Wolseong-ri, Chungcheongbuk-do; and the Busocheon from the Sanjeonghosu hot springs resort (40 m^3/day) in Sanjeong-ri, Gyeonggi-do. Fish sampling was conducted in three consecutive months (December 2015 to February 2016) during winter season. Fish were collected from the mixing zone (MZ), where wastewater effluent enters and mixes with the stream, and from two other locations upstream and downstream of the effluent discharge point, as follows: *Carassius auratus* from the Eungcheon; *Zacco platypus* from the Mihocheon; and *Zacco koreanus* from the Busocheon. In case of Eungcheon stream, fish were collected approximately 620 m upstream and 540 m downstream of the discharge point. For Mihocheon stream, fish collection was conducted approximately 160 m upstream and 530 m downstream, while they were 100 m upstream and 300 m downstream in Busocheon stream. Additionally, the Busocheon located at higher altitude (approximately 172 m) than other two streams (approximately 90 m).

Fish sampling was undertaken in various habitats at the sampling sites, including riffles, runs, and pools, using a casting net (5×5-mm mesh, 15 times per site), and skimming net (4×4-mm mesh, 40 min per site). Following collection, 10 individual fish >4 cm in total length were selected randomly from each sampling site. *Z. platypus* and *Z. koreanus* were approximately 6 cm in length, whereas *C. auratus* were approximately 8 cm. Three fish were used in each oxidative stress and histopathology study. Fish care and handling were performed following the guidelines of the Institutional Animal Care and Use Committee of Korea University. For histological examination, the gills (5–10 gill filaments/sample, 6 samples/fish), liver (3 mm tissue/sampling, 6 samples/fish), and kidneys (3 mm tissue/sampling, 6 samples/fish) were removed, keeping the tissue as intact as possible, and fixed in the field with 10% neutral formalin solution. For biochemical analyses, tissues from the gills, liver, and kidneys of the respective fish species were dissected out, flash-frozen with liquid nitrogen, and placed in an ice craft packed with dry ice on site. These samples were transferred to the laboratory within 2 h and stored at −80 °C.

2.2. Water sampling and chemical analyses

Water samples were collected from the same sites as fish sampling, transported on ice in polyethylene containers to the laboratory, and stored at 4 °C throughout the entire study period. Dissolved oxygen (DO) concentration, water temperature, pH, and electrical conductivity (EC) of the samples were measured using a multiparameter water quality meter (YSI 556, Yellow Springs Instruments, OH, USA) in the field.

All water parameters were analyzed according to the Standard Methods for Examination of Water Quality, Ministry of Environment, Korea [16]. The water quality parameters measured were suspended solid (SS), biochemical oxygen demand (BOD), chemical oxygen demand (COD), total nitrogen (TN), and total phosphorus (TP). Water pollutants measured were Cu, Pb, As, Hg, CN, Cr(VI), Cd, Ni, Zn, Se, dichloromethane, trichloroethylene (TCE), perchloroethylene (PCE), phenols, benzene, 1,2-dichloroethane, chloroform, organophosphorus compound, carbon tetrachloride, di(2-ethylhexyl) phthalate (DEHP), 1,1-dichloroethylene, 1,4-dioxane, vinyl chloride, acrylonitrile, and bromoform.

2.3. Oxidative stress analyses

LPO was analyzed according to the method of Barata et al. [17], using a malondialdehyde (MDA) kit (NWK-MDA01, Northwest Life Science Specialties, Vancouver, WA, USA). Tissue samples were homogenized in a motor-driven Teflon homogenizer with 1.15% KCl at pH 7.4 (100 mM phosphate buffer). The homogenates were then centrifuged at 1788 × *g* for 10 min at 4 °C. LPO levels was evaluated by measuring production of MDA reacting with thiobarbituric acid at 60 °C for 1 h, using a microplate spectrophotometer at 532 nm (BioTek Inc., Winooski, VT, USA), according to the manufacturer's instructions.

CAT activity was measured based on the method of Aebi [18]. Tissue samples were homogenized in a motor-driven Teflon homogenizer with 50 mM phosphate buffer (pH 7.8). The homogenates were then centrifuged at 1788 × *g* for 10 min at 4 °C, and the supernatants were retained for further analysis. CAT activity was then determined based on decrease in absorbance at 240 nm due to H_2O_2 consumption ($\varepsilon = 0.0436\ mM^{-1}\ cm^{-1}$).

GST activity was measured as described by Habig et al. [19]. Tissue samples were homogenized with 20 mM phosphate buffer (pH