

Analysis of Heavy Metal Pollutant in Wangi River Pasuruan and Its Impact on *Gambusia affinis*

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Abstract

Pollution that occurred in Pasuruan area's watershed Wangi-Beujeng river, District of Beji, Indonesia has been initiated in 2007 and continues to this day. The activity was caused by many factors such as the industrial (I), household wastes (II) and agriculture wastes (III) as well as the erosion process. The aims of this study to analyzed the heavy metal pollution that occurs in the Wangi river flow and the effect of gill histology and antioxidant activity on gambusia fish. The research method of observation at river flow and sampling for laboratory test. Three sites were assigned for chemical sampling and tissue histological in this study. Fish (*Gambusia affinis*, local name: Gatul) and water were used as indikator from each site to determine of cadmium (Cd), lead (Pb) and mercury (Hg) concentration using *Atomic Absorption Spectrophotometry* (AAS). The study was indicated that the streams previously used by residents for daily activities. Results showed level of cadmium (Cd), lead (Pb) and mercury (Hg) in *Gambusia affinis* exceeded the permissible standard (0.01 ppm Cd; 0.03 ppm Pb; 0.001 ppm Hg) respectively. Based on histological, the tissue showed of damage of chloride cell (CC) which was used in ion homeostasis process and heavy metal route in grill fish. While protease activities, CAT, MDA and peroxidase was increased in each sampling area, with significant different (>0.95) between the three sampling sites

Keywords: Chloride cell, Heavy metal, Toxicity, Wangi river

INTRODUCTION

Pollution of industry wastes will be accumulation of water pollutant [1], [2]. Its substance from industry wastes have within it heavy metal [3]. As research was result contains of heavy metal pollutant a toxic [4] are mercury (Hg) [5], cadmium (Cd) [6], chromium (Cr) [7], lead (Pb) [8], etc. If a toxic expelled to environment can dangerous of organism [7]. Toxic of capacity heavy metal reason enzyme block ways [9], with the result that disturbed of metabolism [10]. Although if heavy metal absorbs at human body can be allergy or carcinogen [11] and deaths if maximum of concentration [12].

One of the case water pollution is Wangi river bleach water condition and surface measurement of putrid taste. Heavy metals increase in intensity with the entry of waste and some other pollutants into the waters [8] and end or settle in the mouth of the river before heading into the sea [5]. Waste contains heavy metals, usually from industry, household and agricultural activities [13], as is the case in the Wangi river.

But, specially study about heavy metal analysis and identification at Wangi river-Beujeng, Beji district pollution case not identified. Whereas, but actually the case of Cd are dangerous to human body [14] and water environment [15]. The first case Cd pollutant at Japan tragedy to come to the surface itai-itai disease as toxic to fisherman [16] and families and deaths arrived 100 human inhabitant [17].

Species of organism various can be accumulated heavy metal in the big quantity as fish, mollusca, plants and microorganism [18]. This study will focus on heavy metal accumulation in fish, and Gatul (*Gambusia affinis*) used as biomonitoring tool from Wangi River. Fish from *Poeciliidae* family [19] are one of the freshwater fish lives in water and wide dispersed on tropical [20]. Its easy adaptation and high tolerant to temperature and salinity [21], moreover water pollutant [22].

Cadmium (Cd), dangerous heavy metal, spread out as environmental contaminant. The main route of Cd were respiratory track and digestion system [23]. Exposure toward environment and biota inside were come from various contamination source especially waste that spill out into environment. This exposure can caused dysfunction of kidney, liver toxicity, genotoxicity

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and apoptosis effect depend on doses, route and duration of exposure[24].

One of Cd toxicity aspect is cytotoxic of ROS-generation which caused damage in macromolecule biology oxidative[25]. In cell, keep the balance between ROS production and antioxidant was important in transport mechanism of ROS[24]. Superoxide dismutase (SOD), Catalase (CAT) and Glutathione peroxide (GPx) are part of antioxidant system that protects cells against ROS. O_2N was shredded by SOD and H_2O_2 then decomposed by GPx and CAT. When ROS generation rate exceeds the cell's antioxidant capacity, severe oxidative stress will result in oxidative damage. In addition to the enzyme index, the central measure of oxidative stress is lipid peroxidation (LPO), as indicated by the level of malondialdehyde (MDA), which can be accumulated as consequence of cell damage[26].

Cd contributes to increase the oxidation reaction of exposed cells. The production of ROS induced by Cd is a response to its toxic effect in many tissue and organs. One of the major mechanisms behind Cd toxicity is associated with oxidative stress. Cd itself is not able to produce free radicals directly, since it has only one oxidative process, however, indirect generation of radicals

involving superoxide radicals, hydrogen radicals and nitric oxide has been reported[25]. Some experiment also confirm the generation of hydrogen peroxide which turn may be significant source of radicals through the chemical reaction of Fenton and Haber-Weiss. ROS has been implicated in chronic Cd nephrotoxicity, immunotoxicity and carcinogenesis. Because Cd interferes with the SH group, it effects the function of many proteins but also the redox status of the cell and hence the cellular level of the active species redox. Sensitivity of the cell relative-withdifferentprotein critical targets, however, it is not well marked[27].

The aims of this study to analyzed the heavy metal pollution that occurs in the Wangi river flow and the effect of gambusia fish on gill histology and some of its antioxidant enzyme activity. Discover environmental condition in the flow of Wangi River, especially in 3 different sites there are industrial area, household area and agriculture area. Those areas are suspected has a great contribution in degradation of environment quality in the Wangi River's flow. The effects of environmental degradation will discovered through *Gambusia* fish by observed the gill histology, heavy metal accumulation and the influence on antioxidant activity.

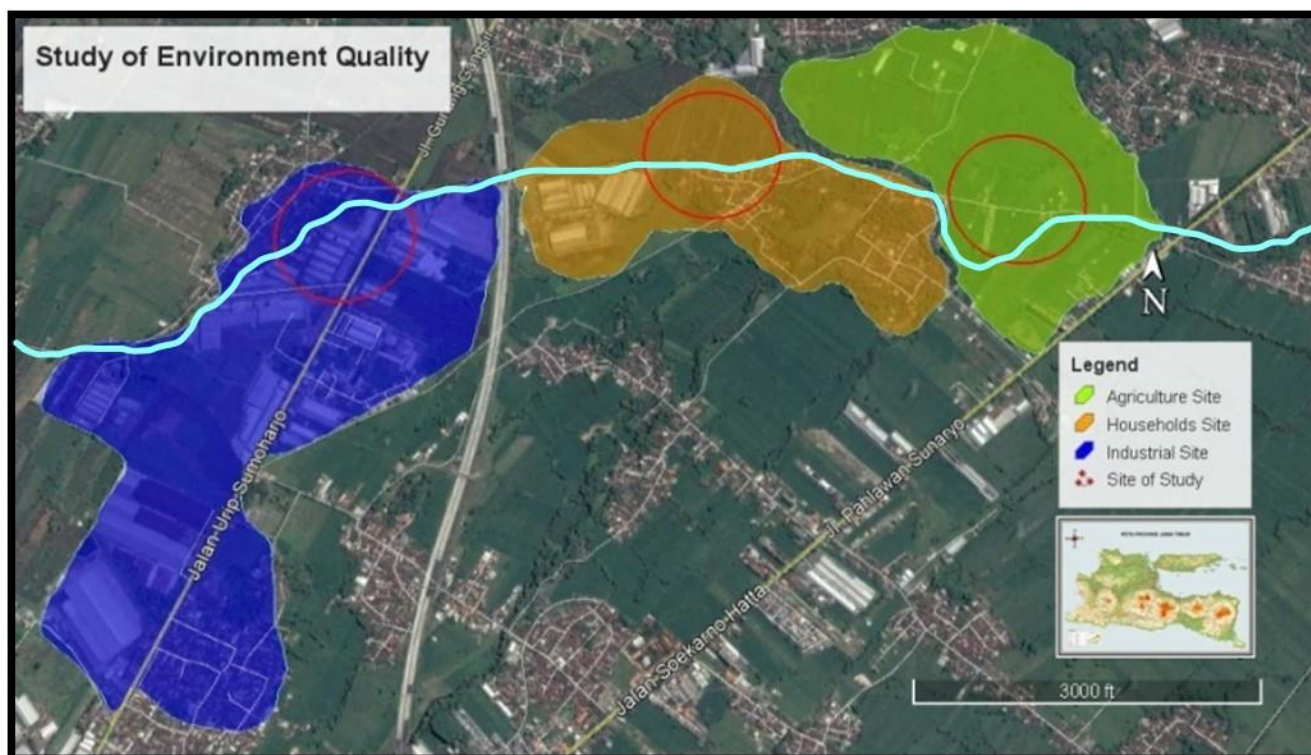


Fig. 1. The Wangi River (blue line) located in East Java, Indonesia. The three sampling areas was marked by the different pointer; (I) Sampling site at industrial wastes; (II) Sampling site at household wastes; (III) Sampling site in agriculture wastes.

MATERIAL AND METHODS

Study Area

The Wangi River in East Java has flow from the spring at ArjunoMount to The Prigen waterfall. The river flows through the following district, which define the prominent zones; Pandaan, Beujeng, Beji and Bangil. Water, sediment and fish samples were taken from seven different sites, but only three different sites were influenced by wastes that used for further investigation.

The chemical-physical characteristics of aquatic environment was listed in Table 1, providing several water quality parameters, including dissolved oxygen (DO), temperature, pH, chemical oxygen demand (COD) and biological oxygen demand (BOD) [13]. The Wangi River of Beujeng were influenced by wastes are characterized by a relatively higher BOD and COD and a lower amount of dissolved oxygen (DO).

Sample Collection

The water, sediment and fish was collected from Wangi River, Pasuruan in three different site (Fig 3). The flame atomic absorption spectrometry from Atomic Absorption Spectrophotometry (AAS) [28] type AA6200 (Specification; Double-beam system (using chopper mirror); Aberration-corrected Czerny-Turner monochromator; Holographic grating; Shimadzu corporation- 2014-Japan). The fish samples were collected from Wangi River, Pasuruan and cleaned then washed with distilled water. Sample collection with method Irianto and Austin on [13] modified, body fish dried were dried at room temperature for two weeks. The dried tissues were digested as following: 1.0 g of each was dissolved in 1M nitric acid (10 ml), boiled to complete the dissolution and filtrated. The obtained precipitate was washed with nitric acid (1 M) and transferred to 25 ml volumetric flask and fill up to the level with de-ionized water.

Preparation of gill

The gills were immediately removed, packed in plastic bags and transported to the laboratory in ice cooled box where they were stored at -20°C. Latter the gills were dried at 105°C in a gravity oven to constant weight [29]. The dried fish gills for five fish from each sampling station were mixed and homogenized by grinding with a pestle and mortar into a fine powder and placed in well labeled plastic bags.

Histological observation

Gill tissues were carefully excised modified from [30], fixed in 10% buffered formalin for 24 hours, followed by dehydration with ethanol and toluene series and embedded in paraffin. Approximately 4mm-thick serial sections were obtained and stained with hematoxylin and eosin (H&E) for observation with a light microscope (Olympus BX51).

Hydrogen peroxide levels

The tissue hydrogen peroxide level was determined according to the methods that provided by Philip J. [31] using HRP and phenol red. The values were expressed as nanomole of hydrogen peroxide per milligram protein.

Catalase activity

Catalase activity (EC 1.11.1.6) was estimated by the method described [32]. Homogenates (4%) of tissues were prepared in cold phosphate extraction buffer (50mmol, pH 7) using a glass Teflon homogenizer and then centrifuged at 3,000 rpm for 15 min. To the supernatant (1 ml), 10 mmol of hydrogen peroxide in 2 ml of phosphate buffer (50 mmol, pH 7) was added as a substrate for catalase to initiate the incubation. The decrease in absorbance of the sample was measured at 240 nm using UV-visible spectrophotometer (Hitachi, model no. U-3310). The values were expressed as millimole of hydrogen peroxide decomposed per milligram protein per minute. The value was compared with the absorbance standard kit CAT merk stressmarq Canada, to obtain an estimate of the same protein value in the treatment sample. 10 µg/ml of SPC-115 was sufficient for detection of Cu/Zn/Cd CAT in 20 µg of colorimetric immunoblot analysis.

Estimation of protease activity

Protease activities in the tissues were estimated by the ninhydrin method as described by [33]. Homogenate (4 %) was prepared in cold phosphate extraction buffer (50 mmol, pH 7) and centrifuged at 3,000 rpm for 15 min. To 2 ml of the supernatant, 0.5 ml of 1 % casein and 2 ml of 0.1 mol phosphate buffer (pH 5) were added. The contents were mixed well and incubated at 37 °C for 30 min. The reaction was stopped by adding 2 ml of 2 % ninhydrin reagent. Again, the contents were mixed thoroughly and placed in a boiling water bath for 20 min. The solution was cooled and made to 10 ml with diluents (distilled

water and n-propanol in 1:1 ratio). The optical density of the color developed was measured using a spectrophotometer at 570 nm against a reagent blank. The protease activity is expressed as a micromole of tyrosine equivalents per milligram protein per hour.

Lipid peroxidation estimation

MDA, the secondary product of lipid peroxidation, was estimated in the tissue homogenates utilizing the colorimetric reaction of TBA [34]. It gives an index of the extent of

progress of lipid peroxidation, and since the assay estimates the amount of TBA reactive substance, e.g., MDA, it is also known as TBARS test. Tissue homogenates (16 %) were prepared in cold 50 mmol Tris–hydrochloric acid (HCl) (pH 6.8) extraction buffer. To 0.8 ml of the homogenate, 2 ml of 15 % trichloroacetic acid (TCA) was added and centrifuged at 5,000 rpm for 15 min. To the entire supernatant, 0.7 ml of TBA reagent (1 %) was added, and the test tubes were covered with aluminum foil followed by incubation in a shaking water bath for 60 min at 100 °C.

Tabel 1. Water Quality in three different areas of Wangi–Beujeng River

Parameter	Area I (Industrial Area)	Area II (Household Area)	Area III (Agriculture Area)
DO (mg/L)	7.86	7.92	7.94
pH	6.5	6.7	7.1
Temperature (°C)	28-30	28-31	29-30
COD (mg/L)	45	30	53
BOD ₅ (mg/L)	30	18	42
Detergent	22.2;374	35.39;460	27.17;365

Tabel 2. Heavy metal in three different areas of Wangi–Beujeng River

Parameter	Area I (Industrial Area)	Area II (Household Area)	Area III (Agriculture Area)
Cadmium (Cd)			
- Water	0.019	0.009	0.015
- Sediment	0.386	0.235	0.286
- Fish	0.036	0.061	0.048
Mercury (Hg)			
- Water	0.010	0.015	0.009
- Sediment	0.125	0.174	0.081
- Fish	0.019	0.018	0.012
Lead (Pb)			
- Water	0.020	0.035	0.027
- Sediment	0.320	0.524	0.422
- Fish	0.031	0.039	0.032

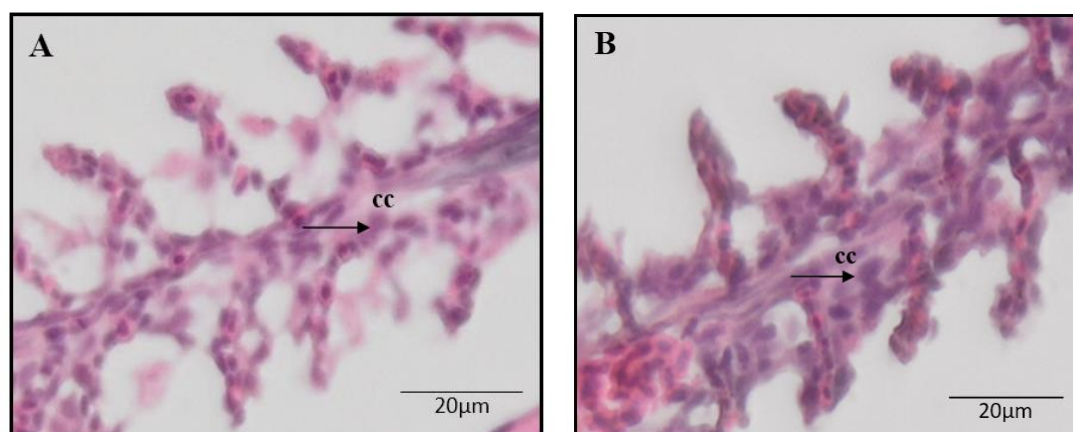




Fig. 2. Gills structure of fish. A) Industrial area, B) Household area, C) Agriculture area. Chloride cell (cc) from three different areas was changed. Severe damage of chloride cell showed in gill from industrial area.

The tubes were then transferred to ice-cold water for 10 min, and the absorbencies were read at 532 nm against a reagent blank. The rate of lipid peroxidation (LPO) was expressed as nanomoles of TBARS formed per milligram of protein using a molar extinction coefficient of $1.56 \times 10^{-5} \text{ mol}^{-1} \text{ cm}^{-1}$.

Statistical analysis

Data correspond to the average of three replicates with ANOVA analysis SPSS [35]. The data obtained were analyzed statistically by following Duncan's and Tukey's multiple range test.

RESULT AND DISCUSSIONS

Quality of Water Environment

Water pollution observation data based on change level BOD_5 , COD, DO, temperature, pH, Detergent and metal observation (Pb, Cd and Hg) is presented in Table 1 and Table 2. The result of water quality from three different sampling areas was showed in Table 1. Level of DO, pH and temperature were in normal condition for the life of aquatic organism. However, the COD and BOD_5 levels were in quite alarm as it shows well above the limits for the life aquatic organism. Tolerance of COD between 20-30 mg/L while the limits for BOD_5 is 8-15 mg/L. Meanwhile, field observation showed COD; 45 mg/L (I), 30 mg/L (II) and 53 mg/L (III) while BOD_5 ; 30 mg/L (I), 18 mg/L (II) and 42 mg/L (III).

Small proportion of detergent can be classified as having a medium toxicity (10-100 mg/L) and a few have very low toxicity (up to 10,000 mg/L). Detergent can cause damage to the gill respiration

epithelium such as enlargement and vacuolation of the cell with dystrophic to necrobiotic changes. The clinical signs of poisoning include respiratory disorder and later by inactivity. The characteristic in the patho-anatomic examination are increase amount of mucus on the skin and in the gill, and congestion to oedematous swelling of the gill apparatus [36].

The concentration of heavy metals (Cd, Hg and Pb) determined in water, sediment and fish at three sampling area given in Table 1. The range of Cd was 0.36-0.061 mg/L with NO significant different (>0.95) between the three sampling sites, while the range of Hg was 0.012-0.019 mg/L and 0.031-0.039 mg/L for Pb. The highest concentration of the heavy metal in fish was recorded for Cd, followed by Pb and Hg, where Cd recorded higher concentration was collected from household area (Fig. 1). The same trend was found in the level of Pb.

Water sample from three different sites showed (Table 2) concentration of Pb was highest compared to Hg and Cd. The result indicated that Pb in water and sediment samples (Table 2) were higher than Cd and Hg. But in fish samples observations showed that Cd was higher than Pb and Hg. It was indicated that the industrial area contributes the highest heavy metal waste and the type of metal Cd with the highest concentration in the waters and fish samples. Fish can accumulate cadmium from the water and eating foods contaminated with cadmium (contaminated food chain). It is important to note that bioaccumulation/magnification occurs when a substance cannot be easily metabolized or

excreted[16]. Diverse industrial wastes have aggravated the problem of water pollution as and this problem becomes complex with of non-degradability of inorganic pollutant like heavy metals[37].

However in small amount, exposure of heavy metal in long period can cause accumulation in aquatic organism body. General effects caused by accumulation of heavy metal are damage of central nervous system and parenchymatous organs. Very small concentration of Cd may produce specific effect after long exposure period. Hg compound can cause damage to some vital tissue and organ in fish and may also have a harmful effect on reproduction[16].

Histology of gill fish

Appearance on histology of gills of *Gambusia* fish taken from samples of industrial, household and agriculture areas showed gill damage (Fig. 2). The damage was clearly seen in the chloride cell. From three different areas, Industrial area showed severe damage in the chloride cell compare to Household and Agriculture area. Chloride cells play an important role in the homeostasis process of fluid entry into the gills fish. This process allows heavy metal to be first absorbed into the body through a chloride cells. Observation result has indicated that damage occur when organism was exposed by waste containing with heavy metals[38]. The statement of [4] asked of gill destined for histopathologic can be either excised from the carcass as whole gill arches or individual filaments, or examined in whole-body sections, in the case of small fishes. The histomicrographs indicated to follow this approach can lead to inappropriate diagnoses of lamellar epithelial hyperplasia (LEH), lamellar fusion, lamellar loss or atrophy or chloride cell hyperplasia.

Heavy metal induced Oxidative Stress

The result data showed that catalase and protease enzyme activity in industrial area (0.562 ± 0.192 ; 0.932 ± 0.049) greater than other areas (0.462 ± 0.114 ; 0.877 ± 0.112 , 0.265 ± 0.064 ; 0.937 ± 0.133) (Fig 3). Data's showed a high effect of industrial waste on the activity of CAT and protease enzymes. Catalase and protease can be endogenous antioxidants which function to neutralize free radicals[39]. While the activity of H_2O_2 and MDA tends to decrease in industry area (2.167 ± 0.242 ; 21.278 ± 2.749) and increase in other area (6.141 ± 0.604 ; 28.222 ± 5.49 , 18.235 ± 0.66 ; 25.222 ± 12.4) (Fig 3). Data's showed a low effect of industrial waste on the activity of H_2O_2 and MDA,

lack of the role of H_2O_2 and MDA in reducing free radicals, with significant different (>0.95) between the three sampling sites. The happened because of the increase in antioxidants with the entry of heavy metals into the fish body. Such a statement[37] asked the heavy metal intake in body of living organism can occur through the air, water and food consumed or it can be said that accumulates heavy metals in the body of a living thing through the food chain. Organism needs heavy metal as essential metals in metabolic process and also as co-enzyme factors but in very small amount. When organisms absorb the metal more than the safe limits, the body will be harmful, because it will be poison that can disrupt the metabolic[6].

But H_2O_2 , MDA and protease activity in household and agriculture highest industrial, could be the influence of household and agriculture waste containing detergent and pesticides higher so that the effect on existing antioxidants. In the statement [15] as the respiratory system, the absorption of molecular oxygen and transformation into the reactive oxygen of the compound, which a represented of defense mechanisms of inflammation. The physiologically important role of oxygen reactive compounds with a certain concentration capable of modulated reactive oxygen sensitive signals and improving function immunologic cellular [22]. The occurrence of increased and decreased enzyme work is greatly influenced by environmental conditions. The heavy metal in our bodies will also trigger ROS as a result of the deactivate of antioxidant enzymes such as Superoxide dismutase (SOD), Catalase (CAT), and Glutathione Peroxidase (GPOD), which function as antioxidants [41]. The formation of ROS in our body is also caused by oxidative stress, ROS will easily damage peroxide fat from the pleated membrane, cell membrane from phospholipid, and lipoprotein by spreading in chain reaction [42], [43].

Heavy metals are harmful polluting substances, the metals that enter into our body through our digestive system will react with the element of sulfur and enzymes in our body so that the enzyme will not work properly but also heavy metals that enter into our body will also react with the Cluster carboxylate ($-COOH$) is also amino ($-NH_2$) in amino acids [37]. Free radicals are basically unpaired molecules in their chemical structures so that these free radicals will look for couples to bond. Basically all biomolecules pair up to achieve stability, so to achieve this stability free radicals will look for other free electrons to bind to achieve stability.

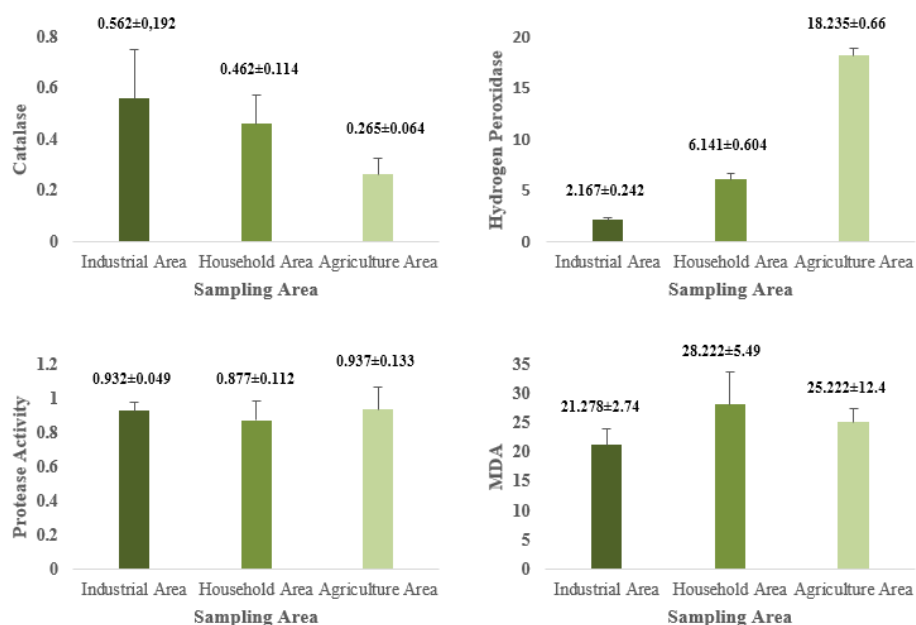


Fig. 3. Indicator of oxidative stress activity. Catalase (CAT, $\mu\text{mol} \cdot \text{min}^{-1} \cdot \text{mg protein}^{-1}$), Hydrogen peroxidase (H_2O_2 , $\mu\text{mol} \cdot \text{mg protein}^{-1}$), Protease activity (PA, $\text{mmol} \cdot \text{min}^{-1} \cdot \text{mg protein}^{-1}$) and Lipid peroxidation estimation (MDA, $\text{mol}^{-1} \text{cm}^{-1}$) from industrial, household and agriculture area was increased. The data's suggest a significant difference in oxidative stress activity against heavy metal waste discharges.

The nature of oxygen (O_2) is essentially an electron acceptor so that it will receive free electrons even when it reaches stability, thus forming superoxide (O_2^-) these free radicals which bind with oxygen are called Reactive Oxygen Species (ROS), $\text{X} + \text{O}_2 \rightarrow \text{Y-X} + \text{O}_2^-$ (The process of occurring oxygen species) [37], [40].

ROS can attack all types of biomolecules such as nucleic acids, proteins and amino acids later on interfere with metabolism. DNA damage is a consequence of the modification of genetic material resulting in cell death, mutagenesis, carcinogenic and aging [31], [39], [44] in our body, ROS is generated by metabolic processes which will be used programmatically to disable cells. ROS consist of Superoxideradical (O_2^-), Hydrogen Peroxide (H_2O_2), Hydroxyl Radical (OH^\cdot) and Singlet Oxygen (O_2^1) which increase when exposed to UV rays, ionic radiation and pollution (heavy metal) [9], [15], [22], [45]. Heavy metal toxicities occur when it has exposed and accumulated in living organism through food and water (drinking water) and pollution from wastes.

The observation on indicator of the oxidative stress; CAT, H_2O_2 and MDA activity in fish samples from industry, residential and agriculture areas has increased (Fig. 3). This indicate that exposure to waste containing heavy metals be able to activate

the performance of enzyme that contain of activity antioxidant in self-defense against foreign matter. Heavy metal pollution is one of the greatest national health problems with referring to peoples eating fish foods, it requires special and intense effort at all levels; individual, groups, national, and international.

Heavy metals are inorganic chemicals that are non- biodegradable, cannot be metabolized and will not break down into harmless forms since they leave biological cycles very slowly[4]. Elements such as cadmium, copper, lead and zinc are considered most dangerous in the ecotoxicological aspect[17]. Metal ions can be incorporated into food chains and concentrated in aquatic organisms to a level that affects their physiological state and causes drastic environmental impact on all organisms[30]. Such health risk may over shadow the cardiovascular benefits from the consumption of certain farmed fish, Moreover, aquaculture products are sometimes banned due to rejection of export consignments.

CONCLUSION

Based on histology, the tissue showed of damage of chloride cell (CC) which was used in ion homeostasis process and heavy metal route in grill

fish. While protease, CAT, MDA and H₂O₂ was increased in each sampling area, with significant different (>0.95) between the three sampling sites.

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