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Intricacies of Salinity Effects on Zebrafish Physiology: Acute Salinity Stress Induces Mortality

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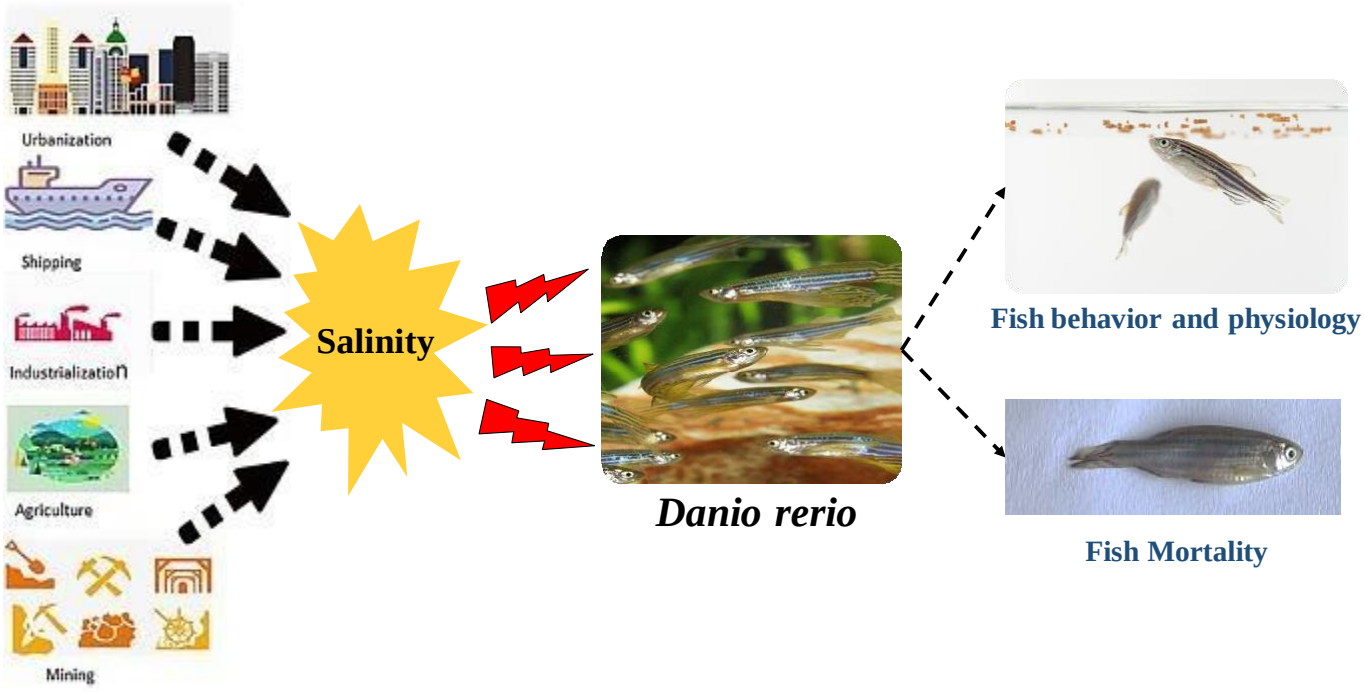
Abstract

Water parameters are essential in maintaining the good health of aquatic organisms. Fluctuation in any of the parameters is a stress factor for the organisms. Among them, salinity acts as a significant factor in stress. Zebrafish have substantial stress responses to variations in salinity, which cause changes in physiology and behavior. Fish experiencing fluctuations in the salinity content of their aquatic surroundings are said to be experiencing salinity stress. Excessive salinity can dehydrate freshwater fish and lead to an imbalance in ions; however, abrupt salinity reductions in saltwater fish can induce an influx of water and a loss of ions. Fish exposed to prolonged salt stress are more sensitive to illness, have lower general fitness, and have a lower chance of surviving. The acute toxicity and chronic effects of extreme salinity 6g/L,7g/L,8g/L,9g/L, and 10g/L are effectively seen in Zebrafish's heart tissue. This study enlightens how water quality parameters like salinity affect specific physiological and molecular events in Zebrafish. In summary, exposure to sub-lethal salinity concentrations influences Zebrafish physiology, resulting in mortality.

Keywords: Zebrafish, fish health, salinity, toxicity, mortality

Graphical abstract



Introduction

Stress is a condition that causes decreased fitness due to external factors that test an organism's homeostatic vitality and threaten species' survival (Chu et al., 2019). The anti-stress response system is initiated by activating the hypothalamus, which activates the neuroendocrine system, followed by the metabolic and physiological systems. Stress negatively impacts behavior, development, reproduction, immunological function, and illness resistance (Das et al., 2024a). The degree of reaction in physiological and behavioral systems differs among animals and individuals (Schulte et al., 2014). Adults, juveniles, and even fish larvae adapt well to environmental changes due to stress (Wedemeyer et al., 1990). Because of their unique physiologies, all creatures, including fish, develop and grow differently (Mallya et al., 2007). Endocrine, neurological, metabolic, and ecological connections all have a role in physiology. Physiological influences, alone or in combination, can influence or alter human physiology, particularly early development (Portz et al., 2006). Salinity, among other external elements, is vital to fish because it influences osmolality and metabolism (Majumder et al., 2024). It alters activity, structure, and physiology, eventually influencing fish growth, habits, survival, and dispersion. Although some fish can give birth to new ones, most fish require in vitro fertilization and encounter several hurdles during fertilization and early development in their aquatic habitat. Furthermore, environmental risks, pollutants, and external variables contribute to lower egg production and juvenile survival rates (Khan et al., 2019). The most common environmental stressors impacting ecosystem structure and function are abiotic stressors (temperature, cold, foreign substances, etc.) and biotic stressors (pathogens, predators, invasive species, etc.). Abiotic stress is caused by physical or chemical attacks from the environment on fish, whereas biotic stress is caused by biological attacks that a fish may encounter throughout its existence

(Singleman et al., 2014). Several physical, chemical, and biological stresses pose a potential hazard to freshwater ecosystems. As a result, various stressors continually endanger freshwater ecosystems (Jiang et al., 2005). Zebrafish is one of the most popular model organisms used for research. It is a tropical fish that lives in rice paddies in Asia. Critical ethical concerns surrounding human experimentation have led to the use of animal models in medical research (Panigrahi et al., 2020a, 2020b, 2020c). In Latin, Zebrafish are commonly known as "*Danio rerio*". As a result, it is commonly known as a zebrafish because its striped body resembles a zebra (Panigrahi et al., 2021a, 2021b, 2021c). The stripe is dark blue and horizontal, flowing the length of the body from the caudal fin to the gills (Sahoo et al., 2021a, 2021b). Zebrafish are widely used for research work because of several benefits. Zebrafish are very robust and easy to care for. Since they occur naturally in ponds, they can easily grow in ideal conditions by changing the environment. Also, they are very inexpensive (Sahoo et al., 2023; Sahoo et al., 2024). However, it requires more space than other model organisms, such as flies (Panigrahi et al., 2024). This model is also vertebrate, which makes it better than others (Zurlo et al., 2010). Zebrafish have a high fertility rate, laying hundreds of eggs each week (Jung et al., 2020). The generation time is also very short, giving scientists an infinite supply of this model (Patro et al., 2023; Das et al., 2024b). This shortens the entire experimental process and is basically useful production. Zebrafish share 70% of their genes with humans, and 84% of disease-associated genes have zebrafish counterparts (Khan et al., 2019). The genome of Zebrafish has been fully sequenced, with over 140,000 genes mutated to study their role in development and disease. Zebrafish are particularly useful as animal models due to their low cost, short breeding time, and relative similarity to humans (Delcourt et al., 2018). Zebrafish share many of humans' genes, tissues, and organ systems. Because Zebrafish are more like humans than invertebrate models (such as the nematode *Caenorhabditis elegans* or the fly *Drosophila melanogaster*), discoveries made in Zebrafish are more likely than discoveries made in invertebrate systems can be directly applied to Humans.

Methodology

Zebrafish facility

Hundreds of adult wildtype zebrafish, aged 5-6 months post-fertilization, were obtained from the Central Institute of Freshwater Aquaculture (ICAR-CIFA). These Zebrafish had an average weight of 0.9 ± 0.1g. They underwent an eight-week acclimation period in a 30L stock tank fitted with high-quality filters. The water parameters in the tank were carefully maintained, with a pH range of 7±0.2, a temperature of 27±1°C, and a conductivity of 490-510 S/cm **(Table 1)**. Following the established protocol, the fish were subjected to a 14-hour light/10-hour dark cycle, with lights switched on at 9:00 AM (Avdesh et al., 2012). Throughout the trial, the Zebrafish were fed commercial processed dry floating feeds (Optimum Tropical Fish Food - Mini Pellet) twice daily. To prevent infections, the setup underwent periodic cleaning on a weekly basis. After the acclimation period, the main experiment commenced.

Feeding

Fishes were fed twice a day with a commercial floating feed.

Water quality parameters

Table 1: Different water quality parameters such as pH, temperature, salinity, dissolved oxygen.

Sl. No.	Parameter	Ranges	Device used
1	Salinity	0.2 mg/L	Conductivity meter
2	pH	6.8-8.2	pH meter
3	Temperature	27-32 °C	Normal thermometer
4	Dissolved oxygen	3.9-8.5mg/L	Light and dark bottle method

Stress Reagent

Sodium chloride was used to prepare different concentration of salinity.

Experimental design

Ten eight-inch glass bowls were taken. The bowls were named C, E1, E2, and E3, E4, E5, E6, E7, E8, and E9, respectively, 3 litres of water were filled in each bowl. The collected specimens (Zebrafish) were in each bowl (3 adult fish per bowl). The fish were left to acclimate to their environment for 3-4 days. They were fed twice a day and their behaviour was observed. After seven days, it was replaced with the following prepared solutions (the water in the controlled bowl was replaced with normal tap water). 3 litres of solutions were prepared in 3 different containers by adding 1g/L, 2g/L, 3g/L, 4g/L, 5 /L, 6g/L, 7g/L,8g/L,9g/L and 10 g/L to replace the water of E1, E2, and E3, E4, E5, E6, E7, E8, and E9, respectively. They were kept under observation for 7 days.

Results and discussion

Behavioral study

No fish fatalities occurred among the naïve ones in the acclimation period preceding stress exposure. Throughout the trial, we monitored their behavior and physical characteristics and contrasted the stressed Zebrafish with the control group. We recorded the fish's post-stress survival duration **(Table 1)**. Our observations determined that the crucial limits for zebrafish mortality are higher salinity concentrations of 6g/L,7g/L, 8g/L, 9g/L, and 10g/L, respectively. Fish died after being exposed to different concentration levels, both alkaline and acidic **(Table 2)**. Fish with extreme salinity values significantly damaged the cardiac system, as swollen heart tissue was seen. Fish in high salinity conditions were hypoactive, arranging themselves diagonally and pointing their heads toward the water's surface **(Table 2)**. They then grew listless and occasionally trembled violently before passing away. By contrast, the fish were restless when exposed to a salty environment, which resulted in fast swimming and tail slapping.

Table 2: Mortality record of fish after exposed to salinity stress.

Experimental sets	Salinity	Zebrafish (N=3)	Mortality after stress(after exposure to stress)		
			Fish1	Fish2	Fish3
Control	0g/L	3	---	---	---
Set I	1g/L	3	---	---	---
Set II	2g/L	3	---	---	---
Set III	3g/L	3	---	---	---
Set IV	4g/L	3	---	---	---
Set V	5g/L	3	6th day(122hr)	6th day(128hr)	6th day(134Hr)
Set VI	6g/L	3	5th day(98hr)	5th day (106hr)	5th day(112hr)
Set VII	7g/L	3	4th day(76hr)	4th day(84hr)	4th day (92hr)
Set VIII	8g/L	3	3rd day(56hr)	3rd day(65hr)	3rd day(70hr)
Set IX	9g/L	3	2nd day(32hr)	2nd day(40hr)	2nd day(48hr)
Set X	10g/L	3	1st day(15hr)	1st day(20hr)	1st day(26hr)

Additionally, it was observed that fish exposed to salinity 8g/L, 9g/L and 10g/L initially had slow swimming patterns and a pale appearance after about an hour. There was decoloration of stripped line on the skin of zebrafish due to the extreme stress levels. Decrease the spontaneous activity of the fish towards taking feed. Fish showing higher opercular ventilator movements, with possible open mouth (Hyperventilation) and Mouth and opercular movements at the water surface, resulting in the intake of water and air (gulping), Rotation around a long axis; erratic movements, often in Bursts (Corkscrew swimming), Fast reflex expansion of mouth and operculate not at water surface assumed to clear ventilator channels (coughing) **(Table 3)**. Between 24 and 48 hours of exposure, all specimens died at 8g/L, 9g/L and 10 g/L.

Table 3: Symptoms observed in zebrafish after exposure to different salinity concentrations.

Experimental sets	Salinity	Symptoms observed in zebrafish after stress exposure
Control	0g/L	Greyish skin color with striped lines, Normal behavior
Set I	1g/L	Lighten skin colour and hypoventilation, decrease spontaneous activity, and swim normally.
Set II	2g/L	Lightened skin color, hyperventilation, Decreased spontaneous activity, and normal swimming behavior.
Set III	3g/L	Slightly change in skin colour, normal ventilation Decrease in feeding activity and abnormal swimming behavior.
Set IV	4g/L	Slightly change in skin colour, normal ventilation Decrease in feeding activity, abnormal swimming behavior
Set V	5g/L	Lighten skin colour, hyperventilation Decrease in spontaneous activity, abnormal swimming behavior
Set VI	6g/L	Lighten skin colour, hyperventilation Decrease in spontaneous activity, swimming at the bottom.
Set VII	7g/L	Hyperventilation, Coughing, Abnormal skin pigmentation(lightened),
Set VIII	8g/L	Loss of schooling / shoaling behavior, Abnormal surface distribution/behavior
Set IX	9g/L	Abnormal skin pigmentation(lightened), Gulping, Corkscrew swimming, mucus secretion, Aggression
Set X	10g/L	Abnormal horizontal orientation, Loss of buoyancy control, Abnormal surface behaviour, hyperventilation

Comparative studies of zebrafish exposed to different salinity concentrations

All the zebrafish exposed to different salinity levels showed different morphological changes compared to the control. There were clear blue fluorescent lines on the body in control, while in highly acidic exposure, these lines were not seen in the body of the zebrafish **(Figure 1)**. In the alkaline water exposure, stripped lines were present on the bodies of zebrafish **(Figure 1)**. The black eye lens is slightly discolored into white at a high salinity. The discoloration of the eye lens is not seen in fish at lower concentration levels.

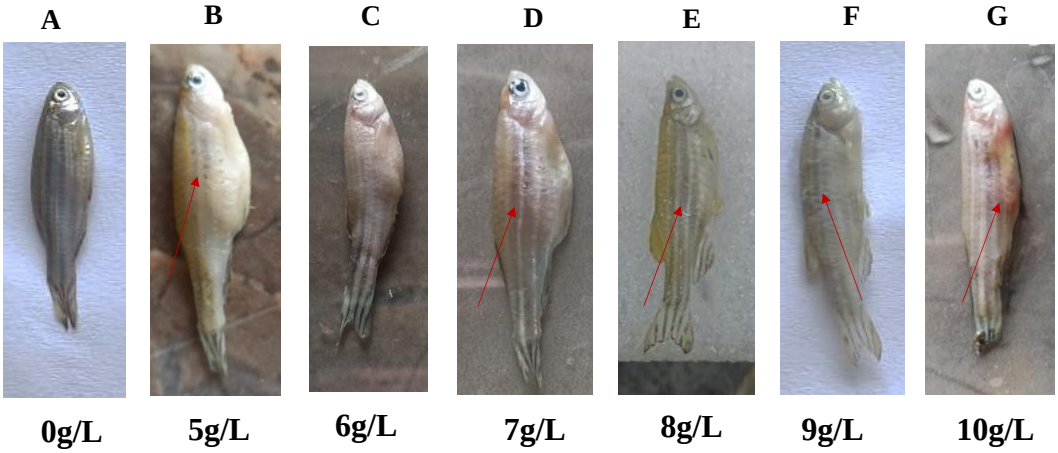


Figure 1. ***Dorso-ventral side view of fish The red arrow shows the discoloration of pigmented lines in the skin of zebrafish.***

After the exposure to the stress environment, a brown colour was seen on the bodies of the zebrafish, which implies that the tissues present in that area were affected by stress-inducing factors **(Figure 2)**.

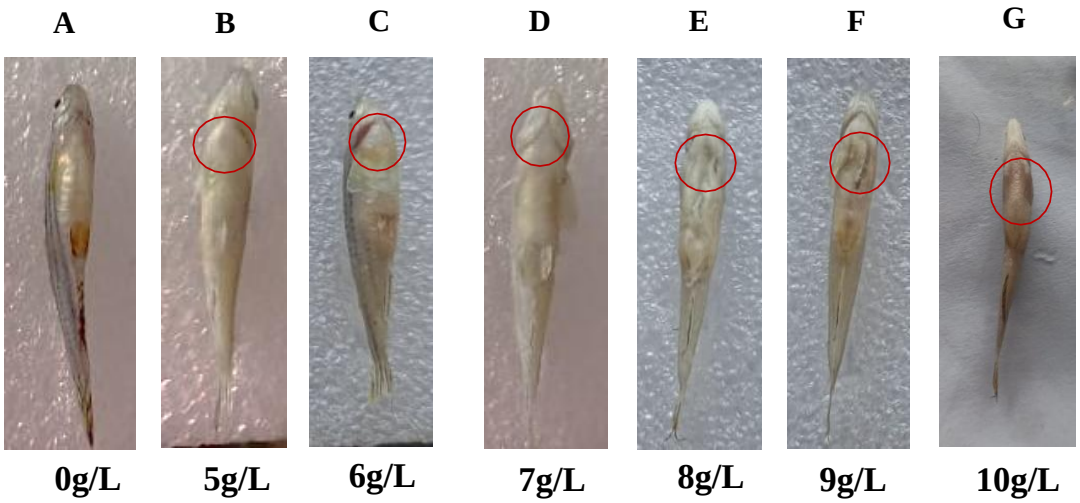


Figure 2. Ventral side view of fish: The red circle also has black spots in the fish after exposure to salinity.

During the observation days, fish showed different activities before death. When the fish was dead, it was identified by checking their response to stimuli and the movement of different parts, mainly gills. Once a fish was found dead, the immediately dissected fish was separated into different parts and generated pictures for analysis **(Figure 3)**. All the differences seen in the fish body are compared with the control zebrafish. A distinct difference was seen in the case of heart at higher concentration of salinity **(Figure 4)**.

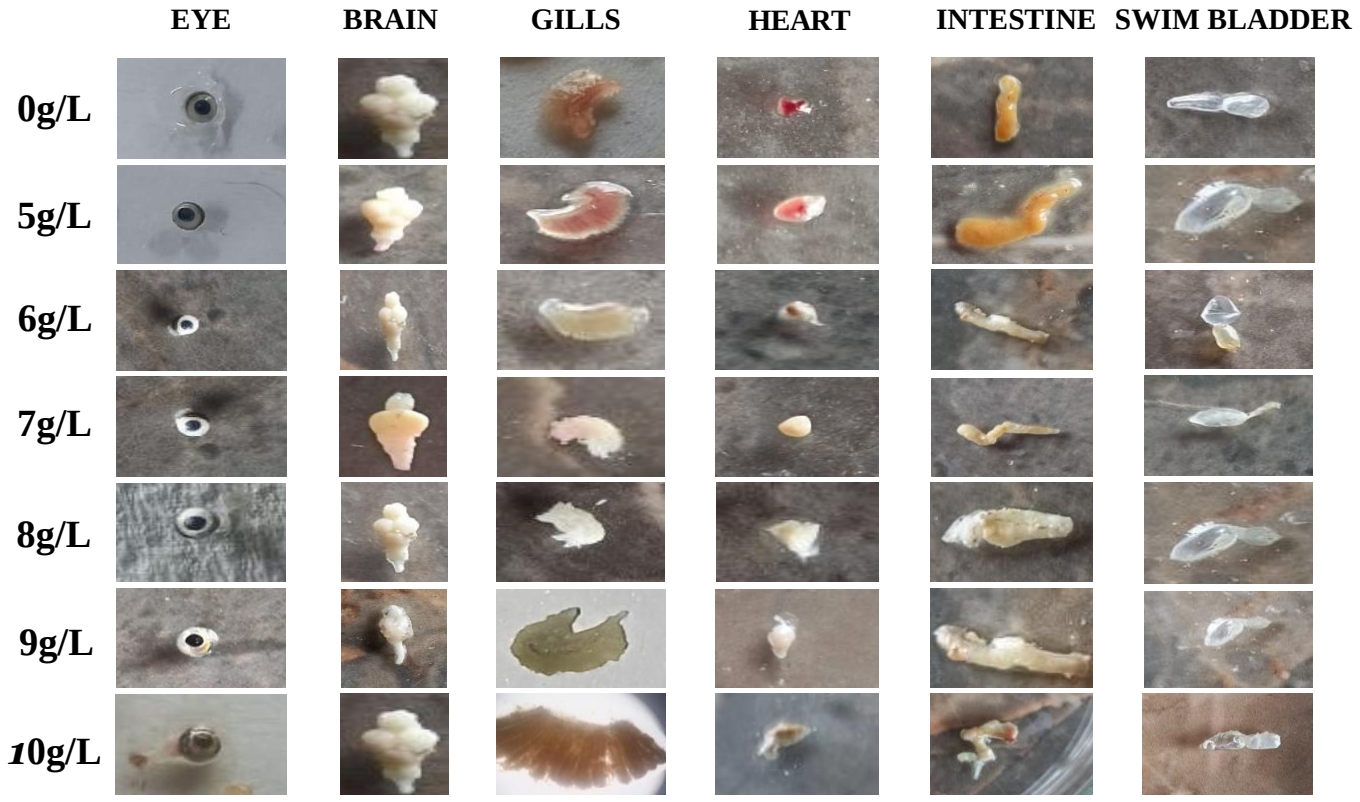


Figure 3. ***Comparative anatomy of zebrafish exposed to stress: The dead zebrafish (exposed to stress) were dissected and separated into different parts of the zebrafish, as shown in an image. Different body parts like the eyes, brain, gills, heart, intestine, and swim bladder of zebrafish are exposed to different salinity stress.***

Subsequently, the staining images of the gills were compared for different salinity. After the dissection, the collected gills were stained by the H&E staining process and seen under the microscope in the scale bar of 10x. In dissection, zebrafish's gills were highly acidic as the primary and secondary laminae were visible **(Figure 4)**.

Figure 4. ***Histological study by H&E staining: Representative images are a view of zebrafish gill following hematoxylin and eosin staining Magnification and scale bar 10x correspond to different stress.***

As an effect of exposure, the fish ended its density at the stage of mortality. Due to the stressor conditions, the fish died at different percentages and different salinity concentrations. The mortality percentage is slightly higher in 10ppm conditions than in comparison to control 0ppm **(Figure 5)**. There was no mortality in the case of control. Detailed information on mortality percentages in the different pH is described below **(Figure 5)**.

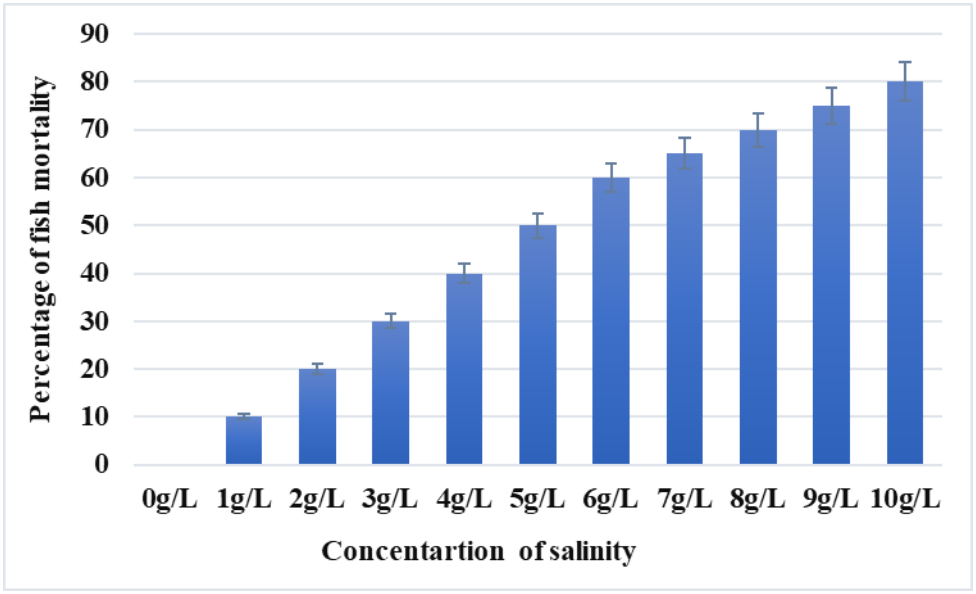


Figure 5. ***Showing the relation between the concentration of salinity and percentages of fish mortality***

Conclusion

The primary purpose of this study was to give preliminary data on the exposure of Zebrafish (*Danio rerio*) to the effects of salinity stress. The findings of this study revealed a definite link between specific salinity exposures and changes in zebrafish physiology. Fish undergo genetic changes due to the impact of salinity. The primary goal of the current study was to present early findings about the susceptibility of Zebrafish (*Danio rerio*) to the effects of salinity stress. This investigation showed a clear connection between some salinity exposures and alterations in zebrafish physiology. Additionally, the degrees of these modifications depend on exposure duration and concentration. Fluctuations in the salinity concentration cause serious physiological issues that worsen fish health, cause secondary infections, and ultimately cause fish death. There are also noticeable behavioral changes, as well as increased stress levels. Further research will shed light on the molecular processes behind the interplay between abiotic stressors and the physiological mechanisms in eukaryotic model organisms, potentially addressing the Life below water challenges as framed by the United Nations Sustainable Development Goals (SDGs) such as SDG 14.

Statements and Declarations

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Conflict of interest

The authors declare that they have no conflict of interest.

CRediT authorship contribution statement

All the authors have substantial contribution for the preparation of the manuscript. GKP: conceptualized and conceived the idea. RD and GKP: conducted experiments, data curation and writing. All the authors have read and approved the final manuscript before submission.

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