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Original Research

Ecological Risk Assessment of Urban Streams Using Fish Biomarkers of DNA Damage and Physiological Responses

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Abstract

Ecological risk assessment was carried out in two urban streams, viz. Gap Stream (GS) and Miho Stream (MS), in the Geum River Watershed during July 2013-October 2014. The techniques used in this study included multi-level fish biomarkers of DNA damage based on single-cell gel electrophoresis (SCGE) coupled with the study of physiological responses based on 7-ethoxyresorufin-*O*-deethylase (EROD) and acetylcholinesterase (AChE) activities in fish species. Biomarker values of tail DNA (tDNA), tail length (T_L), and tail extent moment (TEM) in impacted zone (I_z) were 2.0-3.6-fold greater than in controls (C_z). Nucleus image analysis showed that the nucleus had circular particle forms in the C_z as compared with a longitudinal oval form with broken particles from the nucleus in the I_z. Physiological response analysis of EROD and AchE activities indicated that their levels were higher in the I_z than in the C_z. Such DNA damages and greater physiological responses in the I_z were attributed to chemical contaminants discharged from the wastewater disposal plants and industrial complex. This combination of DNA damage and physiological responses approach can be used as a key tool for early-warning detection of chemical contaminants and concomitant risks to the ecological health of urban streams.

Keywords: ecological risk, single-cell gel electrophoresis, heavy metal, DNA damage, 7-ethoxyresorufin-*O*-deethylase

Introduction

Ecological risk assessment (ERA), based on molecular-level or biochemical biomarkers, has been highlighted in ecotoxicological and genotoxic research [1-2] dealing with the evaluations of aquatic ecosystems impacted as a result of exposure to one or more chemical stressors such as anthropogenic pollutants [3-4]. The detection approaches of deoxyribonucleic acid (DNA) damage at the level of an individual eukaryotic cell have been previously applied to various research areas such as plant sciences [5-6] and mammal toxicology studies

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[4]. Recently, single-cell gel electrophoresis (SCGE), also known as the comet assay, has been widely applied in DNA damage research as a cost-effective method [7-8] as well as being a simple and sensitive dose-response technique for measuring deoxyribonucleic acid (DNA) strand breaks in eukaryotic cells of a species [9-10].

The comet assay measures the single/double-stranded DNA breaks, alkali labile sites, DNA cross-links, base or base pair damage, and apoptotic nuclei in the cells of an organism. The approach of SCGE was originally introduced by Östling and Johanson [11] and later modified by Singh et al. [12]. Since then, aquatic ecologists have used the standard dose-response monitoring approach for detection of molecular-level damages in aquatic organisms [13-14]. In addition to this novel approach, change in physiological responses such as acetylcholinesterase (AChE) activity [15-16] and 7-ethoxyresorufin-O-deethylase (EROD) activity [17] estimation could be used as an excellent barometer (biomarker) in detecting the cause-effect problems at the cellular or sub-cellular levels. Therefore, due to the above-mentioned reasons, such approaches can be considered as integral parts of an early warning (alarm) system, which can provide significantly reliable clues regarding the imminent threats and existing issues in aquatic ecosystem health [18-20].

Previous research on aquatic ecosystem health diagnosis mainly focused on the population responses of aquatic communities such as fish, macroinvertebrate and periphyton [21-24] in relation to various chemical stressors, physical habitat disturbances because of anthropogenic activities as well as channel modifications [25-28]. Such ecosystem risk assessment approaches at the community level were widely applied to reveal the extent of disturbances in aquatic ecosystems in North America [29], Europe [30], Africa [31-32] and Asia [33-34]. In addition, the risk assessments in streams and rivers were originally carried out at the population and organ/tissue level [18, 35-36]. Several disadvantages of these risk assessment studies according to the bioindicator level used were, however, pointed out as their inability to act as an early detection system that could be relied on as in the case of impending severe pollution crises in aquatic ecosystems. Thus, the use of biomarkers such as SCGE, AChE and EROD may provide an excellent nexus leading to unveil some significant clues to identify the causal mechanisms potentially responsible for any type of damage at molecular, cellular, and sub-cellular levels [35, 37-39].

Despite the numerous advantages of early warning detection of problems in ecosystem health, very little is known about DNA-level and physiological-level risk assessments in stream or river ecosystems in Korea. Adams [40] pointed out that the combination of comet assay and physiological assays using fish samples (sentinel species) plays a significant role in risk assessments of aquatic ecosystems. They may act as very effective biomarkers used in detecting the organic matter, toxic chemicals, and heavy metal pollution [41-44] in urban streams. Moreover, the comet assay is known as a biomarker that is considered one of the best barometers used in assessing DNA damage by oxidative stress responses [12, 45].

Keeping in view the grander importance of the novel techniques as well as the significance of early detection and warning systems in urban streams, the key objectives of this investigation encompassed the analysis of DNA damage using two different sentinel species from benthic and water-column fish communities dwelling in the target urban streams. It also included the measurements of Tail DNA (*t*DNA), tail length, and tail extent moments in the control zones (pristine region) to compare with the impacted streams using the comet assay technique. In addition, physiological activities of fish, based on EROD activity and AChE activity, were measured and compared with chemical conditions in the sampling streams.

Experimental

Sampling and Sampling Sites

The sampling was conducted in two urban streams, viz. Gap Stream (GS) and Miho Stream (MS), during July 2013-October 2014. Gap Stream is one of main tributaries in the Geum River watershed, and courses through the epicenter of metropolitan Daejeon. The sampling sites were selected in two zones, which are represented as upstream and pristine region (control zone, C_z) and downstream and polluted region (impacted zone, I_z), based on major point sources within the watershed. The control zone (C_z) is mainly surrounded by forest (>about 65 %), some paddies (about 20 %) and ordinary fields alongside the stream route, and has riffle-pool morphology with gravel as the dominant substratum. The impacted zone (I_z) is largely influenced by Daejeon Industrial Complex (IN CO) besides a wastewater disposal plant (WDP), and merges with Yudeung Stream, which is also running through Daejeon (Fig. 1). Principally, the WDP discharge capacity is approximately 9×10^5 m^3 day^{-1} and the effluents find their way to the impacted zone (I_z) in the Gap Stream directly. Fish species and their taxonomic classification were carried out based on their salient body features as described by Kim and Park [47]. The fish sampling was performed in accordance with the modified wading technique [27, 48-49] for assessing stream condition, and the method was derived from the classic Ohio EPA technique [50].

Laboratory Analysis of DNA Damage Detection and Physiological Responses

Single-cell gel electrophoresis (SCGE) analysis (comet assay) was conducted based on the approach modified from the methods of Singh et al. [12]. After the blood samples were centrifuged for 5 minutes at

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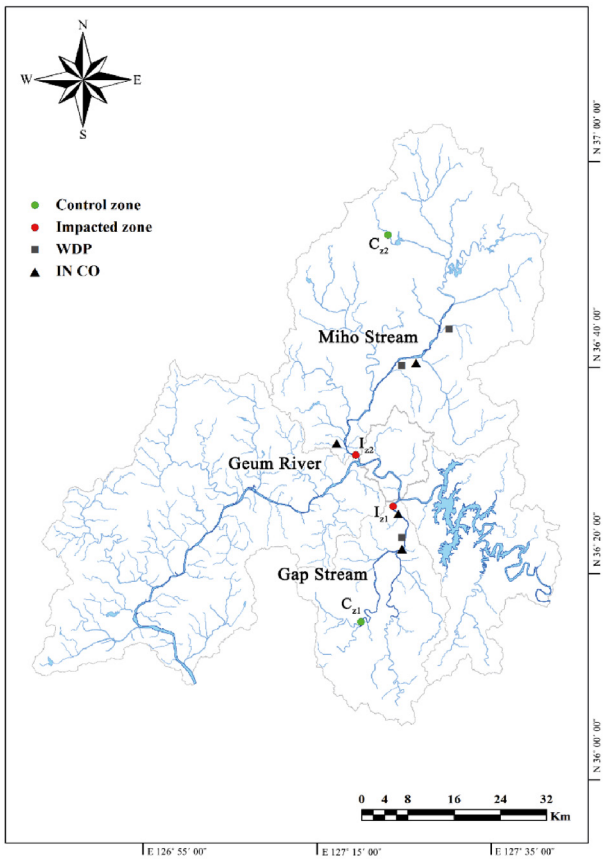

Fig. 1. Watershed maps and sampling zones in Gap Stream (C_{z1}, I_{z1}) and Miho Stream (C_{z2}, I_{z2}); in the figure, the abbreviations of "C_z" (C_{z1}, C_{z2}) and "I_z" (I_{z1}, I_{z2}) denote the control and impacted zones, respectively; also, "WDP" and "IN CO" indicate major point sources of waste water disposal plants and industrial complexes, respectively.

3000 rpm, the samples with 0.65% agarose were layered on the pre-coated slide glass using 1% agarose, and then treated by lysis buffer for 3 hours at 4ºC. The sample slides treated with the lysis buffer were conducted by electrophoresis (25 V, 300 mA) in the buffer solution for 40 minutes. To determine DNA damage, the lengths of nuclear DNA fragments (tail length, T_L, μm) and the proportion of DNA in the tail (tDNA, %) were analyzed. The tail extent moment (T_{EM}) was also estimated as T_L × tDNA/100 using a Komet 4.0 comet image analysis system. The analysis of 7-Ethoxyresorufin-*O*-deethylase (EROD) activity using liver samples was conducted by the approach modified from the methods of Kennedy and Jones [51]. The formation rate of the product from ethoxyresorufin was calculated from the peak area of different concentrations of resorufin. Acetylcholinesterase (AChE) activity was analyzed using an ABC kit at the Korea Institute of Ocean Science and Technology (KIOST), based on colorimetric assay of Ellman et al. [52]. The frozen 20 mg brain sample was mixed with 1 mL buffer and homogenized. Then the sample was centrifuged (10,000g, 4ºC) for 10 minutes. Protein quantitative analysis was carried out using the BCA protein assay kit. The microplate reader method was used based on the absorbance measurements at 415 nm.

Heavy Metals and POPs Chemicals Assessment

Bottom sediment samples of the two sampling streams were procured for heavy metal analysis. The top 5-cm layer of sediments was scooped using sterile stainless steel equipment into autoclaved disposable containers, simultaneously. All extracts were analyzed using an atomic absorption spectrometer (Analyst 800, PerkinElmer, Shelton, CT, USA). The persistent organic pollutants (POPs) were also estimated from the water samples at the different zones of the two urban streams. In total, we analyzed 24 POP chemicals, including agricultural pesticides (1), aromatic hydrocarbons (1), EDCs (2), herbicides (8), insecticides (3), metabolites of medicines (1) and heavy metals (8). The laboratory assessments of POPs chemicals and heavy metals were conducted as described by Eaton and Franson [53] and Segner et al. [54].

Data Analysis

For the data analysis of biomarkers and bioindicators, an independent two-sample t-test was used for sample

Table 1. Mean values of tail DNA (*t*DNA), tail length (T_L), and tail extent moment (T_{EM}) in two habitat-type fish collected in the control zone (C_z) and impacted zone (I_z) of the sampling streams; each value is expressed as mean±standard error (SE).

Fish type / Location	n	Total length (mm)	Weight (g)	Tail DNA (%)	Tail length (μm)	Tail extent moment
a) Benthic Species (*Pseudogobio esocinus*)						
C_z	14	134.36±4.74	20.37±2.16	21.55±0.50†	43.34±0.89†	12.90±0.50†
I_z	17	120.18±5.88	17.04±2.81	43.90±0.58*,†	92.42±1.31*,†	46.40±1.04*,†
b) Water-column Species (*Zacco platypus*)						
C_z	8	110.88±6.01	14.42±2.43	16.60±0.51†	28.56±0.80†	7.19±0.38†
I_z	10	94.70±1.89	7.31±0.74	32.25±0.70*,†	66.67±1.80*,†	28.02±1.29*,†

* : $p<0.001$ (significantly different between the C_z and I_z)
† : $p<0.001$ (significantly different between benthic and water-column species)

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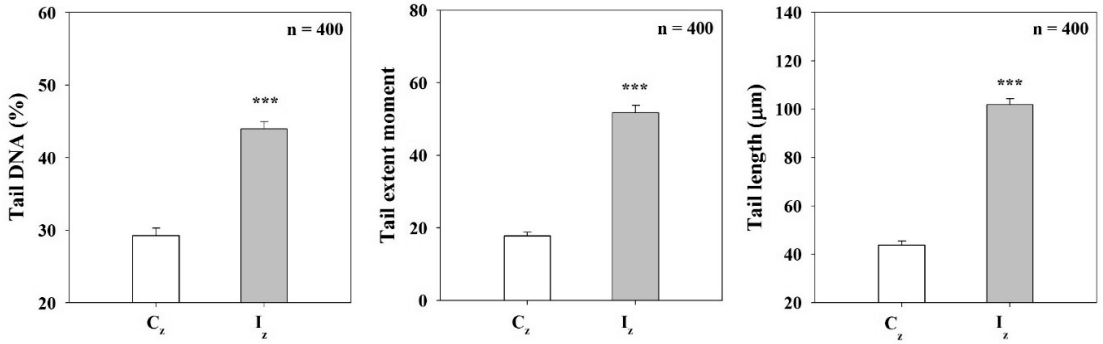

Fig. 2. Overall mean values of tail DNA (*t*DNA), tail extent moment (T_{EM}), and tail length (T_L) analyzed from two habitat-type fish (water-column species vs. benthic species) in the sampling streams; asterisks (***) indicate a significant difference at $p < 0.001$ statistically between the C_z and I_z by independent two sample *t*-test.

comparison between sampling zones and two habitat-type species. Simple linear regression analyses were conducted to examine the relationships among the environmental factors. Statistical analyses were performed at the significant level of $p<0.05$ using the SPSS statistical package (Ver. 21.0).

Results and Discussion

DNA Damage Assessment

The detections of deoxyribonucleic acid (DNA) damage based on single-cell gel electrophoresis (SCGE) was analyzed at the level of two sentinel fish eukaryotic cells as shown in Table 1. Mean tail DNA (tDNA), tail length (T_L), and tail extent moment (T_{EM}) of benthic species, *Pseudogobio esocinus* and water-column species, *Zacco platypus*, were 1.5 times greater in the impacted zone (I_z) than in the control zone (C_z), and the difference was statistically significant ($p<0.001$; Table 1).

In benthic species, biomarker values of tDNA, T_L, and T_{EM} in the I_z were 2-, 2.1-, and 3.6-fold,

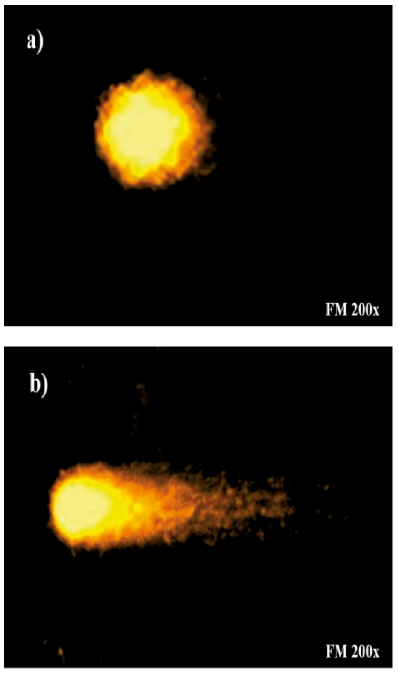

Fig. 3. Nucleus image analysis of blood samples from *Zacco platypus* as a sentinel species in the control zone (C_z; a) and the impacted zone (I_z; b) of the sampling streams (Fluorescent microscope 200x).

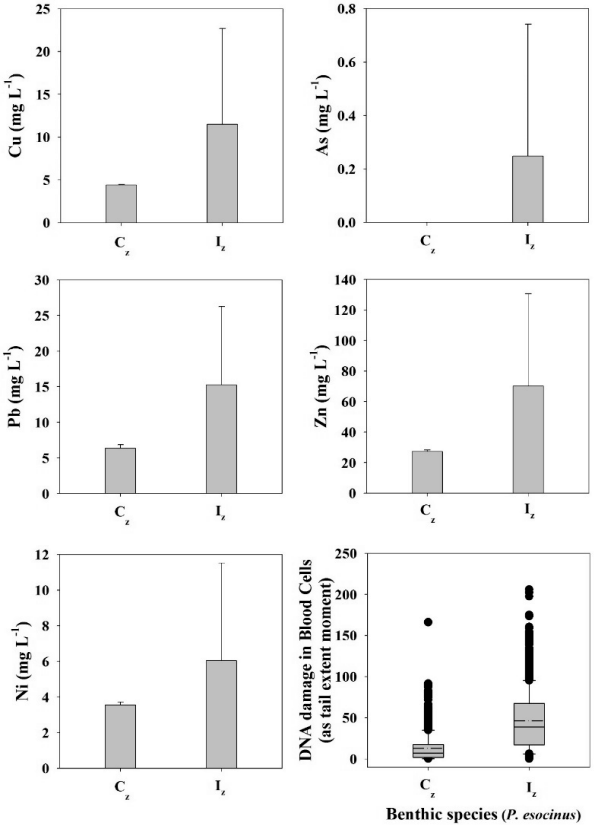

Fig. 4. Heavy metal analyses from bottom sediment samples and DNA damage as tail extent moment in *Pseudogobio esocinus* from the sampling streams; statistical Mann-Whitney U-tests of all heavy metals were not significant in the level of $p = 0.05$ between the C_z and I_z.

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Table 2. Persistent organic pollutants (POPs) in the water samples of the control zone (C_z) and Impacted zone (I_z); "ND" indicates "not-detected" from the analysis.

Chemicals of the POPs		C_z	I_z	
			Mean	Range
Agricultural Pesticides	Hexachlorobenzene (ng/L)	N/D	0.09	0.04-0.24
Aromatic Hydrocarbon	Fluoranthene (ng/L)	N/D	1.35	0.8-1.9
EDCs	Bisphenol A (ng/L)	N/D	5.50	0-22
	Nonylphenol (ng/L)	N/D	27.75	11.5-68
Herbicides	Alachlor (ng/L)	N/D	17.43	2.81-57.83
	Metolachlor (ng/L)	N/D	118.25	42.7-328.9
	EN/Dosulfan I (ng/L)	N/D	11.74	7.6-65.3
	EN/Dosulfan II (ng/L)	N/D	4.10	1.67-23
	Atrazine (ng/L)	N/D	N/D	-
	Simazine (ng/L)	N/D	4.45	4.73-31.53
	MCPA (ng/L)	N/D	12.75	0-51
	2,4-D (ng/L)	N/D	10.00	0-31
Insecticides	Carbofuran (ng/L)	N/D	42.75	18-135
	Chlorpyrifos (ng/L)	N/D	N/D	-
	Acenaphthene (ng/L)	N/D	0.88	0.8-1.9
Metabolite of Medicine	Fluorene (ng/L)	N/D	0.95	0.8-2.2
Metals	Cd (mg/L)	0.07	0.02	-
	Cu (mg/L)	4.38	11.51	-
	As (µg/L)	N/D	1.02	0.74-1.34
	Pb (mg/L)	6.35	15.27	-
	Zn (mg/L)	27.34	70.29	-
	Ni (mg/L)	3.55	6.05	-
	Cr (µg /L)	N/D	0.12	0.09-0.17
	Hg (µg /L)	N/D	1.33	0.6-2.0

respectively, than in the C_z (p<0.001), while in water-column species the biomarker values in the I_z were 1.9-, 2.3-, and 3.9-fold, respectively, than in the C_z (p<0.001). The magnitude of DNA damage was greater in the benthic species than the water-column species (Table 1). Our results of DNA biomarker indicate that the DNA damages were greater in the I_z than in the C_z, and that the damage was greater in bottom-feeding fish species with polluted sediments than the water-column fish.

According to single-cell gel electrophoresis of blood samples in two sentinel species with different habitat types, mean tDNA (%), T_L (µm), and T_{EM} had 29.3±1.0%, 43.8±1.6 µm, 17.8±1.0, respectively (Fig. 2). The value of I_z in tDNA was significantly higher (p<0.001; 1.5 times) than the value of C_z (44.0±1.0 %). Also, values of I_z in T_L were significantly higher (p<0.001; 2.3 times) than the value of C_z (101.8±2.4 µm), and a similar outcome between C_z and I_z was found in T_{EM} (51.7±2.0; 2.9 times). Numerous research on comet bioassays in polluted streams reported that the main causes of DNA damage in eukaryotic cells of aquatic organisms are closely associated with heavy metals, polyaromatic hydrocarbon (PAHs), agricultural chemicals (pesticides, herbicides) and genotoxicants [55-59].

The magnitude of DNA damage in the blood cells is shown by the results of single-cell gel electrophoresis (SCGE; Fig. 3). Distinct morphological differences in nucleus shapes were contrasted in image analysis of the control zone (C_z) and impacted zone (I_z). The shape of the nucleus in the C_z, based on the single-gel electrophoresis, had circular form and no damage from deoxyribonucleic acid. In contrast, the nucleus in the I_z had a longitudinal oval form and was composed of small particles broken from the nucleus and scatted longitudinally in the image

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(Fig. 3). The scattered nucleus in the I_z indicates that the nucleus moved toward the positive (+) polar region by the principles of electrophoresis. The image analysis supported the facts that chemical pollutants influenced DNA damage of fish eukaryotic cells [50-54]. Our results were corroborated by previous research of SCGE bioassays that the magnitude of DNA damage has a functional relationship with concentrations of xenobiotic pollutants [55-57], and the chemical impacts are evident when the breakage of deoxyribonucleic acid is severe, the head part of the nucleus is shorter, and the tail part is longer longitudinally [60-61].

Heavy Metals Assessment

The analysis of heavy metal concentrations in sediment samples showed that I_z had higher values than that of C_z in all heavy metal parameters of Cu, As, Pb, Zn, and Ni (Fig. 4). High heavy metal values of I_z from the sediments agreed with total mercury concentrations in fish tissues. Statistical analysis of Mann-Whitney U-tests, however, showed that there were no significant differences in the heavy metals of Cu, As, Pb, Zn and Ni between the control and impacted zones (Fig. 4). The reason turned out to be high variabilities between the samples and the low number of observations in the metal analysis. In the comet assay of DNA damage (tail DNA, tail moments, DNA tail length), the total number of observations were 400 samples, but the number of the heavy metal samples in the sediment were very few (n = 6). In addition, sample variation in the sediments was highly contingent on the silt or sand percentages at the study locations. Therefore, the outcomes of these metals showed no significance between the control and impacted zones [62].

Persistent Organic Pollutants (POPs) Assessments

In the meantime, the analysis of persistent organic pollutants (POPs) showed that the hazard chemicals in the water samples were not detected in the control zone, but significant amounts of chemicals were detected in the impacted zone as shown in Table 2. The herbicide concentrations of metolachlor averaged 118.25 ng/L in the I_z, compared to "not detected" in the C_z. Also, bisphenol-A and nonylphenol, which are known as endocrine-disrupting chemicals (EDCs) in waterbodies, were 5.5 ng/L (range; 0-22 ng/L) and 27.75 ng/L (range; 11.5-68.0 ng/L), respectively in the impacted zone, while the chemicals were not detected in the C_z. Also, heavy metals of Cr and Hg in the water samples were 1.12 ug/L and 1.33 ug/L, respectively in the impacted zone, unlike the no-detections in the control zone. The persistent organic pollutants might have influenced the DNA damages and physiological responses of fish. In fact, Adams et al. [18] and Adams [40] pointed out that agricultural herbicide, pesticide and the EDCs resulted in repressions of RNA expression and synthesis and the DNA damage in fish, thus the alterations of these molecular levels influenced the fish population and community in the end. Similar results are shown in the paper of [63-65]. Our results and these references suggested that the persistent organic pollutants potentially influenced the DNA damages and EROD activities.

Detection of Physiological Activities Based on 7-ethoxyresorufin-O-deethylase (EROD) Activity

Physiological activities based on 7-ethoxyresorufin-*O*-deethylase (EROD) activity were analyzed using two sentinel species of benthic fish: *Pseudogobio esocinus* and the water-column dwelling fish *Zacco platypus* (Fig. 5). The response of EROD activity was greater in the water-column species; the response of water-column dwelling species was 11-fold higher in the I_z than the C_z (p<0.001). Also, the response of benthic species was 2.1-fold higher in the I_z than the C_z (p<0.001). These outcomes indicate that the physiological response of the I_z increased up to 3.8 times in the EROD activity on average, compared to the response of C_z, and the statistical difference was significant (p<0.01). Adams et al. [18] pointed out that such increases in EROD activity are frequently attributed to physical factors of an aquatic ecosystem such as water temperature, pH, and physical disturbance, and occurred by some exposures of certain chemical contaminants such as polycyclic

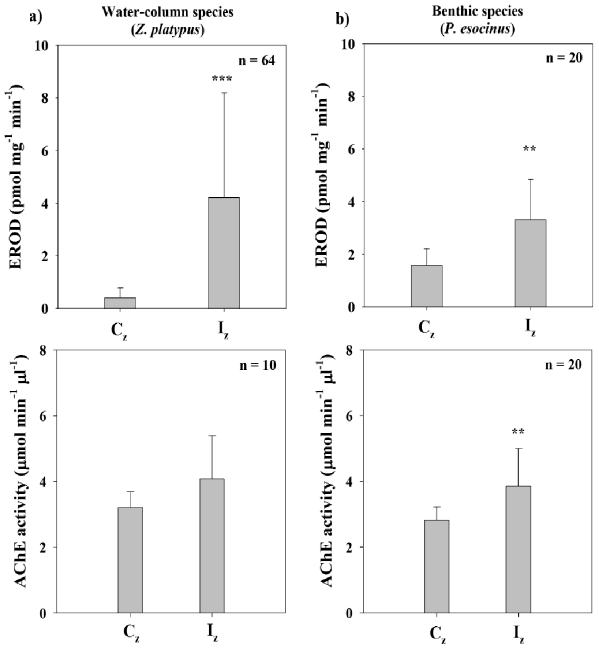

Fig. 5. Physiological responses of two different biomarkers of 7-ethoxyresorufin-*O*-deethylase (EROD) activity and acetylcholinesterase (AChE) in the water-column (*Zacco platypus*) and benthic (*Pseudogobio esocinus*) species in the control (C_z) and impacted (I_z) zones of the sampling streams; a) panel and b) panel indicate water-column species and benthic species, respectively (*** = p<0.001 and ** = p<0.01 as significantly differences between the C_z and I_z)

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aromatic hydrocarbons (PAHs) and polychlorinated biphenyls (PCBs) [66-67]. Therefore, we believe that the increases in EROD activity in the I_z seemed to be influenced by habitat modifications or some chemical pollutants, which are directly discharged from urban regions, industrial complexes and sewage disposal plants. In fact, the impacted zone of our streams has big point sources of wastewater disposal plants (WDP) discharging about 9×10^5 m^3 day^{-1}, and the Daejeon industrial complex, so the effluents increased the activity of EROD in the polluted zone.

Detection of Physiological Activities Based on Acetylcholinesterase (AChE) Activity

The physiological response of acetylcholinesterase (AChE) activity was measured in the benthic fish *Pseudogobio esocinus* and the water-column-dwelling fish *Zacco platypus* (Fig. 5). The response of AChE activity in the water-column species was approximately 1.4-fold higher in the I_z than in the C_z, and the statistical difference in the response was significant ($p<0.05$) between the two zones. In the meantime, in benthic species the response of C_z and I_z was 2.81±0.41 and 3.86±1.14 μmol min^{-1} $μl^{-1}$, respectively, indicating a greater response of 1.4-fold in the impacted zone ($p<0.01$). These responses of AChE were similar to the response of EROD activity, but showed higher sensitivity than the EROD activity in both water-column species and benthic species. The physiological response of AChE is generally increased primarily by neurotoxins [66-71]. Our measured AChE activity showed significantly greater response in the I_z. We did not measure the neurotoxins in our study. It was considered that AChE activity had increased due to increased exposure of contaminants, which can influence the nervous system as passing through urban areas, industrial areas and sewage disposal plants [62, 71-73].

Conclusions

We carried out the ecological risk assessment in two streams by the combined application of DNA damages in selected fish species used as biomarkers at multi-levels as well as studied the change in their physiological responses. The obtained results of applied biomarkers of tDNA, T_L, and TEM in the I_z showed as 2.0-3.6-fold greater as compared to the control zone (C_z). Furthermore, the nucleus displayed circular particle formations in the C_z in comparison to the longitudinal oval forms with broken particles in the nucleus from the I_z. Similarly, the physiological responses signposted the higher levels in the I_z than in the C_z. Such discrepancies in the biomarkers of DNA damages as well as physiological responses in the I_z could be linked to chemical discharges from WWTPs and industrial complexes. Overall, this combined approach of using the DNA damages and physiological responses could become a vital tool in early-warning detection of impending chemicals in urban streams.

Author Contributions

Bae DU collected the data, carried out the laboratory procedures and prepared the manuscript with Atique U. Yoon JH and Lim BJ. helped in data analysis and manuscript preparation. An KG supervised the whole study.

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Conflicts of Interest

The authors declare no conflicts of interest.

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