Reproductive performance of Zebra Fish *(Danio rerio)* exposed to palm oil mill eflluent in chronic toxicity

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Abstract. Palm Oil Mill Effluent (POME) is potentially harmful to the aquatic environment. POME contains high organic material including COD (Chemical Oxygen Demand), BOD (Biochemical Oxygen Demand) TTS (Total Suspended Solid) and various type of heavy metals. of zebra fish *(Danio rerio)*. Reproductin has an important role in producing new individuals which directly affect the population. Impaired reproductive performance potentially impairs juvenile production optimization. The present study investigated how sub-chronic toxicity of POME impact the reproductive performance used Completely randomized Design (CRD) in three treatments and four replicates based on value of LC50-96 hours (5.156 ml/l): Control (0 ml/L), treatment A 10% POME (0,5 ml/L), treatment B 20 % POME (1 ml/L). The fecundity, relative fecundity, GSI, and egg diameter were analyzed. Data was analyzed with Analysis of Variance (ANOVA) and followed with Least Significance Difference (LSD) test. Results showed that fecundity in treatment A (149 ± 38.70) and treatment B (85± 11.35) were significantly decreased compared to the control (219 ± 42.38) ($P<0.05$). While relative fecundity significantly decreased in treatment B (0.33 ± 0.13) rather than control (0,87 ± 0,14). Significantly decline is also observed on GSI in tretment A (4.79 ± 2.55%) and treatment B (2.55 ± 0.21%) compared to control (6.96 ± 1.70%). While the egg diameter only shows a significantly decline in treatment B (0.57 ± 0.18 mm) compared to control (0.71 ± 0.27 mm).

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1 Introduction

Indonesia is the largest palm oil producer. Since 1980, palm oil production in the form of CPO (Crude Palm Oil) has continued to increase with an average growth of 11.13% per year [1]. POME contains high concentrations of Biochemical Oxygen Demand (BOD) ranging from 25000-65714 mg/L, Chemical Oxygen Demand (COD) of 44300-102696 mg/L, and suspended solids ranging from 18000 to 46011 mg/L [2]. Previous studies revealed that POME cause significant decrease in plankton diversity and disrupts the physiology and reproduction of fish [3][4].

Fish is a ideal bioindicator for assessing pollution due to its sensitivity [5]. POME reported cause the alteration in reproduction of Nile tilapia (*Oreochromis niloticus*) including decreased reproduction hormonal as well as decreased gonad somatic index (GSI) and spermatocrit [4]. Exposure to POME reduced the concentration of estradiol, testosterone and progesterone in Nile tilapia *(Oreochromis niloticus)* [6]. Zebrafish *(Danio rerio*) embryos exposed to POME resulted in mandibular malformations, head and eye malformations, and yolk sac edema[7]. Exposure to POME has a negative impact on the hatching rate, survival rate, and heart rate of Nile tilapia. Hatching and survival rates decrease significantly with increasing POME concentration.Besides, malformation and heart rate also increased significantly as the concentration of POME increased [4].

Study related to the impact of POME on fish reproduction has previously been reported on Nile tilapia *(Oreochromis niloticus).* Exposure to 3.130 mg/L POME causes a significant decrease in GSI, and disorientation of gonad shape [8]. In addition to, exposure to several heavy metals (Fe, Cu, Pb and Cd) in Nile tilapia *(Oreochromis niloticus)* caused several degenerative impact and a decrease in the number of seminiferous tubules in the testes and shows deformation or changes in normal shape and the presence of severe lymphocyte infiltration in the ovaries [9].

To date, zebrafish have been used in several toxicity tests [10][11][12]. Previous studies reported the changes in reproductive capacity, increased DNA damage, and alteration in gene expression of zebrafish *(Danio rerio)* due to uranium exposure [13]. Exposure to Hg caused various side effects in fish at physiological, histological, biochemical, enzymatic, and genetic levels. Certain fish species are more sensitive to Hg toxicity than other fish species. Therefore, Hg-induced toxicological pathology in fish is influenced by factors such as species, age, environmental conditions, exposure time, and exposure concentration [14]. Exposure to mercury during the early stages of life disrupts the balance of sex hormones and gametogenesis by changing the expression of mRNA for genes involved in the hypothalamic, pituitary, gonadal axes, resulting in lower fecundity values [15]. To best our knowledge, information underlying the impact of POME on fish reproduction is still very limited. Hence, this study aims to investigated the effect of POME exposure in chronic concentration on several of reproductive variable (gonadosomatic index, fecundity, and oocyte diameter) of zebrafish.

2 Material and Methods

2.1 Study Area, POME Collection and Fish Acclimatization

This research was conducted from August to September 2023 at the Zoology Laboratory, Ar-Raniry State Islamic University, Banda Aceh. POME was obtained from the palm oil factory located in Nagan Raya Regency, Aceh Province. The POME was transported to the laboratory and stored in a cool room at < 4 ◦C to avoid biodegradation. As many as 45 adult female zebrafish *(Danio rerio)* with an average length of 2.7 ± 1.16 cm and weight 0.34 ±

0.26 gr were purchased at fish trader located in Banda Aceh. Adult female zebrafish are characterized by morphological characteristics of larger body size, bulging stomach, reddish urogenital opening, and paler body color [16]. Acclimatization of fist test was done for seven days. During acclimatization, the fish were fed with commercial feed (Wonder Tropical/ Protein content of 40%) twice daily (09.00 and 17.00 Indonesian Western Time) at a satiation level. The feeding was stopped 24 h before the toxicity test.

2.2 Sub Chronic Toxicity Test

The research design was a Completely Randomized Design (CRD) with four treatments followed by three repetitions for each treatment. POME is prepared to the concentration required for the sub-chronic test, namely 10% and 20% of the acute concentration (LC_{50} 96 hours; 5.156 ml/L)[10]. The detail of sub-chronic concentrations of each treatment including control (0 mL/L POME), Treatment A (0.5 mL/L POME), and Treatment B (1 mL/L POME). Preference test was conducted in aquarium (20×15×20 cm) with 3 L of volume. Each aquarium was filled with five zebrafishs. Fecundity, GSI, and egg diameter from each treatment was measured it the end of exposure period. Preference test lasting for 28 days.

The GSI was calculated using the following formula [17]:

$$GSI = 100x\ (\frac{Wg}{Wt})$$

Where, GSI is the gonadosomatic index, Wg is the gonad weight, and Wt is the total body weight. The total fecundity (F) was calculated using the equation as follows [18, 19]:

$$F = \frac{X}{Q} x\ Wg$$

Where F is fecundity, X is the partial number of eggs of the gonad, Wg is the gonad weight and Q is the partial weight of gonad. Relative fecundity (RF) was measured using the equation as follows [20]:

$$RF = \frac{F}{W}$$

Where, RF is relative fecundity (egg/g), W is the body weight and F is the total number of eggs of the gonad. The measurement of oocyte diameter was done on fish with stage IV gonad development. Gonad developmental stage IV of zebrafih was indicated by its morphology referred to [18]. The number of oocytes measured for every treatment consisted of 150 eggs using a microscope equipped with a micrometer (magnification 40X) (Figure 1).

$$D = \sqrt{Dv \times Dh}$$

Where, D is Egg diameter (mm), Dv: Vertikal egg diameter (mm) and Dh: Horizontal egg diameter (mm) [21].

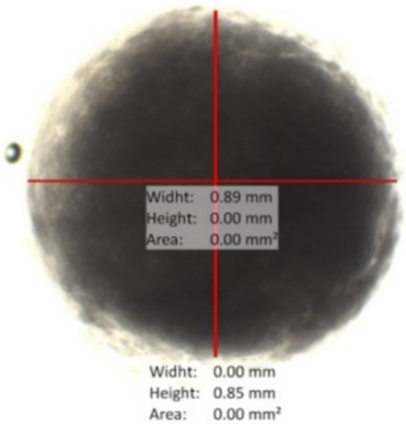

Fig. 1. Guidance in measuring the diameter of zebrafish eggs

3 Result and Discussion

3.1 Fecundity

The fecundity values ranged from 85 to 219 eggs/individual. The highest fecundity value was found in the control (219 eggs ± 42.38), followed by treatment A (149 ± 38.70 eggs), and treatment B (85 ± 11.35 eggs). There was a significant difference infecundity value between control and treatments. The highest relative fecundity was recorded in control (0.87 ± 0.14 eggs), followed by treatment A (0.54 ± 0.25 eggs) and treatment B (0.33 ± 0.13 eggs). There was a significant difference between the control and treatment B, but no significant when compared to treatment A (Table 1).

Table 1. Fecundity and relative fecundity of Zebrafish *(Danio rerio)* between treatment

Paramenters	Control	Treatment A	Treatment B
Fecundity (egg)	219±42,38[a]	149±38,70[b]	85±11,35[b]
Relative fecundity (egg/mg)	0,87± 0,14[a]	0,54±0,25[ab]	0,33±0,13 [b]

* Different letters on the same row indicate significant differences (P<0.05).

A decrease in fecundity might correlated with gonad development alteration, characterized by increased follicles/germinal cells. Previous research revealed that a decrease in fecundity might correlated with a decrease in the GSI. Exposure to mercury ($Hg^{2+)}$ at a concentration of 15µg/L in the early stages of zebrafish (*Danio rerio*) results in disruption of gonad development which is characterized by an increase in early stages follicles/germ cells and a decrease in late stage follicles/germ cells [22]. In addition, exposure to Carbamazepine (CBZ) and Gemfibrozil (GEM) 0.5 and 10 µg L^{-1} caused oocyte atresia and changes in ovarian histology which was a direct effect on oocyte development which caused a decrease in fecundity of zebrafish [23].

3.2 GSI

The result showed that the highest average GSI value was observed in control (6.96 ± 1.70%), while the lowest value was in treatment B (2.55 ± 0.21%) (Figure 2). There was no significant

difference in GSI between the control treatment A (4,797 ± 2,55%). However, a significant difference was observed between the control and treatment B.

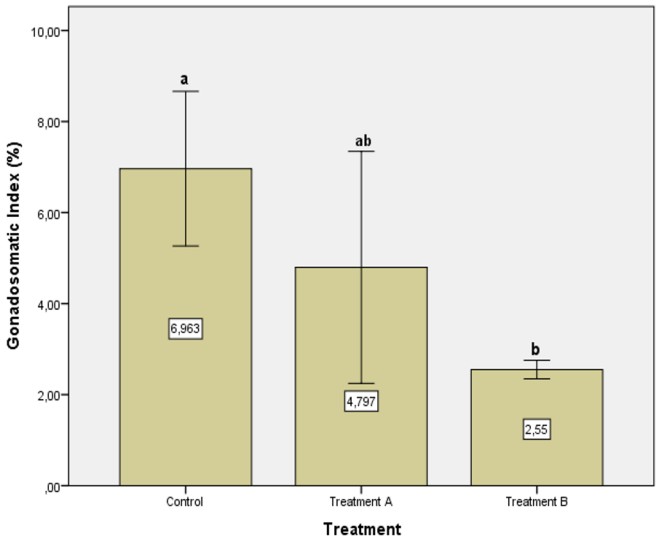

Fig. 2. The value of GSI of Zebrafish *(Danio rerio)* between treatment. (*different letters indicate a significant difference of P<0.05).

The GSI value was decreased as the concentration of POME increases. The low value of GSI was linked to the oocyte's diameter shrinking. Our study showed that the average diameter of oocytes seemed to shrink more when the concentration of POME in exposure media increased. Smaller oocyte weight had been decreased gonad weight so that the GSI values became low. According to [24], the shrinking diameter of oocytes was caused by disturbance in the vitelogenesis process due to pollutant exposure. Such exposure has caused damage to liver tissue so that the process of vitelogenesis becomes disrupted [25]. Fish exposed to mercury (Hg) showed inhibited testicular and ovarian growth and delayed gonad development. Exposure to POME triggered a decline in gonadosomatic index and shrunk the oocyte diameter of female Nile tilapia *(Oreochromis niloticus*) [8]. GSI decreased significantly in zebrafish with exposure to endocrine-disrupting chemicals [10]. Heavy metals are endocrine disruptors that can cause hormonal imbalances that affect various physiological processes, such as reproduction [26]. Cu exposure for 30 days changed steroid hormone levels and endocrine-related gene expression in the HPG (Hypothalamic-Pituitary-Gonadal) in zebrafish (*Danio rerio)* [27]. Flutolanin influences the content of Gonadotropin-releasing hormone (GnRH), regulating reproductive performance through the synthesis of gonadotropin hormones in the HPGL (Hypothalamus-Pituitary-Gonad-Liver) axis, thereby causing a decrease in reproductive capacity in zebrafish (*Danio rerio*) [22].

3.3 Egg Diameter

The result showed that the average value of zebrafish egg diameter in treatments A and B was smaller than in the control treatment. The average value of egg diameter decreases with the increasing concentration of POME (Figure 3). There was no significant difference in egg diameter between the control (0.71 ± 0.1 mm) and treatment A (0.62 ± 0.06 mm). However,

a significant difference was observed in egg diameter of control compared to treatment B (0.58 ± 0.08 mm).

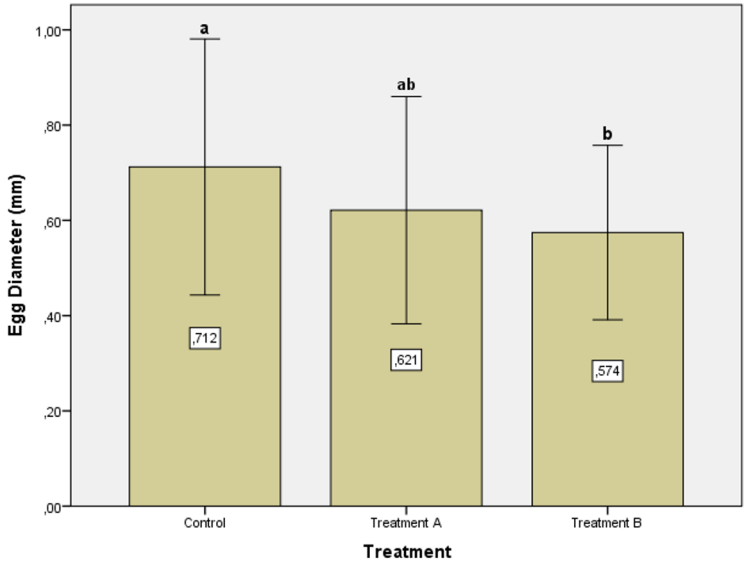

Fig. 3. The value of egg diameter of Zebrafish *(Danio rerio)* between treatment *different letters indicate significant differences of P<0.05

Similar previous research reported that the average oocyte diameter in tilapia *(Oreochromis niloticus)* show a decrease trend when the concentration of POME in the exposure media increase [8]. A decrease in oocyte diameter due to heavy metals has also been reported in tilapia [28]. Water quality plays an important role in fish survival. Inappropriate water quality can have a direct impact on growth, development, metabolism, immune function, behavior, stress, reproduction, and various other disorders.

4 Conclusion

Exposure to POME in sub-chronic concentrations for 28 days caused a significant decrease in reproductive performance which indicated by a decrease in the Gonadosomatix Index (GSI), fecundity, and egg diameter of zebrafish *(Danio rerio)* (P<0.05). Fecundity in treatment A and B were significantly decreased compared to the control. While relative fecundity significantly decreased in treatment B compared to control. Significantly decline is also observed on GSI in tretment A and B compared to control. While, the egg diameter only shows a significantly decline in treatment B compared to control.

Acknowledgments

The study was financed by a Research Grant from the Universitas Syiah Kuala under "Penelitian H-Index 2023" Scheme [Grant No : 122/UN11.2.1/PT.01.03/PNBP/2023].

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