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Impact of Temperature on the Growth Performance, Survival Rate, Osmoregulation, Dissolved Oxygen and Physiological Changes of Freshwater Tilapia (*Oreochromis mossambicus*)

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Authors' contributions

This work was carried out in collaboration among all authors. Authors RVK, SB and VU performed material preparation, research work, data analysis, interpretation, and manuscript writing. Authors CAB and PC participated in the manuscript writing, suggestions and critical reviews. All authors read and approved the final manuscript.

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ABSTRACT

For many aquatic species, any changes in water temperature affect their survival. This study was performed to investigate the effect of rising temperature on the growth parameters, protein content, dissolved oxygen content, moisture content osmoregulation, and physiological activity of freshwater Tilapia (*Oreochromis mossambicus*). The experiments were set in plastic fish tanks, the experimental tank placed in direct sunlight and the control tank in shade, and measuring temperature and pH daily, with proper aeration and feed was provided. The study found that increasing water temperature led to higher mortality rates and low survivability percentages in fish, with a higher growth rate in the experimental group with a pH range of 5.7 to 8.2. High dissolved oxygen levels were recorded during the experimental period, with a mean difference of 26.56 compared to the control group, possibly causing restless movement in fish. The study revealed that the alkalinity of water decreases with increasing temperature. The study found that increased salinity led to a significant increase in growth performance, indicating an increase in metabolic rate, and a decrease in alkaline nature. The difference between the mean protein value in the experimental and control groups is 0.896mg/ml. Thermal limitations may negatively impact *O. mossambicus* physiological responses, impacting aquaculture production due to predicted global climate change-induced water temperature changes.

Keywords: Tilapia; Oreochromis mossambicus; water temperature; dissolved oxygen; survival rate; osmoregulation; protein content.

1. INTRODUCTION

Since aquaculture generates more income, jobs, and food necessities, it has a major impact on the socioeconomic conditions of many nations. This industry is susceptible to environmental challenges, though. For example, one of the main effects of global warming on aquaculture productivity is thought to be the rising water temperatures [1,2]. Significant variations in water temperature can have two effects on aquatic life: either they put them at risk of extinction [3,4] or they can help them adapt [5]. Though there have been reports of aquaculture output losses globally owing to changes in water temperature, research on the effects of global warming on aquaculture production is still in its infancy. For example, Aphunu & Nwabeze [6] discovered that global warming had a detrimental impact on the growth and productivity of aquaculture in Nigeria. Meanwhile, a team of scientists found that Brazil's intense heat had stressed out *Oreochromis niloticus* and increased its mortality rate [7], in Indonesia [8]. There have also been reports from Malaysia on the impact of high temperatures on the aquaculture sector [9]. A study among Malaysian aquaculture producers revealed a strong correlation between climatic change and a decline in economic production [2]. For example, elevated water temperatures have been linked to the reported fatalities of farmed red hybrid tilapia (*Oreochromis sp.*) in floating cage culture at Kenyir Lake, Terengganu, Malaysia [10,11-13].

Tilapia is the second species farmed worldwide, behind carps. They are also known as "Aquatic chickens" since they can be found on all continents except Antarctica. Aquaculturists choose this species because of its fast development, trophic level feeding ability, fast tolerance to a wide range of environmental circumstances, and successful reproductive techniques. It is estimated that tilapias began to rise for food consumption approximately 25000 years ago [14]. Tilapia has become the third most important fish in aquaculture, behind salmonids and carps; in 2002, global production exceeded 1,500,000 metric tons [15]. Because of its various health benefits, tilapia is highly prized as a seafood source. These benefits are linked to its abundance of vitamins, minerals, and nutrients, including considerable protein levels and omega-3 fatty acids. It also includes selenium, potassium, vitamin B12, niacin, phosphorus, vitamin B6, and pantothenic acid, according to the USDA National Nutrient Database. The typical temperature range for tilapia is between 24 and 32 degrees Celsius. They live in a range of freshwater and occasionally brackish water environments, such as ponds and shallow streams, rivers, lakes, and estuaries.

Temperature impacts fish biology in several ways, including growth rates, metabolic rates, reproduction, and general performance. The physiology and behavior of fish can be significantly impacted by both short-term and long-term temperature variations.

Lower temperatures can impair immunological function, lower metabolic rates, and inhibit enzyme activity. Higher temperatures can speed up metabolic processes, raise energy requirements, and cause oxygen limitation. Temperature variations can also affect fish species distribution and abundance, which may have an impact on ecosystem dynamics [16].

The present study was to understand the effect of rising temperatures on tilapia fish. The fish were grouped into two (an experiment and a control system). Temperature and pH were measured every day at a regular interval of time (I.e., 8:45 AM, 1 PM, and 5:15 PM) with the help of a thermometer and pH meter. Proper aeration and feed were provided. The experiment systems were set in an area where direct sunlight was obtained and the control system was in an area without direct entry of sunlight. The purpose of this study is to alarm the world about the devastating effects of global climatic changes on the survival, growth and prosperity of the animals in their environment leading to subsequent impact on the world's human development.

2. MATERIALS AND METHODS

2.1 Experimental Design

The experiments were conducted in a plastic fish tanks (500 L). A hundred fish with a total weight of 13.12g were used in the experiment. 50 Fish were kept in the experimental fish tank placed in terrace of the house and 50 fish were kept in the control fish tank placed in shade. Aeration is provided to both test and control. The control setup was arranged in an area without direct entry of light whereas the experiment was in an open Taurus with direct entry of light. A normal fish diet was provided in both conditions water was changed every day to maintain good water quality. The temperature and pH were measured at a particular time of intervals (8.45 am, 1 pm, 5.15 pm) every day. Fishes were examined after one week of experimental study. The experiment was conducted over six months and fish weight was recorded using a digital weighing balance.

2.2 Growth Parameters

The line weight gain, percentage increase in biomass, specific growth rate, feed conversion ratio, and survival percentage were calculated as follows,

1. Increase in biomass = final weight - Initial weight
2. Increase in biomass (%) = (final weight - Initial weight) x 100/Initial weight
3. FCR = Total feed consumed by fish (g)/Total weight gain by fish (g)
4. Survival rate (%) = (Initial number of fishes - final no of fishes) x 100/Initial no of fishes
5. SGR = $\frac{100 \times (\text{In w (t)} - \text{Inw(i)})}{T}$

Where,

wt was the final weight
wi was the initial weight
T was the experimental duration.

2.3 Protein Estimation

Protein estimation was done by using Lowry Method (1951). Fish flesh was separated under both conditions. About 1 gm of flesh was weighed out. The flesh along with 4 ml distilled water was taken in mortar and ground well. It was then centrifuged along with 16ml distilled water in a centrifuging machine at 3000rpm for 10 minutes. The supernatant was discarded and add 4ml 1N NaOH to dissolve the residue. Three test tubes were taken and labelled as B (blank) T (test) and S (standard). 1 ml of the prepared sample solution was to the tube T, 1ml distilled water in B, and 1 ml standard BSA solution in S. Then 5.5 ml of reagent was added to the three test tubes (B, T, S), followed by 0.5 ml of folinciocalteau. The tubes were kept in the dark for 30 minutes. A blue colour developed and density was measured in the calorimeter at 620nm. The amount of protein in the sample was calculated.

Concentration of unknown sample =

$$\frac{OD\ of\ sample}{OD\ of\ standard} \times concentration\ of\ standard$$

2.4 Estimation of Salinity

Pipetted out 1ml of water sample in a conical flask it was then diluted with 4 ml distilled water and added 4 or 5 drops of potassium chromate as an indicator. Titrated against 0.05 $AgNO_3$ taken in the burette, the endpoint was the appearance of a faint but persistent reddish-brown color in the granules. The volume of $AgNO_3$ used was noted.

The amount of acid required to titrate. The bases of given water samples are a measure of their alkalinity. Bicarbonate, carbonate, and hydroxide are considered to be chief bases in natural water samples containing bases that turn yellow by the addition of a methyl orange indicator. The water sample containing measurable carbonate ions turns pink due to the phenolphthalein indicator and measurements in alkalinity.

In the first stage, the water sample in two stages titrated with a phenolphthalein indicator, its carbonate content is converted into bicarbonate.

$$Co_3^{2-} + H^+ \rightarrow Hco_3^-$$

In the second stage of titration the methyl orange indicator is added to the above sample and the titration is continued to the endpoint. During this titration, all bicarbonates are converted into carbon dioxide and water

$$Hco_3^- + H^+ \rightarrow H_2O + Co_2$$

10 ml of water sample was taken in a conical flask and two drops of phenolphthalein were added to it which serves as an indicator. The solution becomes pink and titrated against 0.02N H_2So_4 taken in the burette. The endpoint was marked by the disappearance of the pink colour. The volume of H_2So_4 was noted. Titration was repeated to obtain concordant values.

Added two drops of methyl orange to the same sample taken in the conical flask. It imparted a golden yellow colour to the solution. This solution was titrated against the 0.02N H_2So_4 taken in the burette. The end point of titration was marked by the appearance of a faint orange colour. The volume of sulphuric acid was noted. Titration was repeated to obtain concordant values.

The alkalinity was obtained by adding both phenolphthalein alkalinity and methyl orange alkalinity.

2.5 Estimation of Dissolved Oxygen

Estimation of Dissolved Oxygen was done by Winkler's Method (1888). Fill the 250 ml glass bottle with the water sample from both conditions up. This can be done by slowly lowering the bottle in the water and raising it when filled. Replace the stopper by taking care that no air bubble is trapped in the bottle.

Add 2 ml $MnSO_4$ solution with pipette. Introduce the pipette into the sample and release the reagent at the bottom of the bottle. Add immediately 2ml of alkaline *KI* solution to the sample well below the water surface. Close the bottle and store it well. A yellow-brown precipitate is obtained.

Allow the precipitate to settle down at the bottom of the bottle When the precipitate has completely settled down, open the bottle and add 2 ml of the conc.H_2SO_4. The acid is allowed to run sides of the bottle, close the bottle with the precipitate has completely dissolved. The sample became yellowish brown due to the presence of Iodine which had replaced oxygen.

20 ml of sample was placed in a clinical flask and titrated with 0.025N sodium thiosulfate in a burette After that, a few drops of the sample become pale yellow in colour. Now add a few drops of starch which act as an indicator. The sample turned blue. Add Sodium thiosulphate drop by drop until blue colour disappears and sample becomes clear. This is the endpoint; repeat the experiment for concordant values.

2.6 Estimation of Moisture Content (%) in Body Muscles

The wet tissue is allowed to dry by keeping in a desiccator. The difference between the wet weight of the tissue and its dry weight gives the amount of water present in the fish tissue. The moisture content in the body muscles of tilapia fishes was estimated by drying of small amount of tissue for 3hr at 105°C body muscle sample in an oven. The samples were taken in a pre - weighted glass dish with a cover and kept in an oven. After drying, the fish was transferred with a partially covered lid to the desiccators to cool.

$$\text{Moisture (\%)} = \frac{W_1 - W_2}{E_1 x 100}$$

Where,

W_1 Weight (gm) of the sample before drying
W_2 Weight (gm) of the sample after drying.

2.7 Statistical Analysis

Data are analyzed using statistical way. Data are subject to unpaired t-tests to find the significant effect of temperature on growth, survival, dissolved oxygen, Alkalinity, salinity, and osmoregulation. Data are presented as treatments Mean ±SD.

3. RESULTS

This study was planned to observe the effects of rising temperature on the growth parameters, protein content dissolved oxygen content, moisture content osmoregulation, and physiological activity.

The water temperature increased progressively. In the experiment, the average temperature ranged 17^0C. The minimum water temperature was 24^0C and the maximum 41^0C whereas in the control minimum temperature was 26^0C and the maximum 36^0C. Along with temperature, salinity also increased with a decrease in alkalinity, $P^{H,}$ and dissolved oxygen. In the present study, the mortality rates were very high and survivability percentage was significantly low during experimental conditions.

The mean value of salinity in control was 0.046 whereas in experiment it was 0.065. The growth performance showed a significant increase to the control condition which indicates an increase in metabolic rate, but they died within one week. Salinity and temperature are directly proportional to each other. Along with increasing salinity, alkaline nature undergoes reduction (Table 2).

During the experimental period, the water of basin remained in alkaline condition. The maximum pH 8.4 and minimum 6.3 was observed in control, whereas in experimental group, maximum pH 8.2 and minimum pH 5.7. In the present study the pH ranged from 5.7 to 8.2 this could be considered congenial for the normal activity and may affect their normal functioning. The result showed that increasing temperature causes a reduction in P^H Value (Table 2).

During the experimental period high fluctuations in the levels of dissolved oxygen were recorded. Difference between the mean value of dissolved oxygen in experimental and control group are 26.56. The total alkalinity difference between the experimental and control group is 99.6mg/l. The mean value of total alkalinity in experimental group is 129.60 where as in control is 229.20. The present study indicated the declining nature of the alkalinity of water in the presence of rising temperature (Table 2).

The present study showed a significant relationship between the control and experiment in terms of moisture content. The osmoregulatory effects were studied by measuring the amount of chloride, sodium carbonate/ bicarbonate, and H+ ions. The mean value of chloride ions in the control (tissue) was 0.34, whereas in the experiment 0.65. The mean value of chloride in water was 0.04 (control) and 0.06 (experiment). The result shows an increasing nature of chloride ions in the experiment than control. The one sample t-test showed the chloride ions in the tissue before the experiment. The mean value was 0.62 which means the chloride ion increased in the experiment (tissue) and decreased in control.

The mean value of carbonate and bicarbonate (tissue) in control was 250 mg/l and experiment 150 mg/l whereas in water, 229.20 mg/l and 129.60 mg/l respectively. The one sample t-test conducted before the experiment showed the rate of carbonate and bicarbonate in the tissue which is 230 mg/l. This result shows the increasing nature of ions in control and the decreasing nature in the experiment.

Changes in H+ ion concentration were measured with a pH meter. The results showed a pH of 7.6 in the control and a pH of 5.7 in the experiment (tissue). And pH was measured before the experiment was 8 showed in one sample t-test. The result showed a decreasing nature of pH in the experiment than control. The results show fluctuation may be due to rising temperatures. The variations in the result indicated an imbalance between the ionic or electrolyte (Table 3).

The present study showed that there is an increasing nature in metabolic activity of experimental group. final body weight gain, percentage of weight gain, specific growth rate, feed conversion ratio of tilapia fish increased significantly when the temperature increased and the mortality rate was 100 percentage within one week (Table 4). When fish metabolism speed up the fish’s breathing rate also increases requiring more oxygen. Temperature increases cause a decrease in oxygen which leads to the restless movement of fish. The experimental fishes showed high rate of feeding and were noticed to spend more time in surface water for gasping air. This is the clear sign of distress.

Table 1. Water quality parameters before experiments

Ions	Mean ± SD
Chloride	0.0623760±0.106214
Carbonate and bicarbonate	230.00±5.66
P^H (H^+Ions)	8±0.274

Values are mean ± S.D

Table 2. Water quality parameters

	Control	Experiment
Temperature	30.230±3.162	35.863±3.873
pH	7.667±1.482	5.740±3.008
Alkalinity	229.20±1.010	129.60±1.67
Salinity	0.046800±0.011300	0.065520±0.01325
Dissolved oxygen	56.200±0.758	29.640±0.410
Chloride	0.046800±0.011300	0.065520±0.011325
Bicarbonate and carbonate	229.20±1.010	129.60±1.67

Values with different letters in each column are significantly different at (P < 0.01). Values are mean ± S..

Table 3. Estimation of Fish tissue sample

	Control	Experiment
Moisture content	13.879480±0.009478	18.1666500±0.010600
Chloride	0.345640±0.006070	0.628380±0.106042
Bicarbonate and carbonate	250±2.00	150±2.51

Values with different letters in each column are significantly different at (P < 0.01). Values are mean ± S.D

Table 4. Growth parameters of Tilapia fish (1 Week)

GROUP	Initial weight	Final weight	BWI	PWI (%)	SGR	FCR	SR (%)
Control	4.316	4.39	1.714	3.4	0.172	0.42	20
Experiment	4.183	4.36	0.177	17.7	0.061	4.384	100

Body Weight Index (BWI), percentage of weight gain (PWI), Specific growth rate (SGR), Food conversion ratio (FCR), and Survival rate (SR)

4. DISCUSSION

Thermal pollution is the degradation of water quality by any process that changes ambient water temperature. When water used as a coolant is returned to the natural environment at a higher temperature, the sudden change in temperature decreases the oxygen supply and affects ecosystem composition. Global warming could affect most terrestrial ecoregions. Increasing global temperature means that ecosystems will change; some species are being forced out of their habitats (possibly to extinction) because of changing conditions, while others are flourishing. This study was planned to observe the effects of rising temperature on the growth parameters, protein content dissolved oxygen content, moisture content osmoregulation, and physiological activity of Tilapia (*Oreochromis mossambicus*).

Fish show a substantial correlation between their metabolic rate and temperature. The metabolic rate often increases with temperature, increasing energy requirements. Fish prioritize growth and reproduction at the ideal temperature range, allocating energy differently under different temperature regimes. On the other hand, metabolic changes may take place in extremely hot environments to preserve energy and preserve vital physiological processes [17]. The experiment showed a gradual increase in water temperature, salinity, and mortality rates of Tilapia, with a low survivability percentage, compared to the control temperature range of 26-36°C.

The mean value of salinity in the control was 0.046 whereas in the experiment it is 0.065. The growth performance showed a significant increase to the control condition which indicates an increase in metabolic rate, but they died within one week. Salinity and temperature are directly proportional to each other. Along with increasing salinity, alkaline nature undergoes reduction. At 25 °C, a negative linear correlation was seen between muscle water content and salinity, which was probably caused by passive water loss as a result of an increase in ion input. However, muscular water loss may be advantageous because it keeps the circulatory system from being fatally dehydrated and permits survival while salt excretion systems are activated. This may be indicated by the fact that plasma osmolality was maintained at 25°C throughout the range of salinities examined. When tilapias were exposed to 35°C, the amount of muscle water stayed constant. The reason for this is yet unknown, however, Handeland et al. [18] found that muscle water drops in Atlantic salmon (Salmo salar) were greater at 18.9 compared to 9.1 or 14.4°C.

In the present study, the rate of metabolism can be determined by the amount of protein present in the tissue. The difference between the mean value of protein in experimental and control groups is 0.896mg/ml. The study of biochemical parameters in magur has revealed information on the physiological changes occurring in fish in response to decreasing or increase of water temperature from the acclimation temperature of 25^0C. Higher levels of serum protein in fish exposed at lower temperatures (20 – 10^0C) than those in control (25^0C) indicated temperature-induced stress in fish after 12 h of exposure [19].

A reduction in pH will have impacts on the entire oceanic system, with high latitude cold water oceans affected earlier and more severely than warm water oceans. Concentrations of atmospheric CO2 and other radioactively active trace gases have risen. Such atmospheric modifications can alter the global climate and hydrologic cycle, in turn affecting water resources [20]. Climate change can occur over evolutionary and ecological time scales as a result of natural and anthropogenic causes [21]. During the experimental period, the water of the basin remained in alkaline condition. The maximum pH of 8.4 and minimum of 6.3 was observed in the control, whereas in the experimental group, the maximum pH was 8.2 and the minimum pH was 5.7. Lagler [22] considered water having a pH range of 7.0 and 8.5 as favorable for fish. The pH of pond water undergoes wide fluctuations. The pH of water changes throughout the day, becoming more acidic right before dusk and more alkaline in the afternoon. Swingle [23] reported optimum growth of fish at a pH ranging between 7.5 and 8.5. In the present study the pH ranged from 5.7 to 8.2 this could be considered congenial for the normal activity, and may affect their normal functioning. The result showed that increasing temperature causes a reduction in P^H Value. Jhingran [24] reported that pH is determined by the relationship between free carbon dioxide and carbonates. The pH can also affect fish health. The optimum pH level for carp culture is 7.5 to 8.5 [25]. In general, tilapia can survive in pH ranging from 5 to 10(do best in a pH range from 6 to 9). But in the present study, the experimental group's pH ranged between 5.7 and 8.2, fishes showed an increased growth rate than the control with 100% mortality rate within 1 week.

The temperature of the water is one of the most important abiotic variables affecting the amounts of oxygen in ecosystems that depend on water. The temperature increases in an inverse relationship with the amount of oxygen in the air. The solubility of oxygen diminishes with increasing water temperature [26]. In research studies by Trewavas [27], Nile tilapia grew better when aerators were used to prevent morning DO concentrations from falling below 0.7 to 0.8 mg/l compared with an unaerated control pond. Jhingran [24] suggested that the concentration of dissolved oxygen above 7ml/l is suitable for productive pond water. During the experimental period high fluctuations in the levels of dissolved oxygen were recorded. The difference between the mean value of dissolved oxygen in the experimental and control group is 26.56. Dissolved oxygen is considered one of the most important aspects of aquaculture. It is a crucial factor in natural waters for the growth and survival of fish. It is needed by fish to respire and perform metabolic activities. Thus, low levels of dissolved oxygen are often linked to fish kill incidents. In this experiment, the fish died within 1 week may be due to this lowered value of dissolved oxygen. The lowered value of dissolved oxygen along with an increase in temperature affects the normal conditions of fish. Reduced value of dissolved oxygen along with increased metabolic rate may cause an increase in respiration.

The total alkalinity difference between the experimental and control groups is 99.6 mg/l. The mean value of total alkalinity in the experimental group is 129.60 whereas in the control is 229.20. The present study indicated the declining nature of the alkalinity of water in the presence of rising temperatures. So the water loses its alkaline nature in the presence of rising temperature and becomes more acidic, which may affect the normal functioning of aquatic organisms and finally result in the destruction of the whole world. The acidic condition of water may lead to a change in the normal functioning of the fish, ultimately resulting in death.

Freshwater fishes are hypertonic to their water environment and therefore water is continually diffusing into the fish through the gill membranes into the blood. In the present study, osmoregulatory effects were studied by measuring the amount of chloride, sodium carbonate/ bicarbonate, and H+ ions. The mean value of chloride ions in the control(tissue) was 0.34, whereas in the experiment 0.65. The mean value of chloride in water was 0.04 (control) and 0.06 (experiment). The result shows an increasing nature of chloride ions in the experiment than control. The one sample t-test showed the chloride ions in the tissue before the experiment. The mean value was 0.62 which means the chloride ion increased in the experiment (tissue) and decreased in the control. Dysregulation between 15 and 35^0C may also result from modifications in the membrane's integrity. It has been shown that acclimation temperatures above the optimal range for rainbow trout (*Oncorhynchus mykiss*) and Nile tilapia (*Oreochromis niloticus*) cause holes in the membranes that enhance water and ion permeability. The altered lipid structure is what causes these holes [28]. The increase in muscle water at high temperatures may also be explained by these types of membrane disruptions. Both lipid domains connected to regular enzyme activity and breaches that allow solutes and water to get through can be affected by phase changes at either end of the membrane [18,29].

The study found that carbonate and bicarbonate concentrations in tissue increased in the control group and decreased in the experiment group, indicating an imbalance between the ionic and electrolyte. The pH meter showed a decreasing nature in the experiment, possibly due to rising temperatures. Animal physiologists soon realized that the projected increases in temperature can have significant effects on the function of many animals because their body temperature is largely dependent on ambient temperature. This group encompasses the overwhelming majority of animals and includes most of the vertebrates [30].

The present study showed that there is an increasing nature in the metabolic activity of the experimental group. When fish metabolism speeds up the fish's breathing rate also increases requiring more oxygen. Temperature increases cause a decrease in oxygen which leads to the restless movement of fish. The experimental fishes showed a high rate of feeding and were noticed to spend more time in surface water for gasping air. This is a clear sign of distress.

5. CONCLUSIONS

Significant variables that affect fish physiology, behaviour, and ecological interactions include temperature, pH, salinity, and dissolved oxygen. Fish populations are impacted differently by each aspect, which might include anything from patterns of dispersion and general survival to metabolic and reproductive processes. This study reveals that Salinity increased in tandem with temperature and decreased with PH, dissolved oxygen, and alkalinity. In the current investigation, the survivability % under experimental conditions was much lower and the fatality rates were very high. Climate change and human activities challenge fish populations by altering the temperature. Therefore, it is essential to consider the interactions between temperature, pH, salinity, and dissolved oxygen and develop strategies to mitigate their impacts on fish populations and aquatic ecosystems. Continued research, monitoring, and implementation of appropriate management practices are crucial for maintaining healthy fish populations, supporting biodiversity, and preserving the balance of our water bodies. By understanding and addressing the effects of temperature, pH, salinity, and dried oxygen on fish, we can make informed decisions to protect and sustain our valuable aquatic resources.

DISCLAIMER (ARTIFICIAL INTELLIGENCE)

Author(s) hereby declare that NO generative AI technologies such as Large Language Models (ChatGPT, COPILOT, etc) and text-to-image generators have been used during writing or editing of manuscripts.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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