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Effect of extreme high and low water pH on physico-chemical parameter and ALT, AST activity of *Labeo rohita* and its recovery

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Abstract
The chronic or sub-lethal toxicity test was performed on fish, *Labeo rohita* (weight 5.05±0.02g, length 7±0.03 cm) with thirteen different pH values such as 4.5, 5, 7 (Control), 9.5 and 10. Fish were fed pelleted feed with 35% crude protein. The alanine-amino-transferase (ALT) activities were lower at pH 4.5 and pH 10, and higher at pH 9.5. While Aspartate-amino-Transferase (AST) activities were lower at pH 4.5 and pH 5, and higher at pH 7 (control). The ALT activity recovered after 14 days recovery test in tap water (pH 8.2) of the fish group exposed to pH 9.5. While no AST activity recovery observed. Dissolved oxygen (DO) was highest and there was no ammonia-nitrogen (NH_3-N) at pH 7 (control). While the alkalinity contents in water were highest at basic pH 9.5 and pH 10.

Introduction

Labeo rohita (Hamilton, 1822) is a commercially important fish for aquaculture in India. It is a prevalent and commercially demandable fish in our country. Pollution from different industries and runoff fertilizer and other chemicals from agricultural land due to rainfall or floods can lead to pH changes in aquaculture ponds. Excessive inputs of supplementary feed, manures, and inorganic fertilizers can also change the pH level in aquaculture ponds. Changes in pH affect the *Labeo rohita* (Hamilton, 1822) fingerlings and lead to the modulation of biochemical stress. Therefore, the study aims to examine ALT, AST activity of Rohu (*Labeo rohita*) exposed to extreme lowering and rising water pH levels and fish recovery from adverse impacts. The present study was conducted to determine the effect of water pH on the ALT, AST activity and physico-chemical parameters of *Labeo rohita* under captivity.

Fish can experience severe oxidative damage-induced stress due to various ambient conditions in the aquatic system, such as dissolved oxygen (DO), pH, and ambient water temperature (Mukherjee *et al*., 2019) [26]. Alteration in the pH of the aquatic environment is an enormous problem worldwide, with severe consequences on tissue hypoxia and oxidative damage. Such alteration in pH often occurs in ponds, rivers, estuaries, or sometimes near-shore areas where water is generally regularly mixed with various anthropogenic and industrial wastes (Mukherjee *et al*., 2019) [26]. Exposure of aquatic animals to various abiotic stresses from either endogenous or exogenous sources enhances tissue hypoxia, which in turn disturbs respiratory physiology, cellular function, and sometimes the organism's survival (Mukherjee *et al*., 2017b) [27]. Earlier studies have revealed that acute exposure to acidic stress in the aquatic environment can even reduce the fertility of the fish, resulting in decreased species growth (Chowdhury *et al*., 2020) [4]. To survive in extreme conditions of pH change and associated oxidative damage, aquatic animals might have evolved a broad array of biochemical and physiological modifications. However, changes in physical factors affect the organism at the molecular level during the process of acclimatization and adaptation under stressful conditions (Mukherjee *et al*., 2017a, b) [28, 27]. In this scenario, researchers examined the brain tissue of two different carp to illuminate the role of critical neurological and antioxidative biomarkers during the adaptive response under acute pH

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change in the aquatic system (Mukherjee *et al*., 2019) [26]. Aquatic species' physiological and biochemical responses are influenced by pH fluctuations in their environment, and a significant pH shift can result in unexpected oxidative reactions in the aerobic metabolic process, which can lead to oxidative stress (Kim *et al*., 2021a, 2021b; Wang *et al*., 2009) [12, 17-18, 35].

In aquatic organisms, excessive reactive oxygen species (ROS) formation brought on by pH variations damages DNA, proteins, and lipids, resulting in toxicity that can lead to the onset of inherited disorders (Kim *et al*., 2019b; Wang *et al*., 2002) [36]. Fish physiological responses are influenced by various aquatic environmental conditions, including pH, temperature, salinity, and photoperiod (Kim *et al*., 2018a, 2018b, 2019a) [16, 14, 15].

Materials and Methods
An experiment was conducted for 30 days using 500 L FRP tank containing 300 L water with five sub-lethal pH values (pH 4.5, 5.0, 7.0, 9.5 and 10.0) including one control (pH 7.0) and fifty fingerlings of *Labeo rohita* to evaluate the impacts of extreme low and high pH in water on the other water quality parameters and biochemical stress enzymes of the fishes. The pH of each experimental tank was maintained using 0.5 N HCl and 1.0 N NaOH and measured through a pH meter (EU-Tech). The fingerlings were fed commercial fish feed having 30% protein and 3% fat twice a day (at 10.00 am in the morning and at 4.00 pm in the afternoon) @ 4% of total body weight. The 20% of water was exchanged every seven days interval maintaining the desirable pH in the water using 0.5 N HCl or 1.0 N NaOH solution. Aeration was provided to all the tanks round the clock with the help of an aerator to maintain dissolved oxygen contents. Water samples were collected weekly for the estimation of water quality parameters and fish samples were collected randomly at the end of the experiment (after 30 days) to analyse Aspartate-amino-Transferase (AST) and Alanine-amino-Transferase (ALT) activities. A recovery test was conducted through the transfer of affected fingerlings of *L. rohita* due to extreme low and high-water pH to freshwater (pH 8.2) for recovery for14 days. Important water quality parameters (temperature, dissolved oxygen, free carbon dioxide, alkalinity and ammonia-nitrogen) were estimated weekly following the standard methods (APHA, 2005) [1]. For the estimation of AST and ALT, the sampled fishes were weighed and homogenized the whole body in a chilled 0.25 M sucrose solution to prepare 5% (1:19 w/v) homogenate. The tube was kept in ice to avoid enzyme reactions. The homogenate was centrifuged at 12000 × g for 10 minutes at 40 °C to obtain the supernatant that was used for enzyme analysis. All the supernatants were stored at -20 °C until further analysis. Aspartate-amino-Transferase (AST) activity of samples was assayed as described by Wooton (1964). The substrate was prepared using 0.2 M DL- Aspartic acid and 2.0 mM Ketoglutarate in 0.05 M phosphate buffer (pH 7:4). In 0.05 mL fish sample 0.25 mL of substrate was added. The assay mixture was incubated at 37 °C for 60 minutes. The reaction was terminated by adding 0.25 mL of 1 mM 2,4-dinitrophenylhydrazine (DNPH). In the control the enzyme was added after DNPH (2,4-dinitrophenylhydrazine) solution. The samples were held at room temperature for 20 minutes with occasional shaking. Then 2.5 mL of 0.4 M NaOH solution was added to the sample and the contents were thoroughly mixed. After

10 minutes, the optical density (OD) was recorded at 240 nm against blank. The Alanine-amino-Transferase (ALT) activity was estimated following the methods of the estimation of AST activity except the substrate comprised of 0.2 MD L alanine instead of 0.2 M DL- Aspartic acid (Prusty *et al.,* 2007) [31]. The AST activity and the ALT activity were expressed as μkat/Lat 37 °C.

Statistical analysis
Data obtained were analysed using the statistical software SPSS 22.0 computer program (IBM statistic 22.0). Differences in mean value of the parameters of the test concentrations and controls were subjected to one-way analysis of variance (ANOVA) followed by Duncan's Multiple Range Test to determine level of significance at 5% probability level. Results were expressed as mean±standard deviation.

Results
Water quality parameters
An investigation was conducted on the chronic impact of extreme low (pH 4.5 and pH 5) and high (pH 9.5 and pH 10) exposures to the fingerlings of *Labeo rohita* for 28 days. The data of different water quality parameters of this study was recorded every week. It was found that there were no significant variations ($p>0.05$) of temperature in water at pH 4.5, pH 5, pH 7, pH 9.5, and pH levels during the 7th, 14th, 21st, and 28th day of the experiment (Fig.- 1). The highest dissolved oxygen (6.24±0.03 mg/L to 7.75±0.02 mg/L) was observed at pH 7.0 (control) during all exposure days. The lowest DO was recorded at pH 4.5 (4.73±0.04 mg/L) and at pH 10.0 (5.25±0.03 mg/L) during 7th, 14th, 21st and 28th day of exposures (Fig.- 2). At pH 5.0 the DO contents decreased up to 14th day of exposure (5.85±0.03 mg/L to 5.94±0.03 mg/L). Thereafter, it was increased from 7.14±0.02 mg/L to 7.24±0.03 mg/L. The dissolved oxygen at pH 9.5 was also gradually increased from 5.25±0.03 mg/L to 6.64±0.03 mg/L during 7th to 28th days of exposure. The free CO_2 in water was absent at pH 7.0. The highest free CO_2 4.00±1.00 mg/L and 3.00±1.00 mg/L was found in the pH 4.5 and pH 5 respectively on the 7th day of exposure followed by 21st day (3.33±1.52 mg/L and 3.00±1.00 mg/L respectively). Free CO_2 (0.16±0.28 mg/L) in water at pH 9.5 and pH 10 (0.33±0.57 mg/L) were same on the 14th to 28th day of experiment (Fig.-3). Similarly, free CO_2 in water at pH 10 (0.33±0.57 mg/L) was same on the 14th to 28th day of experiment. There were significant differences ($p<0.05$) in the free CO_2 at pH 4.5 and pH 5.0 with free CO_2 at pH 9.5 and pH 10.0 throughout the experiment. The highest free CO_2 were recorded on the 7th day of exposure at all pH levels (pH 4.5, pH 5.0, pH 9.5 and pH10) except at pH 7.0. The highest alkalinity was found at pH 9.5 (from 240±6.42 mg/L to 245±2.08as $CaCO_3$) and at pH 10.0 (from 242±6.55 to 247±6.65 mg/L as $CaCO_3$). While the lowest alkalinity was found at pH 4.5 (141±4.16to 155±3.05 mg/L as $CaCO_3$). There were no significant differences ($p>0.05$) in alkalinity between pH 9.5 and pH 10.0 and in between pH 5.0 and pH 7.0 during entire period of study. There were significant differences ($p<0.05$) in alkalinity at pH 4.5 from other pH levels. There were also significant variations ($p<0.05$) in the alkalinity at pH 5.0 and pH 7.0 from other pH levels. Similarly, there were significant differences ($p<0.05$) in alkalinity at pH 9.5 and pH 10.0 from other pH levels (Fig.-4). The highest Ammonia-nitrogen (NH_3-N) in

water was found at pH 9.5 and pH 10 (0.05±0.01 mg/Lto 0.08±0.02 mg/L) during all sampling days (i.e., 7th, 14th, 21st, and 28th day) throughout the experiment (Fig.-5). Ammonia-nitrogen in water was not detected at pH 7.0 throughout the experiment. There were no significant differences (p>0.05) of NH$_3$-N in water between pH 9.5 and pH 10.0, and between pH 4.5 and pH throughout the experiment. While it was varied significantly (p<0.05) at pH 4.5 and pH 5.0 with pH 9.5 and pH 10.0.An investigation was conducted on the chronic impact of extreme pH exposure (both low and high) to the fingerlings of *Labeo rohita* for 28 days. In the present investigation, the highest hardness, from 602±2.64 mg/L to 625±2.08, was found at pH 10 (Table 6). In comparison, the lowest hardness (466±2 mg/L to 484±3.78 mg/L) was found at pH 4.5. There were also significant variations (p<0.05) in the hardness of all pH levels from each other during 28 days of the experiment.

Alanine-amino-Transferase (ALT) and Aspartate-amino-Transferase Activities

In this experiment, impacts of extreme low and high pH in water on the Alanine-amino-Transferase (ALT) and Aspartate-amino-Transferase activities in the whole-body mass of rohu fingerlings were observed. The highest Alanine-amino-Transferase activity (0.74±0.04 μkat/L) in the whole body of rohu fish was found at pH 9.5 in water on the 28th day of the experiment. The lowest ALT activities in the whole body of rohu fingerlings were found at pH 4.5 (0.26±0.03 μkat/L) and pH 10.0 (0.27±0.02 μkat/L) in water after 28th days of exposure (Fig.- 7). The ALT activities in the whole body of rohu fingerlings (*L. rohita*) at pH 4.5 (0.35±0.04 μkat/L) and pH 10.0 (0.38±0.03 μkat/L) in water were significantly lower than the ALT activity at pH 7.0 in water (0.56±0.04 μkat/L) on the 14th day of exposure. However, it was slightly higher at pH 5.0 (0.63±0.03 μkat/L) and pH 9.5 (0.62±0.02 μkat/L) in water than at pH 7.0 (0.56±0.04 μkat/L) in water on the 14th day of exposure. Similarly, the ALT activities in the whole body of rohu fingerlings at pH 4.5 (0.26±0.03 μkat/L) and pH 10.0 (0.27±0.02 μkat/L) in water were significantly lower than the ALT activity at pH 7.0 (0.52±0.05 μkat/L) in water after 28th days of exposure. However, it was slightly higher at pH 5.0 (0.54±0.03 μkat/L) and pH 9.5 (0.74±0.04 μkat/L) in water than the ALT activity at pH 7.0 (0.52±0.05 μkat/L) after 28th days of exposure. The ALT activity in the whole body of *Labeo rohita* at pH 4.5 and pH 10.0 in water were not significantly (p>0.05) varied on the 14th and 28th days of exposures. The ALT activity in *Labeo rohita* at pH 5.0 and pH 7.0 in water were not significantly (p>0.05) varied in the 14th and 28th days exposures as capered to initial values. At the 28th day of exposure, the ALT activity in the whole body of *L. rohita* was significantly varied (p<0.05) between pH 9.5 and other pH exposures in water.

The Aspartate-amino-Transferase (AST) activity in the whole body of *L. rohita* exposed to extreme low and high pH regime in water for 28 days. The findings of the present study indicated that the AST activities in the whole body of the fingerlings were not significantly varied (p>0.05) in between pH 5.0, pH 7.0 (control) and pH 9.5 in water on the 14th and 28th days of the exposures. However, it was significantly decreased (<0.05) in the whole body of fingerlings at pH 4.5 and pH 10.0, but it was not significantly varied (p>0.05) between pH 4.5 and pH 10.0 in water on 14th and 28th days of exposures (Fig.-8). The

highest AST activity in the whole body of the fingerlings was recoded at pH 5.0 (3.24±0.03 μkat/L) in water on the 14th day of exposure and at pH 7.0 (3.24±0.04 μkat/L) in water on the 28th day of the study as compare to initial values. The lowest value of AST activity in the whole body of *L. rohita* was found at pH 4.5 (1.94±0.02 μkat/L) and pH 10 (1.96±0.03 μkat/L) in water on the 28th day of exposure (Fig.- 8). There was no significant difference (p>0.05) of AST activity in the whole body of rohu fingerlings between pH 4.5 and 10.0 in water on the 14th and 28th day of exposure.

Recovery of ALT and AST Activity in the whole body of *Labeo rohita* after 14 days

A recovery test was conducted to assess the impairment of ALT and AST activities in the whole body of *L. rohita* fingerlings effected due to exposure at extreme low and high pH in water after 28th day of exposure. The effected fingerlings were exposed to tap water (pH 8.2) for the 14 days. In the control group at pH 7.0, the average range of ALT activity in whole body of rohu fingerlings decreased to 0.51±0.01 μkat/L after 14 days recovery periods. The ALT activities in whole body of rohu fingerlings were decreased to 0.32±0.01 μkat/L, 0.5±0.03 μkat/L, 0.51±0.01 μkat/L, 0.53±0.02 μkat/L, 0.33±0.01 μkat/L in respect to the ALT activities at pH 4.5, pH 5.0, pH 7.0, pH 9.5, pH 10.0 in water respectively (Fig.-9). There was no significant difference (p>0.05) in ALT activity in the whole body of rohu fingerlings at pH 4.5 and 10.0 exposure in water after 14 days recovery test. The ALT activities in the whole body of rohu at pH 5.0, pH 7.0, and pH 9.5 were also not significantly different (p>0.05) from each other after 14 days recovery test. While the ALT activities at pH 4.0 and pH 10.0 were significantly differences (p<0.05) from other pH exposures after 14 days recovery test. The ALT activities in the whole body of rohu fingerlings under extreme low and high pH exposures in water were slightly decreased during 14 days recovery test in tap water (pH 8.2), when pH stresses were removed. Therefore, the 14 days was not sufficient period to remove the stresses due to extreme low and high pH in water.

The AST activities in the whole body of rohu fish after 14 days recovery test in tap water (pH 8.2) were decreased when from the AST activities observed under the exposure at pH 4.5, pH 5.0, pH 7.0, pH 9.5, pH 10.0 in water (Fig.-10). The recovered AST activities in the whole body of rohu fish were 1.76±0.03 μkat/L, 3.16±0.06 μkat/L, 3.25±0.03 μkat/L, 3.22±0.03 μkat/L, 1.79±0.03 μkat/L at pH 4.5, pH 5.0, pH 7.0, pH 9.5, pH 10.0 in water respectively. There was no significant difference (p>0.05) in AST activity in the whole body of rohu fingerlings at pH 4.5 and 10.0 exposure in water after 14 days recovery test. The AST activity in the whole body of rohu fingerlings at pH 5, pH 7, and pH 9.5 were also not significantly varied (p>0.05) from each other after 14 days recovery test. While the AST activities at pH 4 and pH 10 were significantly varied (p<0.05) from other pH exposures after 14 days recovery test. The AST activities in the whole body of rohu fish under extreme low and high pH exposures in water were slightly decreased during 14 days recovery test in tap water (pH 8.2), when pH stresses were removed. Therefore, the 14 days was not sufficient period to recover the stresses due to extreme low and high pH in water.

Discussion

Several studies revealed the influence of water pH 4.8–5.6 on aquaculture fish in a wide range of hardness variations. Water hardness is essential in fish tolerance to acidification (Nelson, 1982; McWilliams, 1982; McDonald, 1983) [29, 24, 25]. The findings of this present study corroborate with the study of Sahu *et al.* (2018) [33], which stated that the effect of pH significantly affected the water quality of *Trichogaster allies* exposed to different pH treatments. Townsend and Baldisserotto (2001) [34] observed fingerling mortality only in acidic waters with pH values less than 4.0 and hardness levels of 30 mg/L as $CaCO_3$. Similar observations were made in white sucker (*Catostomus commersoni*) and black skirt tetra (Gonzalez & Dunson, 1987) [10]. The increase in hardness levels from 30 to 70 mg/L was enough to reduce the mortality of silver catfish at pH 3.75. McDonald *et al.* (1980) [23] found increased hardness levels (by adding Ca++) from 6.0 to 54.0 mg/L as $CaCO_3$ also decreased ion loss and mortality of rainbow trout exposed to pH 4.0 – 4.5 from 50 to 11%. The relationship between water pH and hardness influences fish physiology, where increasing water hardness could trigger a protective effect for fish exposed to acidic or alkaline pH (Copatti *et al.*, 2019) [5]. Makori *et al.* (2017) [21] reported that increased conductivity, pH, and ammonia decreased fish (*Oreochromis niloticus*) growth. Rainbow trout exposed to alkaline pH in soft water lose ions, which is the primary cause of death. An increase in water hardness allows rainbow trout to maintain plasma ion concentrations at alkaline pH, which could be the exact mechanism involved in improving the survival of silver catfish fingerlings exposed to alkaline pH at higher hardness levels (Yesaki and Iwama, 1992). Boyd (1998) [3] suggests that the desirable range for hardness is 20–150 mg/L as $CaCO_3$. In Southern Brazil, surface waters exhibit pH values from 3.0 to 8.4, and water hardness is usually around 20–50 mg/L as CaCO3. However, several fish farmers use underground water whose pH ranges from 4.0 to 9.5. According to Wood (2001) [37], at highly alkaline pHs such as 10.0 and 10.5, it is necessary to increase water hardness to 300 mg/L as CaCO3 to improve survival. Fromm (1980) [8] reported that an increase in CO_2 concentration increases the adverse effects of water acidification.

Plasma AST and ALT are considered sensitive and reliable indicators for evaluating liver function and damage in fish under stress conditions (Kim and Kang, 2016) [13]. AST and ALT bind with toxicants and are released into the blood circulation. Therefore, a critical increase in the levels of these enzymes represents a stress status in aquatic animals (Malarvizhi *et al.*, 2012; Kim *et al.*, 2020) [22, 11]. Many studies suggests that an elevation of these aminotransferases may be related to hepatic hyperactivity and liver damage (Barcellos *et al.*, 2003; El-Sayed *et al.*, 2007; Lemos *et al.*, 2018) [2, 7, 19]. Whereas, in the present study, the plasma AST and ALT levels of *L. rohita* were significantly reduced at the highly acidic (pH 4.5) and alkaline (pH 10) reared fishes, which is thought to be a functional loss owing to aextreme pH above the threshold that caused by liver damage (Kim *et al.*, 2021) []. Similarly, Malarvizhi *et al.* (2012) [22] reported that the AST and ALT values were inhibited in *C. carpio* subjected to sub lethal drug toxicity indicating a disturbance in cell structure and organelles. In similar, at the extreme acidic (pH 4.5) and alkaline (pH 10) conditions significantly very low plasma AST and ALT were recorded in this study. Meanwhile the fishes reared at circumneutral pH have not shown any difference in the plasma AST and ALT levels. These values were slightly improved at the recovery phase this might be due to the acclimation of fish to the varied pH conditions.
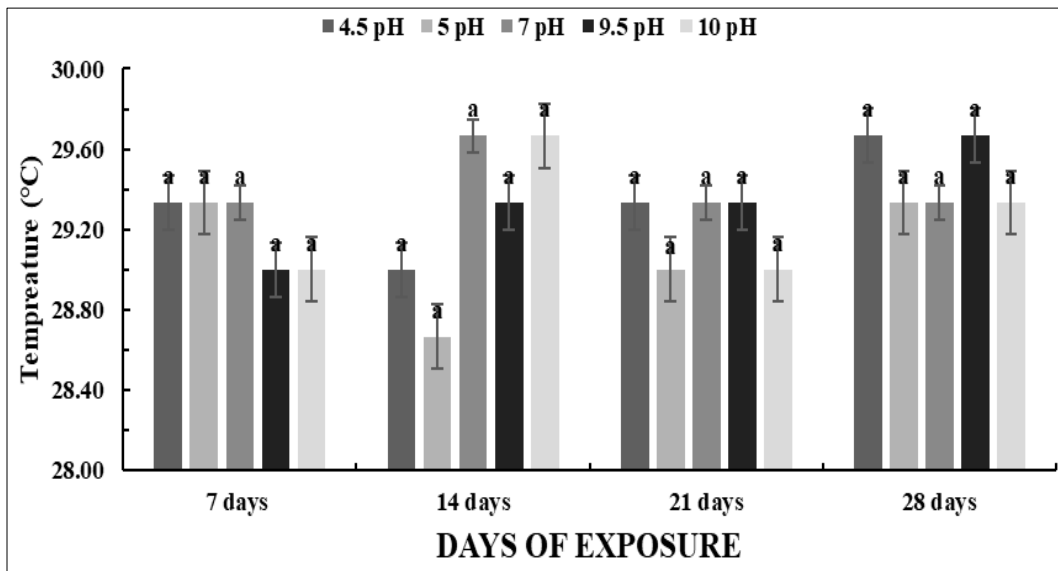

Fig 1: Temperature (°C) in water at different days of extreme low and high pH exposuresto the fingerlings of *L. rohita*(same letter indicates no significant variation)
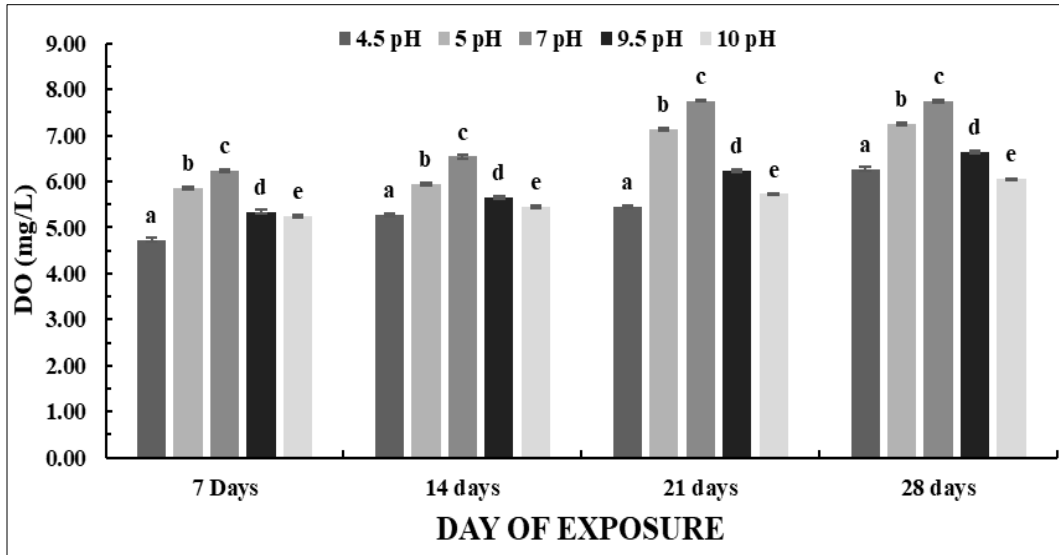
Fig 2: Dissolved Oxygen (DO mg/L) in water at different days of extreme low and high pH exposures to the fingerlings of *L. rohita*(same letter indicates no significant variation)
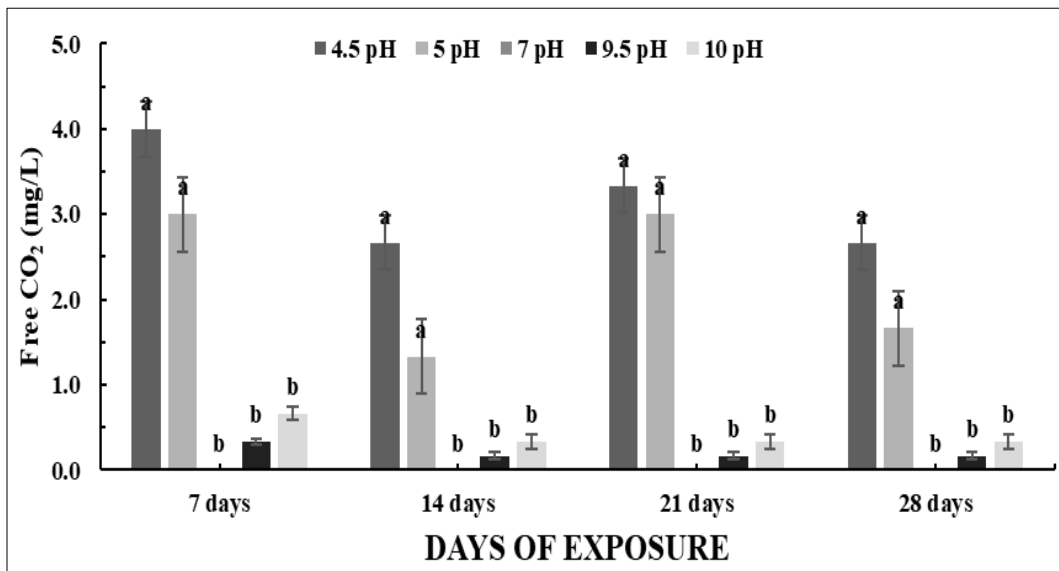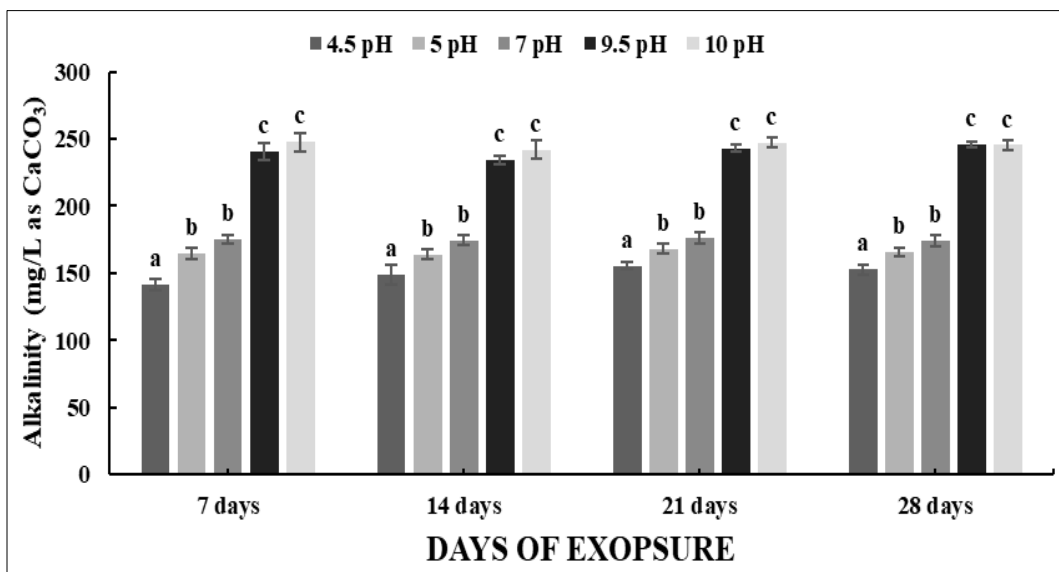

Fig 3: Free CO_2 (mg/L) in water at different days of extreme low and high pH exposures to the fingerlings of *L. rohita*(same letter indicates no significant variation)

Fig 4: Alkalinity (mg/L as $CaCO_3$) in water at different days of extreme low and high pH exposures to the fingerlings of *L. rohita*(same letter indicates no significant variation)
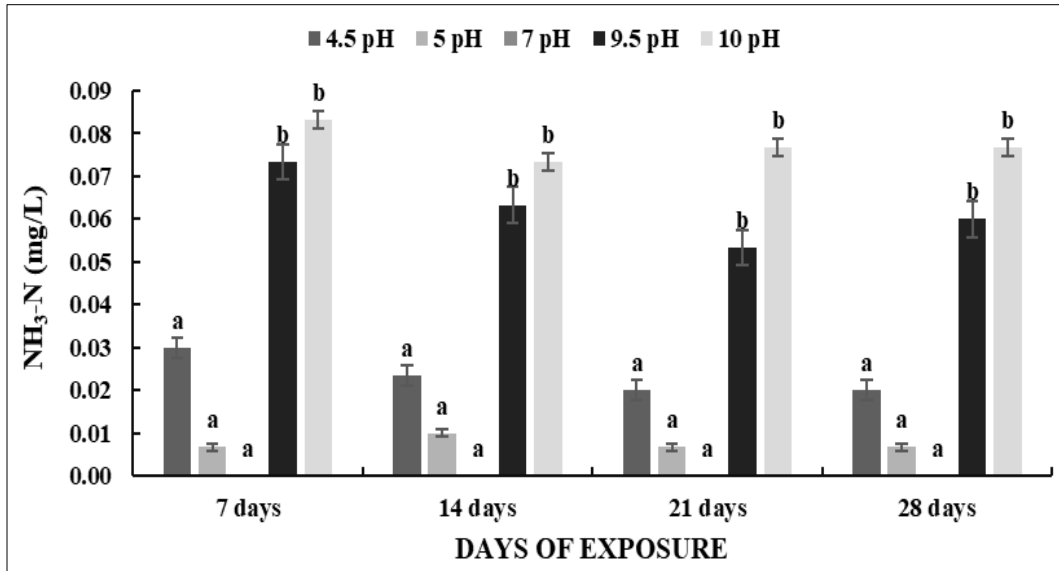
Fig 5: Ammonia-Nitrogen (NH_3-N mg/L) in water at different days of extreme low and high pH exposures to the fingerlings of *L. rohita* (same letter indicates no significant variation)
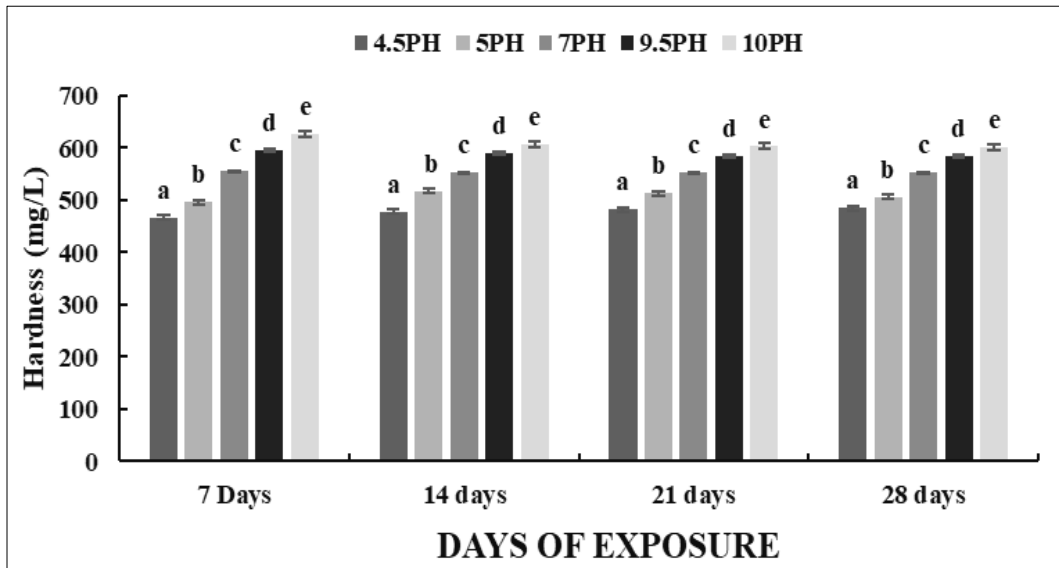

Fig 6: Hardness in water at extreme low and high pH levels during 28 days of exposure to *L. rohita* (Mean±SD, n = 3, same letter indicates no significant variations i.e., $p>0.05$)
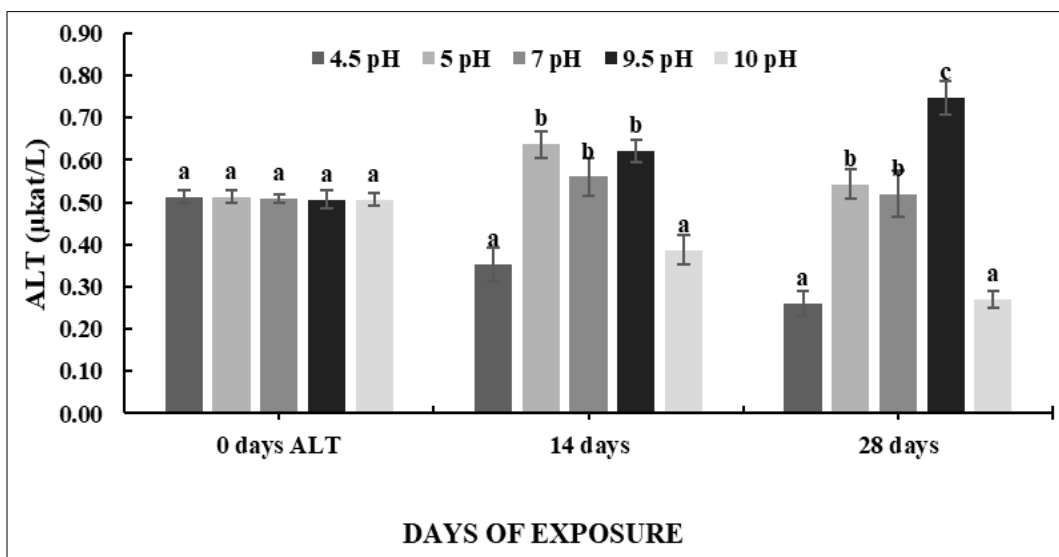

Fig 7: Alanine-amino-Transferase (ALT) activity (μkat/L) in the whole body of *L. rohita* exposed to extreme low and high pH in water at 14th day and 28th day of exposure (Mean±SD, n=3, same letter indicates no significant variations i.e., $p>0.05$).

Fig 8: Aspartate-amino-Transferase (AST) activity (μkat/L) in the whole body of *L. rohita* exposed to extreme low and high pH in water at 14th day and 28th day of exposure (Mean±SD, n = 3, same letter indicates no significant variations i.e., $p>0.05$).
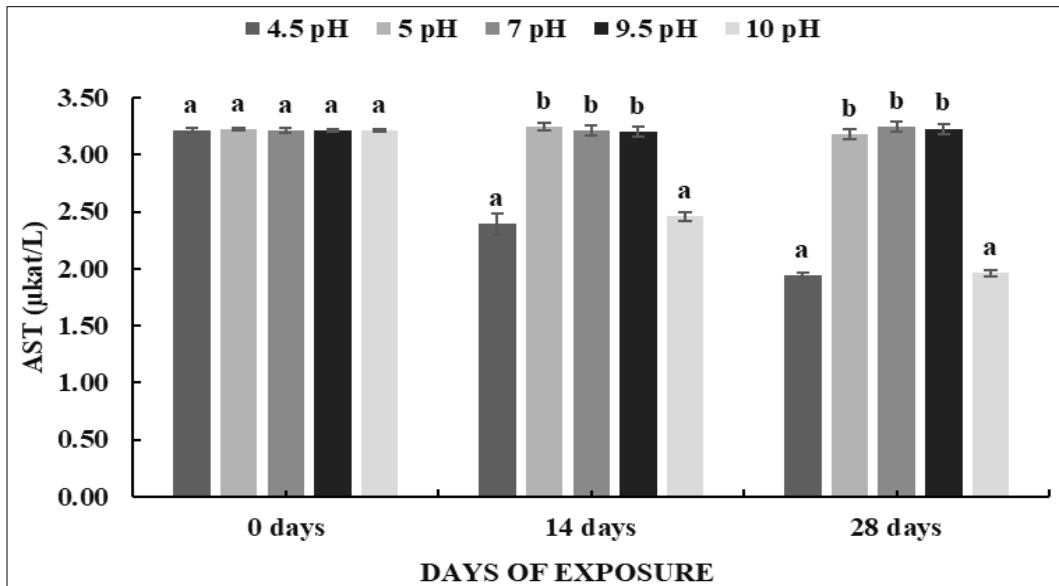

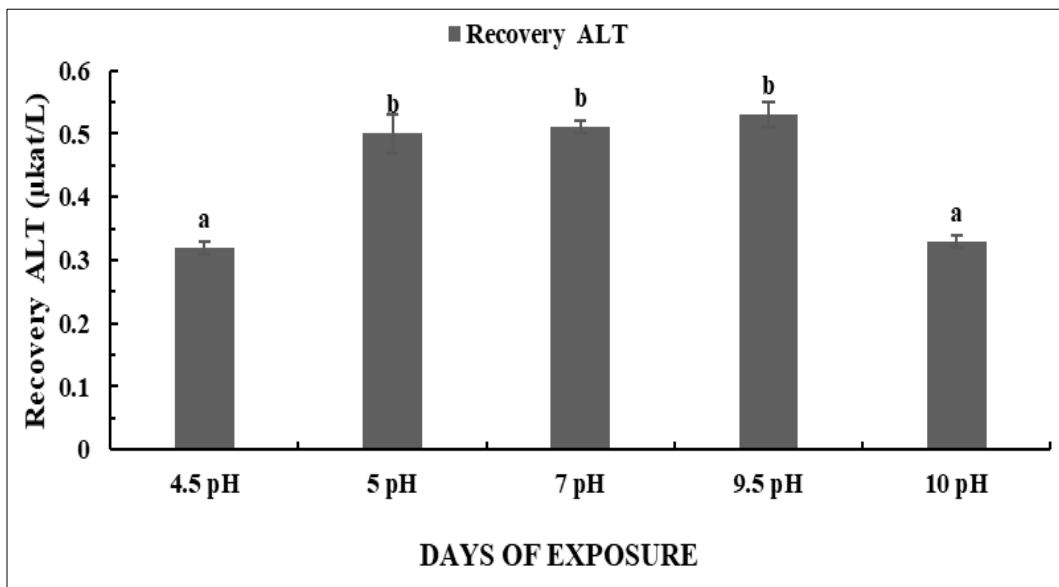
Fig 9: Recovery of ALT activity (μkat/L) in the whole body of *L. rohita*after14 days exposure to tap water (pH 8.2)
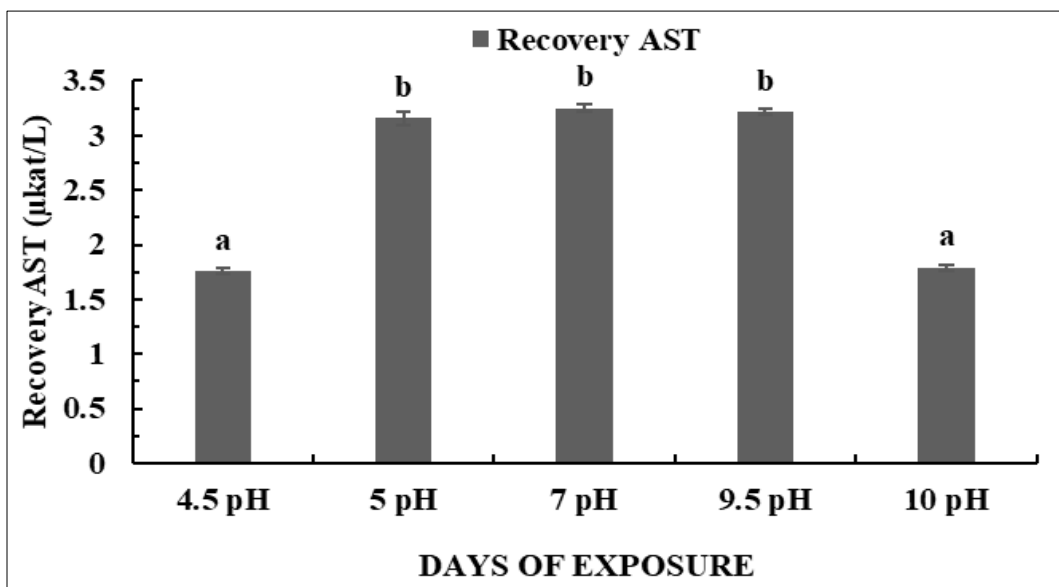

Fig 10: Recovery of AST activity (μkat/L) in the whole body of *L. rohita*after14 days exposure to tap water (pH 8.2)

Conclusion

In the present investigations involving rohu fish (*Labeo rohita*) revealed critical pH levels. Extreme pH levels (both low and high) had adverse effects on fish health. Chronic exposure to extreme pH conditions led to growth retardation and alteration in biochemical stress enzymes (ALT, AST, and CAT) responses. In aquaculture, maintaining optimal pH levels is crucial for fish growth and production. Careful monitoring and adjustments are necessary to achieve successful aquaculture outcomes. The conclusion of current study showed that the pH changes significantly affect the ALT, AST activity and physico-chemical parameters. The biochemical response such as ALT and AST influenced by pH stress conditions. Considering the overall results of this study, the water with extreme acidic (< pH 4) and extreme alkaline (> pH 10) pHs caused pH stress and influenced physiological indicators such as biochemical responses.

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