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Biomarker Evaluation in Nile Tilapia (*Oreochromis niloticus*) to Assess the Health Status of Aquaculture Areas in the Seven Lakes of San Pablo

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The seven lakes of San Pablo have been subjected to different degrees of stresses brought about by intensive aquaculture, sewage discharges, and other anthropogenic disturbances, which could have serious implications on the health of these ecosystems. This study was conducted to assess the health condition of cage aquaculture areas in the lakes using biomarkers in the adult Nile tilapia (*Oreochromis niloticus*). A total of 84 cultured fish samples consisting of 12 individuals per lake were examined to determine the condition factor (K-factor); hepatosomatic index (HSI); splenosomatic index (SSI); and histopathological features of the liver, spleen, and gills. The measured values for K-factor are suggestive of isometric growth and good health condition; however, fish from Sampaloc Lake had relatively lower values in comparison to the other groups. Although all liver samples had normal microstructure, the HSI of fish in Sampaloc and Palakpakin Lakes was significantly reduced than those obtained in fish from Lakes Bunot, Calibato, and Yambo. All spleen samples showed melanomacrophage centers (MMCs), with fish in Calibato Lake having higher SSI compared to the others. The majority of the gill samples showed minimal to mild clubbing of secondary lamellae, thrombosis, and aneurysm. Based on the endpoints considered in this study, Yambo Lake had the most optimal condition for fish culture. Lakes Sampaloc, Palakpakin, and Mohicap – on the other hand – had the lowest environmental status.

Keywords: biomarker, fish, histopathology, lake, organosomatic indices

INTRODUCTION

Environmental monitoring of freshwater systems in the country usually only takes into consideration the physical and chemical measures of water quality. In a recently published study on the Seven Lakes of San Pablo – which consist of Bunot, Calibato, Mohicap, Palakpakin, Pandin, Sampaloc, and Yambo – it was revealed that standards for dissolved oxygen, temperature, turbidity, and pH were generally within the prescribed range of values for the propagation and growth of tropical fish (Paller *et al*. 2017). However, Lakes Mohicap and Palakpakin had eutrophic to hypereutrophic turbidity values that were attributed to surface run-off (Paller *et al*. 2017). Failure to meet water quality standards for fresh surface water would mean that the freshwater resource cannot support aquatic life (DENR 1990). Pollution threats to water quality in the

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lakes include domestic effluent and solid wastes, illegal and crowded fish pens, and overfeeding with artificial feeds (LLDA 2008), which has led to the proclamation of the seven lakes as "threatened" (GNF 2014).

However, the health of fish and other aquatic inhabitants can also reflect the overall health status of the aquatic environment. Physiological and morphological changes in fish may result from exposure to chronic and sublethal levels of pollutants and other stressors in the environment. The assessment of K-factor, organosomatic indices, and organ histopathology in fish has been conducted to monitor aquatic ecosystem health. The K-factor is a measure of various ecological and biological factors such as degree of fitness, gonad development, and suitability of the environment in regards to feeding conditions (Nehemia *et al*. 2012). Organosomatic indices such as HSI, SSI, and GSI pertain to the proportion of organs to body weight, which can provide information on metabolic activity and physiologic responses to internal and external factors (Freyre *et al*. 2009). Histopathological changes in specific organs can provide an assessment of fish health, as it can be integrated with the impact of various stressors like microbial pathogens, toxic compounds, and nutritional and adverse environmental conditions (Valon *et al*. 2013). This study was conducted to evaluate the health status of selected cage aquaculture areas in the seven lakes of San Pablo, Laguna with the use of K-factor, organosomatic indices, and organ histopathology as biomarkers, and the adult male Nile tilapia as bioindicator species. Aside from being an abundantly cultured fish species in the lakes, the Nile tilapia has been used as a bioindicator species in ecotoxicological studies (Parente *et al.* 2004; Sonza and Fontanetti 2006; Fernandes *et al*. 2008; Linde-Arias *et al.* 2008; Firat and Kargin 2010; Seriani *et al*. 2012; Santana *et al*. 2018).

MATERIALS AND METHODS

Study Sites

Sampaloc Lake (area: 104 ha; elevation: 106 masl; depth: 61.75 m) is the largest and most accessible of the seven lakes. Located 1 km from it is Bunot Lake (30.5 ha), which was considered as the most polluted and congested in 2006–2008 by the Laguna Lake Development Authority (LLDA) because of intense aquaculture operations and many illegal settlers along its coastline (Brillo 2015a). Mohicap Lake is relatively "underdeveloped, underutilized, and unstudied." It has the smallest area at 22.89 ha and the lowest elevation (80 masl) (Brillo 2015b). Palakpakin Lake is located adjacent to Mohicap Lake and is the shallowest with a depth of 7.7 m. It has an area of 47.98 ha and an elevation of 100 masl. Aside from aquaculture activities, illegal settlers, and pollution, this lake is also threatened by siltation (Brillo 2016a). Calibato Lake is the deepest lake with a depth of 156 m. It also has the highest altitude at 170 masl and an area of 43 ha (LLDA 2008). Yambo and Pandin are lakes with the same altitude (160 masl) and are contiguous to each other. Pandin Lake (depth: 61.7 m; area: 24 ha), which can be accessed by hiking, has double the depth of Yambo (38 m). Both lakes were better than the others in terms of water quality, as monitored by the LLDA (2008). However, the larger Yambo Lake (30.5 ha) was considered as the reference site in this study as it has the least number of fish cages and fish pens, and the "least populated lakeshore area" (Mendoza *et al.* 2019). Its water quality has also been considered as the highest among the lakes (Brillo 2016b).

Aquaculture is a predominant activity in the seven lakes – particularly in Bunot, Calibato, and Palakpakin (Mendoza *et al*. 2019). The registered fish pens/cages in the lakes totaled to 163 in Sampaloc, 85 in Palakpakin, 75 in Bunot, 49 in Calibato, 25 in Mohicap, 14 in Pandin, and three in Yambo (Brillo 2017). However, there are also reports of unregistered fish cage operations (Brillo 2017). Approximately 3,500–5,000 Nile tilapia fingerlings that are usually sourced from the Bureau of Fisheries and Aquatic Resources are reared by fisherfolk in 10 m x 10 m fish cages and fed with commercial fish feed until the market weight is attained. By contrast, tourism is the main livelihood source of lakeshore communities in Pandin and Yambo Lakes (Zafaralla 2010).

Fish Sampling

Twelve sexually mature male Nile tilapia were obtained in each lake in one or two adjacent fish cages, whose operators were selected by the heads of the local fisherfolk organization based on willingness and capability to provide the required age, sex, and the number of fish in the study. The sampling sites were as follows: southwest of Lakes Bunot and Calibato; northeast of Lakes Mohicap, Palakpakin, and Pandin; southeast of Sampaloc Lake, and northwest of Yambo Lake. Prior to sampling, the fish were fed twice daily with commercial fish feed. Fish were captured using scoop nets and placed in polyethylene plastic bags with water and oxygen. The bags were placed in rectangular plastic containers to provide stability during transport. Transport into the laboratory was accomplished within 2–3 h in an airconditioned vehicle. Fish samples totaled to 84, with weights ranging from 75–450 kg and total lengths varying from 15.3–28.5 cm. Sampling was conducted within the monsoon season, specifically from Jul to Sep of 2017. All procedures performed in fish were approved by the Institutional Animal Care and Use Committee of the College of Veterinary Medicine, University of the Philippines Los Baños (assigned protocol number: 2017-0036).

Organosomatic Indices and K-factor

Fish were immediately euthanized with an overdose of MS222 (300 mg/L). The body weight and length were measured, and the liver and spleen were dissected and weighed thereafter. The following formulas were then used for calculating HSI, SSI, and K-factor:

$$\text{HSI} = \frac{\text{weight of liver (g)}}{\text{body weight (g)}} \times 100 \quad (1)$$

$$\text{SSI} = \frac{\text{weight of spleen (g}}{\text{body weight (g)}} \times 100 \quad (2)$$

$$\text{K} = \frac{\text{body weight (g)}}{\text{body length (cm)}^3} \times 100 \quad (3)$$

Histopathology

Gills, liver, and spleen samples were fixed in 10% phosphate-buffered formalin and subjected to routine processing and embedding in paraffin. Transverse sections of 5-µm thickness were cut, mounted on slides, and stained with hematoxylin and eosin. The slides were examined under light microscopy. Lesions were identified and graded for severity using the scale set by the Organisation for Economic Co-operation and Development (OECD) (2010).

Statistical Analyses

Data on K-values and organosomatic indices were tested for normality using the Shapiro-Wilk test and homogeneity of variances using the Brown-Forsythe test. Data on HSI and SSI were transformed using square root and logarithmic transformation, respectively. Data were then analyzed by analysis of variance, followed by Tukey's test. Significance was set at $P < 0.05$. Statistical analyses were done using SAS version 9.4 (SAS Institute, Inc., Cary, NC, USA). Descriptive statistics was used to compare the prevalence of histopathological lesions in the gills, liver, and spleen of fish among lakes.

RESULTS AND DISCUSSION

K-factor and Organosomatic Indices

The K-factor in Sampaloc fish was significantly lower compared to fish in Calibato, Mohicap, and Yambo (Table 1). Nonetheless, the values were indicative of the robustness of the fish samples in the seven lakes since K-factor that is greater than one is suggestive of isometric growth and good health status (Anani and Nunoo 2016). It is interesting to note that the K-factor in Pandin and Yambo fish were comparable with the values obtained in fish from the other lakes. Although previously classified as oligotrophic, a recent study revealed mesotrophic conditions in the two lakes as indicated by chlorophyll α levels in the water (Mendoza *et al.* 2019). Cultural eutrophication could have influenced the K-factor of fish samples in these lakes.

K-factor is a reflection of the overall health and growth pattern of fish (Anani and Nunoo 2016), and can be affected by factors such as sex, stress, water quality, and food availability. It has been used as a basis for management programs of both cultured fish and fish captured from the wild (Asmamaw *et al*. 2019), with a low K-factor suggestive of diminishing energy reserves. Fish species adaptable to a wide range of environmental conditions such as the Nile tilapia can thrive in polluted and organic matter-rich zones as these increase their feeding sources and, consequently, their K-factor (Araújo *et al*. 2018).

Table 1. Mean ± SD of the K-factor, HSI, and SSI in adult males (*Oreochromis niloticus*) from the seven lakes of San Pablo City, Laguna, Philippines.

Lake	N	K (%)	HSI (%)	SSI (%)
Yambo	12	1.93 ± 0.18^{b}	1.54 ± 0.71^{b}	0.07 ± 0.05^{a}
Pandin	12	1.79 ± 0.19^{ab}	1.31 ± 0.58^{ab}	0.18 ± 0.38^{a}
Calibato	12	1.89 ± 0.17^{b}	1.56 ± 0.85^{b}	0.60 ± 0.23^{b}
Mohicap	12	1.90 ± 0.34^{b}	1.11 ± 0.52^{ab}	0.07 ± 0.05^{a}
Palakpakin	12	1.80 ± 0.20^{ab}	1.00 ± 0.40^{a}	0.13 ± 0.15^{a}
Bunot	12	1.74 ± 0.31^{ab}	1.88 ± 0.54^{b}	0.09 ± 0.04^{a}
Sampaloc	12	1.52 ± 0.16^{a}	0.88 ± 0.30^{a}	0.08 ± 0.12^{a}

Means with different superscripts in a column are significantly different at $p < 0.05$.

The data in this study were higher than the values obtained in *O. niloticus* from two areas in Lake Burullus in Egypt that receive agricultural and industrial effluent (K-factor: 1.45 and 1.48). This is in contrast with fish from the reference site (K-factor: 1.66) (Zaghloul *et al*. 2007) that was lower than those obtained in Yambo fish. Three sites in Taal Lake had Nile tilapia with K-factor of 1.94 (for non-aquaculture sites), and 4.39 and 1.97 (for aquaculture sites) (Bang-asan *et al*. 2016). The higher-than-normal K-factor in the study has been attributed to overfeeding. In another study, K-factor did not vary significantly in fish infected and noninfected with ectoparasitic protozoa (Ridanovic *et al*. 2015), which suggests the lack of influence of certain disease conditions on this parameter.

Similar values for HSI were obtained in most groups with the exception of fish from Sampaloc and Palakpakin Lakes that had lower HSI than the reference group (Table 1).

HSI was likewise decreased in cultured Nile tilapia from Taal Lake (Bang-asan *et al*. 2016) and in agriculture and industrial effluent-impacted Nile tilapia in Lake Burullus (Zaghloul *et al*. 2007). As HSI may be influenced by xenobiotic and parasite load in fish as well as food availability, the reduced values recorded in Sampaloc and Palakpakin could be an indication of low physiological status and energy reserves in fish (Asem-Hiablie *et al*. 2013). It could also be a reflection of lesser environmental quality in these lakes. The human population surrounding Sampaloc Lake was highest among the lakes at 18,500 in 2015 (Mendoza *et al*. 2019). On the other hand, the hypereutrophic Palakpakin Lake had the most turbid waters (Paller *et al*. 2017). It was also found to be polluted with heavy metals (Navarrete *et al.* 2019). The lack of sewage system in communities along the lake was also noted, particularly along its northern bank (Brillo 2016a) where fish in the present study was sampled. In other studies, the reduction of HSI in fish has been attributed to sublethal liver intoxication brought about by chronic exposure to chemical stressors such as sewage effluent (Ma *et al*. 2005) and pollutants (Araújo *et al*. 2018).

Fish from Calibato Lake had significantly higher SSI in comparison to the rest of the samples. Calibato Lake – together with Lakes Bunot, Palakpakin, and Sampaloc – not only have more extensive aquaculture operations than the other lakes but also a substantial number of establishments and settlements along its banks, which could have contributed to the degradation of water quality and excessive algal blooms (Brillo 2017). Increased SSI or enlargement of the spleen may point to a disease state as it is a pathologic response to combat stressors, destruction of blood cells (Akani and Daka 2015), and bacterial or parasitic infection (Dekić *et al*. 2016). Conversely, exposure to acute stress decreased SSI in the common dab (*Limanda limanda*) that was attributed to splenic contraction, causing erythrocyte release into the blood circulation (Pulsford *et al*. 1994).

Histopathological Features of Gills, Liver, and Spleen

The predominantly observed gill lesions were clubbing of secondary lamellae, thrombosis, and lamellar aneurysm (Table 2). Clubbing was found in 50% or more of the samples in each lake and had the highest occurrence in fish from Lakes Sampaloc (Figure 1) and Mohicap. In comparison, thrombosis and aneurysm had a lower prevalence. Thrombosis was seen in 50% or more of the samples from Lakes Pandin and Mohicap, whereas lamellar aneurysm was noted in about half of the fish population in Lakes Pandin and Sampaloc (Table 1). At least three histomorphological gill lesions were seen in fish from each lake except those in Lake Yambo, which only had two. The gill lesions were mainly of minimal severity (Table 2).

The gill lesions reported in this study have been observed in fish exposed to different types of contaminants and irritants (Liebel *et al*. 2013; Sharaf 2013; Yoon *et al.* 2015). Club shaping of the gill lamella is due to hyperplasia – a fish defense mechanism that aims to increase the distance between the external environment and the blood – thus serving as a barrier to inhibit the entrance of irritants, contaminants, or both (Sharaf 2013). Hyperplastic gill lesions are typically reversible when fish is exposed to better water quality (Poleksic and Mitrovic-Tutundzic 1994).

Table 2. Prevalence (%) and mean severity ( ) of observed histopathological lesions in the gills and spleen of the male Nile tilapia from the seven lakes of San Pablo City, Laguna, Philippines.

Lakes	Clubbing	Gill thrombosis	Aneurysm	Splenic MMC[a]
Pandin	66.67	66.67	58.33	100
	(1.16)	(0.75)	(0.5)	(1.67)
Calibato	50	50	16.67	100
	(1.08)	(0.58)	(0.17)	(1.50)
Mohicap	81.81	63.63	9.09	100
	(1.36)	(0.73)	(0.09)	(1.70)
Palakpakin	50	41.67	33.33	100
	(0.67)	(0.42)	(0.33)	(1.83)
Bunot	58.33	41.67	25.00	100
	(0.92)	(0.58)	(0.25)	(1.42)
Sampaloc	83.33	41.67	50	100
	(0.92)	(0.67)	(0.42)	(1.90)
Yambo	75	41.67	0.00	100
	(1.36)	(0.55)	(0.00)	(1.60)

[a]MMC – melanomacrophage center

Thrombosis and aneurysm are types of congestion that occur in different areas: the former occurs in the middle of the lamellae while the latter appears at the tip. Unlike gill epithelial hyperplasia, aneurysms are not as reversible and are indicative of exposure to severe stress (Hassaninezhad *et al.* 2014). Aneurysms in the gill lamellae have been observed in Nile tilapia exposed to treated sewage water and were attributed to injury and the corresponding loss of the support capacity of the pillar cells (Fernandes *et al*. 2008). Congestion in the primary lamellar epithelium, and aneurysms or lamellar telangiectasis may lead to extensive necrosis of the gill filaments (Khalil *et al*. 2015) and

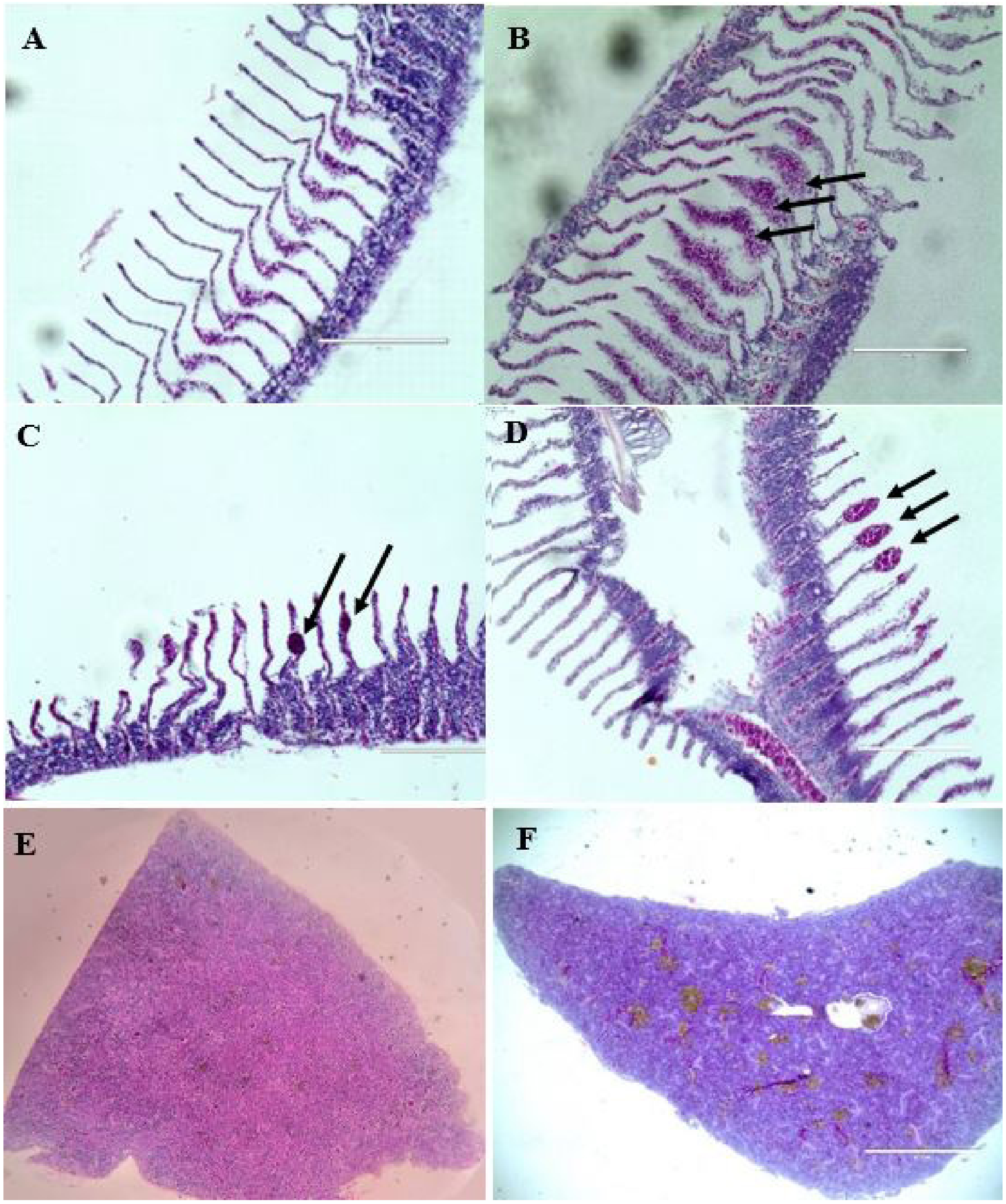

Figure 1. A) Fish gill from Yambo Lake. B) Clubbing or lamellar hyperplasia (arrows) in a fish gill from Sampaloc Lake; scale bar: 200 µm. C) Fish gill from Pandin Lake with thrombosis in the secondary lamellae (arrows); scale bar: 200 µm. D) Fish gill from Sampaloc Lake with aneurysms in the secondary lamellae (arrows); scale bar: 200 µm. (D) Fish spleen from Yambo Lake; magnification: 40x. (E) Brown-pigmented melanomacrophage centers in fish spleen from Sampaloc Lake; scale bar: 200 µm.

impede respiration (Strzyzewska *et al*. 2016).

MMCs were observed in all spleen samples (Figure 1) with a degree of severity that ranged from minimal to mild (Table 2). Other than MMCs, no other microscopic lesions were observed in this organ. The occurrence of splenic MMC has been attributed to the rupture of erythrocytes in the red pulp and eventual uptake of released pigments by macrophages (Tayel *et al*. 2013). An increase in the severity and presence of MMC may be due to the increase in the homeostatic mechanism of the spleen to phagocytose deposits of hemosiderin and other debris, such as melanin granules and lipofuscin residues (Agius and Roberts 2003; David and Kartheek 2015). Hyperactivation of MMC was also observed when Nile tilapia were exposed to water treated with copper sulfate and lead acetate (Osman *et al.* 2009).

The liver samples showed no significant lesions. This observation corresponded to comparable HSI between the reference group and other fish groups. Additionally, vacuoles in the liver parenchyma were within normal limits. Cultured fish have glycogen or neutral fat reserves which appear as extensive irregular vacuolation under the microscope (Mumford *et al.* 2007). In contrast, Nile tilapia cultured in Taal Lake showed liver degeneration as evidenced by the increase in MMC, fatty degeneration, low glycogen reserves, and nuclear alterations (Bang-asan *et al*. 2016).

CONCLUSION

Although the obtained values for K-factor were indicative of the good condition, the presence of microscopic lesions in the gills and spleen – albeit of minimal to mild severity – cannot discount the presence of stressors in the water. This suggests the limitation of using the K-factor as a sole measure of fish health in aquatic ecosystems. Further studies to elucidate the deviations in organosomatic indices of cultured fish from Lakes Sampaloc, Palakpakin, and Calibato from reference values are needed. Although the absence of aneurysms in the gills of fish in Yambo Lake reveals a better health status, the occurrence of other lesions in this organ suggests less than ideal aquaculture conditions in the lake. The biological responses in this study suggest a lower environmental status in the aquaculture areas of Lakes Calibato, Palakpakin, and Sampaloc. Lake Yambo, on the other hand, had the most optimal condition for maintaining the health of cultured fish. Future studies should consider greater sample size, seasonal variation, species differences, and the identification of lake pollutants and stressors that have caused the alterations.

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