JBS
Research Article

Journal of Biological Studies
eISSN: 2209-2560

V. 7, N. 2:31-42, 2024

https://doi.org/10.62400/jbs.v7i2.9125

Effect of pH and Dissolved Oxygen induced antioxidant activity in the liver of zebrafish, *Danio rerio* Hamilton, 1822

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Received 30 November 2023 | Accepted 01 March 2024 | Published 05 June 2024

Abstract

Two aquatic ambient factors, viz. Dissolved Oxygen (DO) and pH were selected for the study on the effect of oxidative stress in the liver of zebrafish. The zebrafish were exposed to four DO saturation levels (20-30%, 40-50%, 60-70% and 80% above) and five pH levels (4.5-5.5, 5.5-6.5, 6.5-7.5, 7.5-8.5 and 8.5-9.5) for different periods (DO, 4h-16h at 4hr interval; pH, 1h-4h at 1 hr interval). The antioxidants studied were catalase, glutathione and superoxide dismutase (SOD). The malondialdehyde (MDA) was assessed as a bioindicator of oxidative stress. Results showed that the fish suffered from oxidative stress in all DO saturation levels (except 80% and above, as control) and pH levels (except 6.5-7.5, as control). However, the highest generation of MDA was observed at 20-30% DO saturation level and 4.5-5.5 pH level. Under 20-30% DO saturation level, the highest oxidative stress level was observed at 12 h and pH 4.5-5.5 at 2 h of treatments. Accordingly, the antioxidant enzymes catalase, glutathione and SOD also showed low antioxidant activity at 20-30% DO saturation level and 4.5-5.5 pH level for treatment periods 12 h and 2 h, respectively. It was also evident from the study that the alkaline pH (8.5-9.5), although showed altered antioxidant activity, but not as high as pH 4.5-5.5, compared to the control.

Key words: Catalase, Glutathione, Super Oxide Dismutase, Oxidative stress, Zebrafish

1. Introduction

Fishes often face oxidative stress, especially due to environmental fluctuations that occur in the aquatic environment, which results mainly from abiotic stress generated by extremities in pH, salinity, temperature, dissolved oxygen (DO), acidity, pollution load, etc. (Bal and

Paital, 2023; Bal et al., 2024; Wu et al., 2018; Bernhardt et al., 2020; Song and Choi, 2021; Booth et al., 2023). Of these, oxygen and pH are two important aquatic ambience factors that often push an organism towards physiological stress conditions. A hypoxic situation is detrimental to the physiology of fish (Menon et al., 2023). Such detrimental effects appear through a reduction in growth rates, fish reproduction and development, and behaviour of fish which may lead to reduced abundance, reduction in immunity, reduced diversity and harvest of fish in aquatic environment (Bols et al., 2001; Breitburg, 2002; Wu, 2009).

In a study on zebrafish, Chowdhury et al. (2020) highlighted that acidic ambience impairs the growth of zebrafish under the experimental condition in the laboratory. In a recent article, Chowdhury and Saikia (2023) reported that oxidative stress resulted damage to mitochondrial biogenesis in zebrafish. So far, the oxidative stress is concerned, Chowdhury and Saikia (2022), in an extensive review, have suggested zebrafish as a potential animal model for studying oxidative stress.

The liver is a major organ often attacked by ROS (Sanchez-Valle et al., 2012). In mammals, when the ROS is excessive the homeostasis is disturbed, which results in oxidative stress and leads to liver diseases and other chronic and degenerative disorders (Li et al., 2014). Oxidative stress is regarded as one of the pathological mechanisms that result in the initiation and progression of various liver diseases, such as chronic viral hepatitis, alcoholic liver diseases and non-alcoholic steatohepatitis (Feng et al., 2011; Singal et al., 2011). With regard to hepatic stellate cells, the proliferation and collagen synthesis of hepatic stellate cells are triggered by lipid peroxidation caused by oxidative stress (Sakagachui et al., 2011; Cichoz-Lach and Michalak, 2014; Wu and Cederbaum, 2009). Several toxicological research is available where chemical toxicants have been reported to induce oxidative stress in fish liver (Farombi et al., 2007; Yadav et al., 2015). However, the effect of DO and pH-induced oxidative stress in fish liver has not been studied yet. In this study, the antioxidant responses of zebrafish were experimented against DO and pH-induced oxidative stress in the liver. The antioxidants evaluated are Superoxide dismutase (SOD), Catalase and Glutathione. Such knowledge is necessary, especially in zebrafish for its wider acceptability as an experimental model and also for the possibility as discussed in Chowdhury and Saikia (2022).

2. Materials and methods

Fish collection and maintenance

Zebrafish were collected locally from a commercial supplier in West Bengal, India. All fishes were stocked for one week in an aquarium in laboratory environment (pH 6.5-7.5, temperature 25-28 °C, DO 7-10 mg/l). Regular washing and cleaning of the aquarium was performed. Continuous aeration was provided to the stocked fish. Commercially available food (Tetra bits complete) was supplemented three times a day.

Stress exposure to zebrafish

After one week of acclimatization, adult zebrafish (weight: 0.7±0.5 g, total length: 3.8±0.2 cm,) in the aquarium were used for oxidative stress experiment. Two different experimental conditions were set in triplicate, viz. four levels of per cent DO saturation (viz. 20%-30%, 40%-50%, 60%-70% and 80% and above) and five levels of pH (4.5-5.5, 5.5-6.5, 6.5-7.5, 7.5-8.5, and 8.5-9.5) (Figure 1). In each experiment, a total of 30 zebrafish were used.

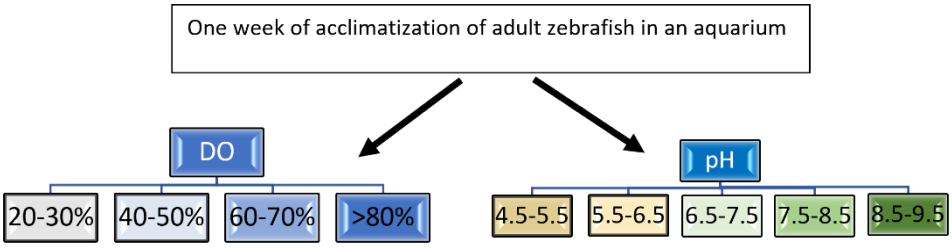

Figure 1. Flow diagram showing the experimental design with DO and pH. There were four different Dissolved Oxygen saturation (%) levels and five different pH levels tested.

The oxygen saturation was regulated by diffusing N_2 gas (25ml N_2 gas per sec) into the aquarium (Butler et al., 1994). The pH levels were measured by a digital pH meter (HI98107P) and were maintained by adding weak organic acid and strong base as independent variables (X) fixing in a pre-tested regression model [$Y = 7.675 - 0.008X$ where Y = pH and X = weak organic acetic acid (µl), $R^2 = 0.997$; $Y = 7.3667 - 0.005X$ (Y= pH and X= Volume NaOH (µl), $R^2 = 0.9774$]. A time-dependent observation on the survivability of zebrafish showed 16 hrs of treatment as the maximum tolerable level of hypoxic stress, beyond which more than 50% mortality of zebrafish occurred. Similarly, after 4 hours of pH treatment, more than 50% mortality was observed in the zebrafish population. It is, therefore, the maximum treatment time has been fixed at 16 hrs for different levels of DO saturation and 4 hrs for pH levels.

Tissue collection and processing

Pooled samples of liver tissue were kept in lysis buffer (RIPA buffer, and protease inhibitor) and then homogenized using microtissue homogenizer in ice (4 °C). The homogenized tissues were then centrifuged in 10000 g for 15 min and supernatant was collected for analysis.

Biochemical assays

The malondialdehyde (MDA) assay was performed according to the method of Aust (1985). For SOD, the antioxidant enzyme SOD assay was performed (Ewing and Janero, 1995), and catalase assay and reduced glutathione quantification were performed using microplate assay kits (G-Biosciences, ITAK1061 and ITAK1006).

Statistical analysis

Student's t-test, ANOVA or one-way MANOVA was performed (wherever applicable) to see any effect between or among the means of the treatments. Both these tests were computed after verifying the normality of the data. The Kolmogorov-Smirnov test of normality was used to test the normality of data. In case of all analyses, α level was fixed < 0.05. SPSS 16.0 was used for all statistical analyses.

3. Results

MDA analysis for each level of pH and DO

MDA within each group of treatments of DO show statistically significant differences (One-way MANOVA, $p>0.05$) (Figure 2A). The maximum mean values for MDA in case of DO saturation levels were exhibited by 20-30% DO saturation during 12hr treatment.

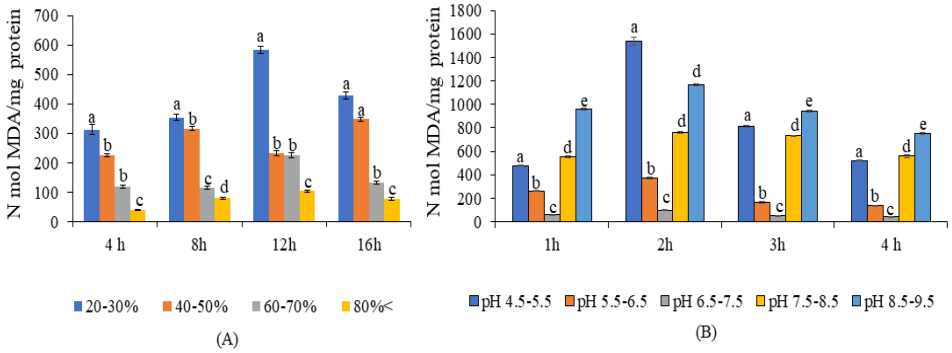

Figure 2. Bar graphs showing the generation of MDA in the liver tissue of zebrafish treated with (A) four DO saturation levels treated for four different periods and (B) five pH levels treated for four different periods. Graphs show mean ±SE. Means (± SE) were compared using One-way MANOVA at $p<0.05$. n=10 for each mean in the figure.

The MDA values of 20-30% DO saturation levels across treatment hours also showed a statistically significant difference (Table 1) and MDA values at 12 hr of treatment can be recorded as the highest MDA value for DO. Similarly, in case of pH, the 2hr of the treatment period for pH 4.5-5.5 exhibited the highest mean (Figure 2B). Further analysis showed this MDA value is statistically significant within the treatment group of pH 4.5-5.5 (Table 1).

Table 1. Comparison of the activity of oxidative parameters (MDA, Catalase, Glutathione and SOD) within the effective DO and pH levels. One-way ANOVA was performed.

Effective treatment	**OP**	**df**	**F**	***p***	***p* ***
20-30% DO	MDA	F (3, 39)	9.84	<0.05	4h,8h,16h
	Catalase	F (3, 39)	64.43	<0.05	4h, 8h
	Glutathione	F (3, 39)	150.61	<0.05	AD
	SOD	F (3, 39)	142.21	<0.05	AD
pH 4.5-5.5	MDA	F (3, 39)	270.93	>0.05	AD
	Catalase	F (3, 39)	2.61	>0.05	NS
	Glutathione	F (3, 39)	36.26	<0.05	1hr, 2hr
	SOD	F (3, 39)	37.89	<0.05	1 hr, 3hr

OP, Oxidative Parameter; NS, Not Significant; AD, All treatments are significantly Different. *Post Hoc Tukey (HSD) test results within treatments which have no statistically significant difference ($p>0.05$).

Effect of pH and DO on antioxidants

Catalase (CAT)

On analysis for each level of DO, reduced generation of catalase was found in all other DO saturation levels in liver tissues compared to the control (i.e. DO saturation 80% and above) (Figure 3A). Out of all the oxygen saturation levels, the 20-30% DO saturation was marked with a significant reduction of catalase than others (Figure 3A). A one-way MANOVA among the treatment periods of DO saturation of 20-30% confirmed its significant decrease in the liver tissues at 12 hr of treatment, compared to the control (Table 1). Within the group (12h) comparison also confirmed that the liver tissues showed minimum catalase level on exposure to the 20-30% DO saturation level. Although the 40-50% DO saturation also showed reduced catalase activity which is statistically insignificant with 20-30% DO saturation at this period, later on, it increased during the 4hr treatment period (Figure 3A). A comparison of catalase activity at 20-30% DO saturation levels was found very low compared to the control (t_9= 39.56, $p<0.05$, Figure 3C).

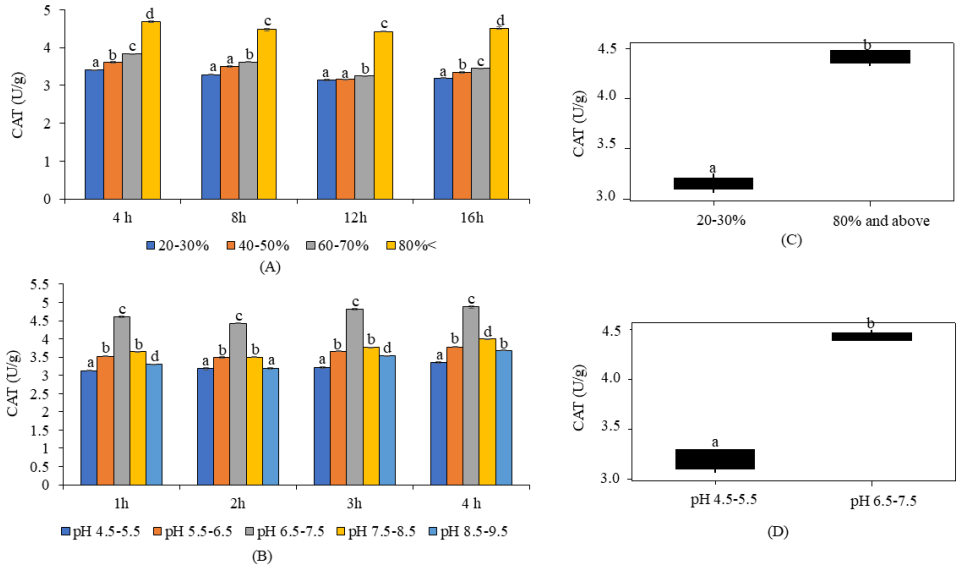

Figure 3. Bar graphs showing (A) Catalase activity for four DO saturation treatment levels across four treatment hours, (B) Catalase activity for five pH treatment levels across four treatment hours. Means (± SE) were compared using One-way MANOVA at $p<0.05$. On the right, boxplots showing Catalase activity of the liver compared with the control for (C) 20-30% DO saturation level at 12hr and (D) pH 4.5-5.5 at 2hr. Means (± SE) were compared using Student's t-test at $p<0.05$. Different lowercase alphabets indicate statistically significant differences at $p< 0.05$, n=10 for each mean in the figure.

In case of the effect of pH levels on catalase, the liver tissue resulted similar pattern as observed in case of DO for all pH treatments. The lowest level of generation of catalase was observed at pH 4.5-5.5 in all the treatment hours (Figure 3B). An analysis within the group (pH 4.5-5.5) showed that such reduction in catalase activity is not significantly different through the treatment hours (Table 1). Like DO, catalase activity at pH 4.5-5.5 at 2 hr treatment was compared with the control (a descriptive analysis was followed where catalase activity at pH 4.5-5.5 at 2 hr was low compared to others treatment periods) (t_9= 21.35, $p<0.05$, Figure 3D).

Glutathione

For DO, all the DO saturation levels of each treatment period showed reduced glutathione levels across the treatments (Figure 4A). Except for 16hr of treatments, in all treatment hours, the 20-30% and 40-50% DO saturation levels show reduced glutathione activity in the liver. However, when compared within the means of DO 20-30% saturation level, the glutathione activity exhibited statistically significant differences (Table 1). The lowest glutathione activity was recorded for 12hr of treatment in 20-30% DO saturation level. Its comparison with the control also maintained a statistically significant difference (t_9= 50.40, $p<0.05$, Figure 4C).

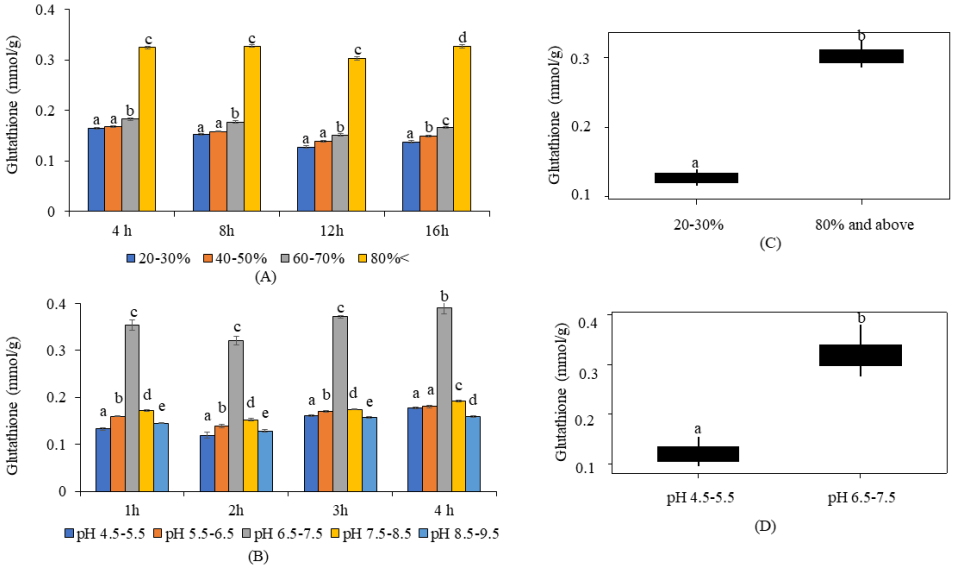

Figure 4. Bar graphs showing (A) Glutathione activity for four DO saturation treatment levels across four treatment hours, (B) Glutathione activity for five pH treatment levels across four treatment hours. Means (± SE) were compared using One-way MANOVA at $p<0.05$. On the right, boxplots showing glutathione activity of the liver compared with the control for (C) 20-30% DO saturation level at 12hr and (D) pH 4.5-5.5 at 2hr. Means (± SE) were compared using Student's t-test at $p<0.05$. Different lowercase alphabets indicate statistically significant differences at $p< 0.05$, n=10 for each mean in the figure.

For pH, within each treatment group and for a particular treatment period, the activity of glutathione was highly reduced at pH level 4.5-5.5 followed by the alkaline pH level 8.5-9.5 (Figure 4B). A comparison across the pH 4.5-5.5 levels for all the treatment periods showed the highest reduction of glutathione activity in the liver when the fish was treated for one or two hours (Table 1). On a descriptive scale, the two-hour of treatment exhibited a lower mean value of glutathione than the one-hour treatment. The glutathione activity at pH 4.5-5.5 during 2 hr of treatment is also significantly different from the control (t_9= 14.71, $p<0.05$, Figure 4D).

SOD

In case of DO treatments, the generation of SOD was found to be significantly low in all treatment groups and all treatment hours for each DO saturation level than the control (Figure 5A). It also clearly showed that out of all the DO saturation levels within a treatment period, the 20-30% DO saturation level showed a significant reduction in SOD level compared to the rest. Within the 20-30% DO saturation level across treatment hours, the lowest generation

of SOD was observed during the treatment for 12hr (Table 1). Accordingly, the SOD activity at 20-30% DO saturation level during 12 hr of treatment is significantly different from the control (t_9= 32.54, $p<0.05$, Figure 5C).

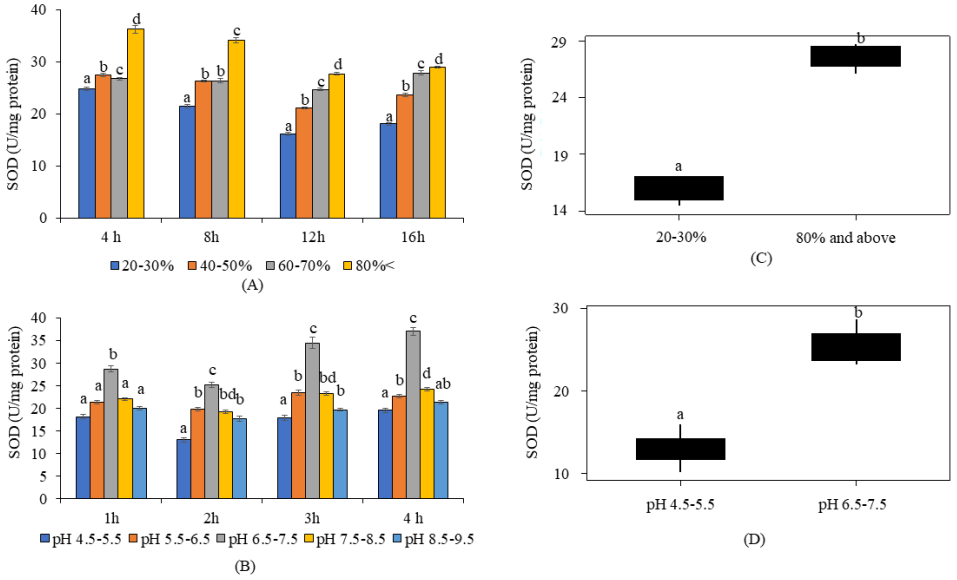

Figure 5. Bar graphs showing (A) SOD activity for four DO saturation treatment levels across four treatment hours, and (B) SOD activity for five pH treatment levels across four treatment hours. Means (± SE) were compared using One-way MANOVA at $p<0.05$. On the right, boxplots showing SOD activity of the liver compared with the control for (C) 20-30% DO saturation level at 12hr and (D) pH 4.5-5.5 at 2hr. Means (± SE) were compared using Student's t-test at $p<0.05$. Different lowercase alphabets indicate statistically significant differences at $p< 0.05$, n=10 for each mean in the figure.

In case of pH, generation of SOD was observed to be low in all treatment groups at pH level 4.5-5.5 (except for 1 hr treatment group, where differences among all DO saturation levels excluding the control are not statistically significant) (Figure 5B). When the generation of SOD at pH level 4.5-5.5 was compared across all the treatment hours, it was found to be significantly decreased at 2hr of treatment (Table 1). For alkaline pH level (pH 8.5-9.5), the generation of SOD was not as low as its generation observed at pH 4.5-5.5. The SOD activity at pH 4.5-5.5 during 2 hr of treatment is significantly different from the control (t_9= 15.33, $p<0.05$, Figure 5D).

Analysis among the treatment groups showed that the skeletal muscle was strongly affected when the fish was subjected to a hypoxic ambience (20-30% saturation) at 12 hours out of all the levels tested for different time durations. Its generation beyond 12 hr of treatment showed a gradual increase.

The treatments with different levels of pH clarified that the fish underwent stress at an acidic ambience (pH4.5-5.5) compared to the rest four pH levels tested. The maximum effect in terms of generation of SOD was observed at pH 4.5-5.5 for 2 hr of treatment. Its generation beyond 2 hr of treatment showed a gradual increase.

4. Discussion

The present study intends to confirm that if zebrafish is subjected to ambiences like different levels of acidic (pH 4.5-5.5, pH 5.5-6.5), alkaline pH (pH 7.5-8.5, pH 8.5-9.5) and DO saturations (20-30%, 40-50% and 60-70% saturation) at different time intervals (1hr, 2hr, 3hr and 4hr for pH; 16hr, 12hr, 8hr and 4hr for DO saturations), the liver tissue undergoes oxidative stress on time dependent manner. Two levels of stressors, concerning DO and pH, are clear from the present study, i.e., DO level 20-30% saturation and pH 4.5-5.5. The MDA results, compared to control are sufficient to establish such observation. Although MDA has been debated for its reliability to act as a bioindicator of oxidative stress (Khoubnasabjafari et al., 2015), its formation through a biochemical pathway under oxidative stress environment still emphasizes it as the first and primary biomarker of oxidative stress (Cordiano et al., 2023). The MDA results from this study indicated that the liver tissue has undergone oxidative stress during the treatments tested. Further, it has also provided direct evidence of higher oxidative stress in the liver of zebrafish during DO saturation level 20-30% and pH 4.5-5.5. Besides, the time at which the oxidative stress was highest in the liver is 12h for oxygen saturation and 2 hr for pH levels. In general, under adverse situations, fish suffer from DO-induced hypoxia at 1-2mg/L (≈12-24% DO saturation level at room temperature, https://www.waterontheweb.org/under/waterquality/dosatcalc.html) for a few hours in ponds (Abdel-Tawwab et al., 2019), but such kind suffering for only 12 hours may cause the oxidative stress-related health issues in fish. Samaras et al. (2023) studied the physiological adaptations through haematological, hormonal, biochemical and osmoregulatory alterations in blood and plasma on European sea bass, *Dicentrarchus labrax* and gilthead seabream, *Sparus aurata* for DO saturation of 40–60%, 60–80% and 80–100%. They found that these fishes face difficulty in growth performances towards the lower range of DO saturation level. However, in the present case, zebrafish could sustain up to a level as low as 20-30% DO saturation (1.65-2.48 mg/L at room temperature, https://www.waterontheweb.org/under/waterquality/dosatcalc.html), but with high oxidative stress in the liver. The MDA levels from the liver also depicted a similar tolerant ability of zebrafish, although, with 50% survivability. Earlier, Mukherjee et al. (2019) studied two freshwater carps *Labeo rohita* and *Cirrhinus cirrhosus* with different pH (5.5, 6, 7.5, and 8) against control (pH 6.8 ± 0.05). They have observed significant alteration in MDA levels, along with the other antioxidants towards pH 5.5. Zahangir et al. (2015) studied the effect of pH on zebrafish and recorded that it undergoes stress (monitored through secondary stress responses) at pH 5.5 towards the acidic side and pH 10.0 towards the alkaline side. Although an alkaline pH of 8.5-9.5 resulted in higher MDA in this study, the concern towards the acidic effect seems to be most significant since it appeared earlier to the alkaline effect. The alkaline effect on MDA was highest at 4hr of treatment. Indirectly, this is evident that in zebrafish, the oxidative stress in the liver can tolerate more alkaline ambience compared to the acidic ambience. In pacu juveniles (*Piaractus mesopotamicus*), alkaline water within pH 8.5 resulted no effect on the antioxidant and growth of the fish (Pllegrin et al., 2020). However, in the present case, the alkaline range of pH 8.5-95 generated oxidative stress as evident from MDA, Catalase, Glutathione and SOD in zebrafish liver, although milder than the acidic range. It could be good evidence for assessing the aquatic ambience quality of fish for management decisions.

It was also clear from the results that the levels of SOD, catalase and glutathione were significantly decreased from a baseline level (at control) for liver tissue at different time intervals treated with each level of pH and DO, other than control. Effect on ambience condition may variably exert oxidative pressure on different tissues of fish. For example, in gibel carp *Carassius gibelio*, the hypoxic situation affected the antioxidant generation in liver,

but not in gills, whereas in silver carp *Hypophthalmichthys molitrix* it affected both the tissues (Falfushynska and Sokolova, 2023). Similarly, in goldfish also, hypoxic conditions prominently affected catalase activity in the liver earlier to the brain and muscle (Lushchak et al., 2001). With such reference and relevance to the present study, the liver in fish may be considered as an early indicative tissue for oxidative stress against alterations in DO and pH in aquatic habitats.

In most cases, oxidative stress in the liver is studied in relation to the pollution caused by toxic substances in water. For example, metal accumulation in *Carassius auratus* (Qu, 2014), grey mullet *Mugil cephalus* (Padmini et al., 2008), catfish, *Clarias gariepinus* (Amin and Hashem, 2012), Common Carp, *Cyprinus Carpio* (Padmanabha et al., 2015) etc. The relevance of such studies relies on the functionality of the liver, which, being a metabolic organ has a significant role in the detoxification of pollutants through metabolic processes from the body. However, apart from that, the liver function itself compensates for any alterations in the aquatic factors, like those of pH and DO for prompt adaptation against the physiological changes. In their recent study, Chowdhury and Saikia (2023) observed severe mitochondrial damage in the liver of zebrafish following low acidic and hypoxic ambience in water. Such damages indirectly explain the possible challenge to the metabolic functions of the fish. Like MDA, the antioxidant levels studied here are low during 12 hr of treatment for a hypoxic environment and 2 hr for an acidic environment. Chowdhury and Saikia (2023) reported the highest mitochondrial damage during these hours in the liver of zebrafish.

Acknowledgments

We would like to thank the reviewers for their valuable comments and suggestions to improve paper quality

Conflict of interests

The authors declare that they have no competing interests.

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