Study On The Effects Of Heavy Metals' Pollution On The Activity Of 7-Ethoxyresorufin-O-Diethylase (EROD) In Two Freshwater Fish Species Of Indonesia

Nur Kusuma Dewi

Abstract: The study aimed to determine the effect of heavy metal pollution on 7-ethoxyresorufin-O-diethylase (EROD) enzyme activity in carp (Cyprinus carpio) and tilapia (Oreochromis niloticus). The samples were taken by purposive random sampling technique. The treatment site was the downstream part of the Kaligarang River, Semarang, Indonesia, which contaminated byheavy metals (concentration of Cd 0.007 ppm, Pb 0.010 ppm, and Hg 0.0006 ppm). A clean reference site in Nyatnyono Village, Ungaran was used as the control site with the heavy metal content of 0 ppm. The EROD activities in carp and tilapia were significantly decreased after Cd, Pb, and Hg exposure. The results showed that the average value of liver EROD activity in carp fish control group amounted to 1.77 ± 0.23µmol/min/mg protein and the treatment group was 0.49 ± 0.24µmol/min/mg protein. Whereas in control group of tilapia fish, EROD activity was equal to 2.08 ± 0.47µmol/min/mg protein and the treatment group was 0.49 ± 0.40µmol/min/mg protein. The results demonstrated that metallothionein was a specific heavy metals biomarker, whereas EROD activity ishighly sensitive to extremely low concentrations of theselected pollutants.In conclusion, the use of multiple biomarkers is recommended to monitor the heavy metal pollutants in the river environment.

Keywords: EROD activity, heavy metals, biomarker, river environment

1 INTRODUCTION

Recently, the industrial, economical, education and culture fields are developing rapidly. Beside the advantages of the rapid development, there are many risks arisen by this issue. The development in those areas is possible to affect the environment, especially the aquatic environment. In river environment, it is well known that pollution has become a great problem. In Indonesia, river is an important compartment of the environment. The discharge of unpurified sewages and wastewater from industries, manufacturers, and housing has resulted in the accumulation of pollutants. The accumulation causes a dramatic decline in its water quality. These pollutants are mainly polycyclic aromatic hydrocarbons (PAHs), radio nuclides, pesticides, detergents, insecticides, and heavy metals. The pollution of river by environmental pollutants leads dangers onto humans and wildlife. The non-organic pollutants in the form of heavy metals, i.e. mercury (Hg), cadmium (Cd), and lead (Pb) are well-thought-out the most dangerous metals in the environment [1]. In fact, the heavy metals are persistent in the natural environment and surprisingly, they are able to accumulate in various areas, for instance, sediment, water, and soil. These metals could inhibit the enzymes, which containing sulfhydryl groups in or near their active sites and could generate the reactive oxygen species [2]. These mechanisms are considered to be dangerous as it leads toxic, carcinogenic and mutagenic effects to animals and humans [3]. Regarding to the contamination cases, in Toyama, the water system used in the rice field had been polluted by cadmium (Cd) and caused the civilian suffered Itai-Itai [4, 5, 6, 7].

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The research study results showed that the water was polluted by Cd obtained from the local mining center in the head of Jint River. The rice in that field accumulated the cadmium for decades and biomagnified in the civilian who lived near the river areas [5, 8, 9, 10]. Also in 2004, the cadmium pollution in river happened to Karanganyar River, Central Java, Indonesia. The rate of cadmium reached 0.21-0.40 mg/kg. Fifteen industries near the river area were suspected to be contributed in the pollution [11]. In aquatic systems, the fish are extensively used as a sensitive experimental model. In fish, there is a broad range of enzyme systems involved in xenobiotic metabolism. By these decades, the use of biomarkers to trace the existence of pollutants has become widely used in environmental monitoring. In 1962, the studies concerning cytochrome P450 in fish were published [12, 13, 14]. The 7-ethoxyresorufin-O-diethylase (EROD) is a family of cytochrome P-4501A (CYP1A) contained in fish liver. A specific biomarker, EROD is now often used as a molecular marker to measure the quality and quantity of pollutants in the water. EROD catalyzes the formation of single cytochrome P-450 (CYP) into cytochrome P4501A (CYP1A) through metabolism of 7-ethoxyresorufin substrates into fluorescent resorufin products [15]. EROD activity can be used as an indication of the function of cytochrome P4501A1 enzyme system, which is responsible for environmental contamination through phase I biotransformation, i.e. polycyclic aromatic hydrocarbons (PAHs) compounds and some dioxin-like compounds such as polychlorinated dibenzo-p-dioxins and furans (PCDD/Fs), and polychlorobyphenyls (PCB). In other words, EROD activity has been used as a marker of activity and induction of CYP1A [15, 16]. CYP1A induction and its relationship with EROD activity has been widely used as a biomarker for exposure to AHR agonists in aquatic environments,as well [15]. As a biomarker, EROD activity is widely used in several field and laboratory studies, such as astudy on bleaching industrial wastewater [17], contaminated sediment [18, 19], and petroleum spills and leaks [20, 21], as well as the monitoring of general contamination [22, 23]. In addition, the presence of several compounds in aquatic environments such as metal and estrogen compounds also affects the response of EROD [15]. However, only few studies have reported the presence of EROD enzyme activity due to exposure to heavy metals in the aquatic environment. Accordingly, this study aimed to determinethe response of the EROD enzyme to the following exposure of heavy metals including Cd, Pb, and Hg in two freshwater fish species of Indonesia, carp (Cyprinus carpio) and tilapia (Oreochromis niloticus). The assessment of heavy metals exposure in these two kinds of fish was conducted in Kaligarang River, Semarang, Central Java, Indonesia. It is a home to a broad range of industrial activities, including food factories, paper factories, textile industries, and home industries. In order to evaluate the effect of pollution in Kaligarang River, biomarker studies are needed for fish treated in the river; such studies would give the first and highly valuable information regarding pollution in this area.

2 MATERIALS AND METHODS

2.1 Chemicals
All chemicals were of analytical grade available commercially.

2.2 Treatment sites and fish species
Kaligarang River lies administratively in 3 municipalities in Central Java Province, precisely in Kabupaten Semarang, Kendal, and Kota Semarang. The mainstream part of Kaligarang is located in Ungaran with a height of 1,750 meters above sea level, whereas the downstream part lies in Laut Jawa (Java Sea). In this study, the treatment site was at the downstream part of the Kaligarang River. The downstream part is the 6th part (Segment 6) of the river which surrounded by several major industries and housing. By using karamba floating net, the fishes were caught and exposed with the heavy metal concentration of Cd 0.006 ppm, Pb 0.010 ppm, and Hg 0.0006 ppm. A clean reference site in the center of the freshwater fish seed, which located in the 1st part (Segment 1) near Nyatnyono Village, Ungaran, Semarang Regency, was used as the control site, with the heavy metal content of 0 ppm. Nyatnyono is a village where tourism and fishing are prevalent, and it has no industrial sites. The treatment stations and the reference site are shown in Figure 1. The study was conducted for 7 months in between the dry season with rainy season. Carp and tilapia were obtained from the freshwater fish seed center in Ungaran using random sampling. A total of 100 carps (Cyprinus carpio) and 100 tilapias (Oreochromis niloticus), each weighing 19-25 g, were used as the samples which treated in the treatment site (Segment 6). Carp and tilapia are the economically important source of protein and are cultured by local fishermen. The liver EROD activities of these fish were compared with those of 100 carps and 100 tilapias placed in the control site (Segment 1).

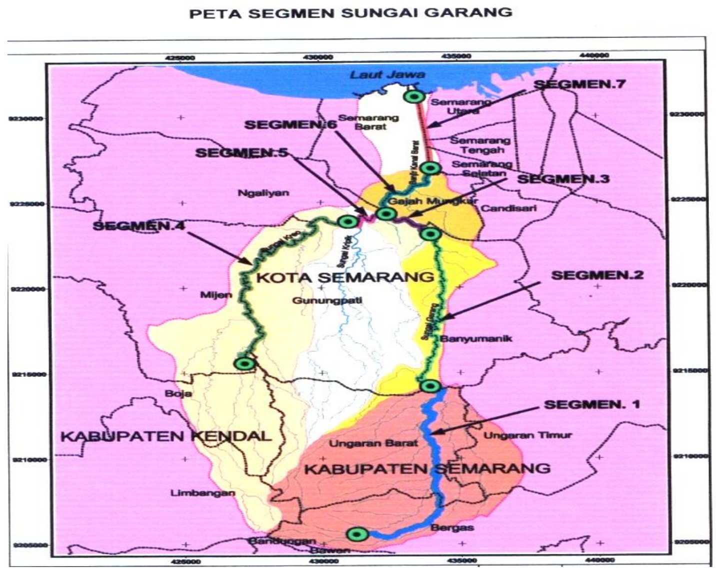

Fig.1.*Treatment site was located in the 6th part (Segment 6) of Kaligarang River, whereas the reference site was located in the 1st part (Segment 1) of Kaligarang River. Fishes were exposed to Cd, Hg and Pb to evaluate the bioindicator.*

2.3 Experimental design
The pre-test and post-test control group design were used as the experimental design [24, 25]. The biological marker shown in fish after the experiment was compared with the control group fish. After 7 months, both the liver of carp and tilapia fishes were taken and the activity of EROD was then measured.

2.4 Preparation of microsomal fractions of livers
After the specimens were weighed and measured, the fish were killed by decapitation and their liver tissues were dissected carefully. The tissue samples were stored in the zip lock plastic bag and were kept in the ice box after labeling.

2.5 Enzyme assays
EROD activities of microsomes were determinedby the spectrofluorometric method [26]. The reaction mixture contained0.1 M potassium phosphate buffer, pH 7.8, 0.1 MNaCl, 1.2 mg BSA, 50–100 μg of fish liver microsomal protein, 0.1 mM nicotinamide adenosinedinucleotide phosphate in the presence of substrate,and 1.5 μM7-ethoxyresorufin. The reactionwas initiated by the addition of substrate,7-ethoxyresorufin was added to the reaction mixture. The reaction begins with the addition of 50 mL of the NADPH regeneration system and incubated at 37 °C for 1 h. The reaction was terminated by the addition of icecold acetone. The acetone blank was prepared with the addition of acetone in the NADPH regeneration system. Standard samples were incubated without the addition of ethoxyresorutin. After centrifugation at 2500 rpm for 5 min, the supernatant fluorescence was measured at a wavelength of 535 nm (excitation) and 585 nm (emission) using Shimadzu RF–594fluorometer (Shimadzu, Japan).

2.6 Statistical Analysis
The data were then performed for coding and statistical description. Initially, the normality of the data was tested using the Kolmogorov-Smirnov test. After normal data was obtained,

the hypothesis was tested using independent t-test and was continued with LSD test. Meanwhile, if the data were not normal,it wasthen tested by the Mann Whitney test followed by Benn Ferroni test. Significant value was indicated by the value of $p < 0.05$. Statistical analysis was performed using SPSS ver 13.0. The results are expressed as mean ±SD of at least three sets of triplet determinations for each data point, and statistical analysis was performed.

3 RESULTS AND DISCUSSION

3.1 The assay of 7-ethoxyresorufin-O-diethylase (EROD)

The activity of liver 7-ethoxyresorufin-O-diethylase (EROD) in carp and tilapia,which treated in the treatment sitesexposed to heavy metals Cd, Pb, and Hg were lower than the fish samples in the clean reference sites. The carp liver EROD activity had approximately three times lower (1.77 ± 0.23 µmol/min/mg protein to 0.49 ± 0.40 µmol/min/mg protein), whereas in tilapia, it had approximately 4 times lower (2.08 ± 0.47 µmol/min/mg protein to 0.49 ± 0.40µmol/min/mg protein) as presented in Table 1.

Table 1 . *Median value EROD activity of control group and treatment group in Cyprinus carpio and Oreochromis niloticus*

Fish species	Sample size	EROD activity (µmol/min/mg protein) mean ± SD	
		Control group (BBI Ungaran)	Treatment group (Kaligarang river)
Carp (Cyprinus carpio)	100	1.77 ± 0.23	0.49 ± 0.24
Tilapia (Oreochromis niloticus)	100	2.08 ± 0.47	0.49 ± 0.40

The Mann Whitney test results of carp fish groups (data not shown) showed that there were significant differences in EROD activity values between control and treatment groups. The mean rank of the experimental group (10.50) was smaller than the control group (30.50). The t-test result of tilapia group, it showed that there were significant differences in EROD activity values between the control group with the experimental group (p value = 0.0003). It was found that the presence of heavy metal exposure decreased the EROD activity in the treatment group compared with the control group. The carp and tilapia liver EROD activitydiagram are presented in Figure 2.The EROD activities between carp and tilapia fish were differ significantly, although the value of EROD activity of carp and tilapia fish in the same treatment groups was low. It was revealed that the low EROD activity value of the experimental group compared with the control group, was likely to occur due to exposure to heavy metals with a high affinity for sulfur (S). The heavy metals attack the sulfur bond in the enzyme active site. This mechanism inactivates the enzyme function. When the enzyme is inactive, it decreased the enzymeactivity, therefore the enzyme's ability to detoxify polluting substances (pollutants) was also declined.

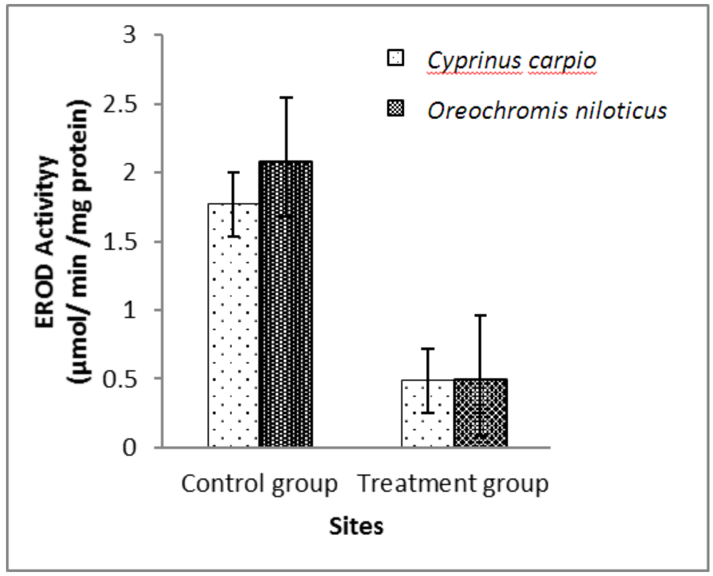

Fig.2. *Hepatic EROD activities of fishes caught from control group (BBI Ungaran)and treatment group (Kaligarang River) in Cyprinus carpio and Oreochromis niloticus*

The metals inhibition of P450 catalytic activity has been observed by others. In vitro study on the effect of Hg^{2+}, Zn^{2+}, Ni^{2+} and Cd^{2+} on EROD activities in the liver microsomal of the leaping mullet showed that all metal ions inhibit the reaction. However, the initial velocity of ions Hg^{2+} and Cd^{2+}on inhibition is higher than other metal ions. The inhibition of EROD activity by heavy metals occurred due to the bonding between metal ions on the enzyme sulfhydril group or generation of reactive oxygen species (ROS)or both [27]. Cytochrome P-450 reductase contains one group of cysteine residues at or near the binding site of NADPHand will bind cadmium or other metals for reduction of reductase enzyme and disable the EROD activity [27]. George and Young [28] reported that administration of $CdCl_2$ with doses below 2 mg/kg led to a decrease in EROD activity after 24 hours of observation. Several studies have reported that heavy metals including cadmium exert toxicity through several mechanisms such as inhibition of enzymes that contain sulfhydryl groups in or near the active site of ROS or free radicals [29]. In a study conducted by Chandrasekera et al. [29] it was reported that the exposure to cadmium from 0.001 to 0.01 mg/L in the fish, was not significantly affected the EROD activity. However, there was a significant inhibition of the EROD activity at concentrations of cadmium 0.1 and 1 mg /L for the provision of 14 days. The exposure to cadmium (concentration ≥ 0.1mg/L) caused inhibition of EROD enzyme activity depends on the concentration and duration of exposure to exposure. Thus, it can be said that a high concentration of Cd in the environment could inhibit EROD activity in tilapia. Carp (Cyprinus carpio) and tilapia (Oreochromis niloticus) were used since they are widely used fishes in environmental biomonitoring studies. EROD activities have been measured in liver samples of Cyprinus carpio and Capoeta tinca in Sarıyer Reservoir (Ozmen et al. 2008) contaminated with organochlorine pesticides. EROD activities in fish which live in contaminated areas (petrochemical hydrocarbons, paper factory bleaching water, and industrial and city sewer system) have been used as biochemical measurement methods and biological monitoring tools to assess pollutionresulting from the activities mentioned [28, 30]. The relationship between EROD induction and exposure to chemicalshas been investigated in over 150 different fishspecies throughout the world [31- 38]. Lavado et al. [39] found a significant difference in their study withcommon carp, 689 pmol/min/mg protein ERODactivity compared to 69 pmol/min/mg protein attheir reference site. For this reason, EROD isdescribed as an early warning system [15]. EROD has been widely applied to detect organic compound contamination in fish. This biomarker has been used as a sensitive indicator of anthropogenic organic compound, such as polycyclic aromatic hydrocarbon (PAH), polychlorinated biphenyls (PCBs), and dioxin. Moreover, the activity of EROD is categorized as a biomarker of exposure, which could be detected directly after the exposure of organic compounds [23, 40-43]. Furthermore, EROD also used as a heavy metals exposure biomarker. However, the detection rate is not significant comparing with the detection rate of organic compound. Ueng et al. studied the effect of $CdCl_2$ exposure in tilapia [44]. The result showed that 2 mg/kg cadmium exposure was not significantly affect the liver EROD activity. According to the result of this study, which the exposure of Cd, Hg, and Pb was significantly affected the EROD activity in fish liver, it represents that EROD activity was a sensitive biomarker to use. In Kaligarang river, the organic compound was also contaminate the water environment, therefore it can be concluded that EROD activity is good for detection of organic compound and the heavy metals contamination. This result also gave an information that EROD activity could not detect the specific heavy metals which contaminate the environment, it onlydetected heavy metals contamination generally. Therefore, metallothionein is better than EROD activity to be used as a biomarker for detection of heavy metals contamination in the aquatic environment. Nowadays, several chemicals have polluted the river environment. These chemicals were resulted from various human activities, such as house and industrial activities. The pollution of chemicals and xenobiotic affect the living organisms as well as the human body. In this study, Kaligarang River was used as the treatment sites, since there is no research on the determination of pollution resulting from industrial and human activities in this area. The results of study could be used to evaluate the quality of Kaligarang River as well as to determine the sensitivity biomarker as an early universal monitoring tool. The temperature of Kaligarang River is various between 26 to 28 ^oC affect the process of metabolism in fish body. Generally, the increase of environment temperature could implicate the resistance of fish body to pollutants. The study results of Miller and Argawala indicated that the accumulation of Cd in fish body was affected by physiological factors, physical and chemical characteristic of Cd, and water temperature [5, 6]. In conclusion, EROD biomarker could be directly used to assess the exposure of heavy metals in the waters, even though it could not detect each kind of heavy metals. The results demonstrated that metallothionein and EROD activity werehighly sensitive to extremely low concentrations of theselected pollutants. However, assessing the toxicity of the pollutants cannot simply depend on the reduction of EROD activity. Rather, the use of multiple biomarkers is recommended for the bio-monitoring of chemical pollutants in the river environment.

4 CONCLUSION

Based on the results of study, it can be concluded that EROD activities in carp (Cyprinus carpio) and tilapia (Orecheomus niloticus) fishes were significantly decreased after Cd, Pb and Hg exposure. 7-ethoxyresorufin-O-diethylasecould be used as a biomarker to assess heavy metal pollutants. However, it was not a specific biomarker. The results of this study provide a useful information as an important proof that fish which live in Kaligarang River could accumulate the heavy metals and might have the health risks if it consumed by humans. Upcoming studies should be conceded to determine the crucial cause of the contamination.

DECLARATION OF CONFLICT INTERESTS

The author declared no potential conflicts of interest with respect tothe research, authorship, and/or publication of this article.

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