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Effects of sublethal arsenic on *Clarias gariepinus* juveniles' behavior, tissue damage, and oxidative stress response

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Abstract

African catfish is the single most cultured fish in Nigeria, and most culture systems depend on surface water abstraction. This has meant continuous exposure to emerging toxicants, including heavy metals. Arsenic is one of the widespread heavy metals present in the aquatic environment posing a threat to humans, animals, and fishes. To assess the effects of sublethal arsenic exposure to juvenile stage *Clarias gariepinus* juvenile, this study evaluated different sublethal doses of arsenic (20, 40, 60 mg/L) based on the estimated lethal (96 hrs-LC50) of 77.1mg/L. Behavioral responses, such as swimming activities, body weakness, surfacing, lethargy, loss of mucus, state of inactivity, and jumping, were observed. To keep track of the effect on tissue damage and oxidative stress response, we conducted enzymes activities assays to measure the activity of ALP, AST, ALT, CAT, and SOD. Results indicated that subacute exposure (96 hrs) to sublethal arsenic strongly distorted the normal behavior of African catfish. In which fish groups exposed to 20, 40, and 60 mg/l showed inactivity, increased mucus production, lethargy, and frequent surfacing. While exposure to sublethal arsenic showed no significant difference in hepatic enzymes activities (ALP and ALT), these exposures were noticeably ($p<0.05$) considering AST. Again, no significant differences in the antioxidant enzyme activities (CAT and SOD) in all fish groups exposed to sublethal arsenic. Overall, while there were no signs of oxidative stress, the significance reported in AST gives the reason to suspect sublethal arsenic concentration impacts African catfish tissue integrity. It is important to note that the behavioral-induced response may pose a risk to aquaculture stocks, as this may ultimately impact fish performance and fitness to aquaculture conditions.

Keywords: Arsenic, Toxicity, Fish, Mortality, Oxidative stress

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Introduction

Production of catfish has increased steadily since the record-keeping period began in 1950, going from 3.030 tonnes to 6 million tonnes in 2020 (FAO, 2022). The efforts to maximize production through technical improvement, innovations in breeding programs, and facility expansion to support higher stocking densities were evident in the strong production growth. The ever-increasing human population has sped up the development of aquaculture as well as urbanization and industrialization. The increased rate of chemical production, which has led to an increase in the variety of pollutants in the aquatic environment, is one effect of this. Oils, phenols, xenobiotics, pesticides, polyaromatic hydrocarbons, microplastics, and heavy metals are some of these (UNEP, 2019). Due to their endurance in the aquatic ecosystem and subsequent bioaccumulation in the food chain, which results in a cocktail of toxicity, heavy metals are undoubtedly one of the most serious contaminants (Banday *et al*., 2019; Javed and Usmani, 2019; Hossain *et al*., 2021; Ullah *et al*., 2021). One of the semimetals discharged into the aquatic ecosystem by human and geological activity is arsenic. It comes in both organic and inorganic forms, the latter of which is more poisonous (Liao *et al*., 2004). For instance, long-term exposure to As2O3 caused Clarias batrachus head kidney enlargement, decreased melano-macrophage population, and increased hemosiderin accumulation (Datta *et al.,* 2009). Following 30 days of exposure to sublethal concentration of inorganic arsenic, freshwater catfish, *Mystus vittatus*, showed impaired protein metabolism, where tissue protein decreases and free amino acid increases (Prakash and Verma, 2020).

This data indicated the adversity underlying the situation of aquaculture in an environment liable to arsenic contamination, which can jeopardize the energy benefit of aquaculture stock. Aquaculture activities are at greater risk of arsenic contamination given the occurrence of this metal in the aquifers and surface water at higher concentrations reaching up to 100–5000 µg/L (Smedley *et al.,* 1996). In developing worlds, where aquaculture operations depend on surface water abstraction, there is a greater risk of arsenic contamination of fish stock. For example, in Nigeria and other developing worlds, a lack of sophisticated technology limits the adoption of open water aquaculture, with the greater assimilative capacity to subdue the effects of emerging contaminants. For example, according to a survey of aquaculture production in Nigeria, 48% of the aquaculture industry practice through flow systems, with only 16% adopting a recirculatory aquaculture system, while 36% practiced static aquaculture (Emmanuel *et al.,* 2014). In this case, most aquaculture systems depend on surface water abstraction, which poses the risk of concentrating contaminants in a small volume of water (Martins *et al.,* 2011; Sibomana *et al.,* 2022).

Given the restricted choice of chosen locations between different industrial activities, these industrial activities often influence the type of heavy metals that may threaten aquaculture stocks. For example, lead (Pb) and arsenic (As) had the greatest concentration in water, while the adjacent industrial activities engaged in ceramics, consumer goods pharmaceutical, heavy electrical engineering, textiles, and vegetable oils production (Rehman *et al.,* 2008). Also, the activities of steel, oil drilling, and refinery companies contributed to the abundant Pb, Cu, and Fe (iron) (Sun *et al*., 2023). Hence, the most susceptible fish stage should be considered in experimental studies to gain insight into the priority of heavy metals as the highest threat. In the present study, we choose the juvenile stages of African catfish since this is the commonly stocked age class in the African catfish industry. This study aims to gain insight into the effects of sublethal arsenic on fish behavior, tissue damage, and oxidative stress response through antioxidant enzyme activities. Also, we are investigating the sublethal level of arsenic to represent the likely condition encountered in the flow through system that offers the opportunity for accumulation avoidance; this is in contrast to stagnant water aquaculture system/RAS, where heavy metals accumulate significantly (Martins *et al.,* 2011; Sibomana *et al.,* 2022).

Materials and methods

Fish culture

Juveniles of African catfish, *Clarias gariepinus* (n = 120, weight = 16.5±4.5g, length = 13.21±0.77cm) were obtained from Yusuf fish farm in Osogbo, Osun state. The fish were acclimatized for 14 days in 50L plastic tanks filled halfway with borehole water and covered with netting material to prevent fish from jumping out of the tank. The fish were fed with commercial feed (30% crude protein) twice daily until apparent satiation. Unconsumed feed and fecal matter were removed, and the culture water was replaced once every two days. The water temperature, dissolved oxygen, and pH level were monitored twice daily with an automatic multi-parameters probe (Hanna HI-9147, HACH Co., Loveland, Co., USA), and their values ranged between 28.5±1.3°C, 6.5±0.4mg/L, and 8.19±0.21 respectively. All these parameters were within the recommended values for the rearing fish (Andrew, 2007). Feeding was terminated 24 hours before exposure to toxic bioassay media to empty fish stomachs and avoid water pollution with their feces.

Procurement and preparation of arsenic

Analytical grade arsenic trioxide - As_2O_3 (CAS No: 1327-53-3, anhydrous) with 98% purity was obtained and used without further purification from the Department of Chemistry, University of Ilorin, Ilorin, Kwara state. The LD50 of arsenic was determined using the Miller and Tainter method (Saxena *et al*., 2009). The lowest tolerated dose that

gave 100% mortality and the highest dose that gave 0% mortality were determined by a hit-and-trial method. Subsequently, 5 doses between these doses were selected, and mortality was recorded as a result of the doses. Correction factor for 0% and 100% mortality group was calculated as:

$$0\% = \frac{0{,}25}{n} X\ 100$$

$$0\% = \frac{n - 0{,}25}{n} X\ 100$$

Where, n = numbers of experimental animals per group.

Probit units were read after converting mortality (%) values to probit values, plotted against log doses, and the LC_{50} value was read as the dose corresponding to probit 5.

Experimental design

Complete randomized design (CRD) consisted of four triplicate treatments, i.e., three treatments and one control. The fish were distributed into twelve 50L plastic tanks containing 20L of water each at a stocking density of 10 fish per tank (Abubakar, 2021) to give four treatments and three replicates. Hence, for this research, the control and exposed groups were separately exposed to sublethal concentrations of arsenic trioxide as follows: Group 1: Fish that were not exposed to any concentration of arsenic (control); Group 2: Fish exposed to 20mg/L (1/4 of 96h LC50) of arsenic trioxide; Group 3: Fish exposed to 40 mg/L (1/2 of 96h LC50) of arsenic trioxide; Group 4: Fish exposed to 60 mg/L (3/4 of 96h LC50) of arsenic trioxide. The fish were not fed throughout the duration of the experiment. Mortalities and behavioral changes were observed and recorded throughout the period of exposure.

Evaluation of survival rate of fish

Survival rate was estimated using the formula:

$$Survival\ (\%) = \frac{Number\ of\ fish\ after\ exposure}{Number\ of\ fish\ exposed} \times 100$$

Assessment of biochemical profile of the fish

The fishes were sacrificed from both the experimental and control groups after the 96h of the exposure period and subjected to laboratory analysis. At the end of the 96hrs experiments, control and test fish samples were randomly collected. The livers were identified and collected in plain bottles. The liver tissues were homogenized in physiological saline water and centrifuged at 3,000rpm for 15 minutes. The supernatants were filtered, and the filtrates were used for enzyme analysis. Using the MERCK kit, alkaline phosphatase (ALP) activity was assessed using the Samanta *et al*., (2014) method (Merck cat. number 1730PDLFT.0045). Briefly, 400 µL of R1 reagent was taken in the test tube, and then 100 µL of R2 reagent was added to this solution. These

two solutions were mixed up and kept for incubation at 37°C for 60 seconds. After that, 10 µL of the sample was added, and an immediately reading was taken from the autoanalyzer for four minutes. Enzyme activity was expressed as IU/L. The activity of aspartate transferase (AST) was determined following the procedure of Samanta *et al*., (2014) by using the Erba kit (Erba cat. number FBCEM0045). In a summary, 500 L of R1 reagent were taken and maintained in a test tube at 37°C for incubation. Following the addition of 50 L of the sample, an autoanalyzer reading was obtained immediately and recorded for three minutes. The measure of enzyme activity was IU/L. Using the Erba kit (Erba cat. number FBCEM0047), tissue alanine aminotransferase (ALT) was tested in accordance with the procedure outlined by Samanta *et al*., (2014). The reading was obtained from the autoanalyzer. All the assays were run in triplicate to avoid the error as much as possible.

Determination of antioxidant parameters

At room temperature, the amount of catalase (CAT) activity was determined spectrophotometrically by keeping an eye on the decline in absorbance at 240 nm brought on by the breakdown of H2O2. The Aebi technique was used to measure the catalase activity (1983). The amount of enzyme that, under assay circumstances, resulted in an absorbance change of 0.001 per minute was referred to as one unit (U) of catalase activity. In a total volume of 3.0 mL, the reaction mixture contained 100 mM sodium phosphate buffer (pH 7.0), 30 mM H2O2, and 100 L of filtrate. Superoxide dismutase (SOD) activity was determined by measuring the inhibition in the photoreduction of nitroblue tetrazolium (NBT) by the SOD enzyme (Wang *et al*., 2023). The reaction mixture had a final volume of 3.0 mL and contained the following ingredients: 50 mM sodium phosphate buffer (pH 7.6), 0.1 mM ethylenediaminetetraacetic acid (EDTA), 50 mM sodium carbonate, 12 mM L-methionine, 50 M NBT, 10 M riboflavin, and 100 L of tissue filtrate. Without using filtrate, a control reaction was carried out. By exposing the reaction mixture to white light for 15 minutes at room temperature, the SOD reaction was carried out. Using a spectrophotometer, absorbance at 560 nm was measured after 15 minutes of incubation. One unit (U) of SOD activity was defined as the amount of enzyme causing 50% inhibition of photochemical reduction of NBT.

Statistical analysis

The data obtained were subjected to a one-way analysis of variance (ANOVA) to test for the significant difference in the means, and the Least Significant Difference (LSD) test was carried out as the post-hoc test. The analysis was performed using IBM SPSS (Statistical Package for Social Sciences) version 25. The confidence level was set at $p<0.05$, and values were expressed as mean±standard deviation. The median lethal concentration was estimated using

EPA Probit Analysis Program (version 1.5).

Results

The median lethal concentration was estimated for juvenile African catfish using EPA Probit ANAlysis Program (version 1.5). The probit analysis estimate of LC_{50} concentration of arsenic oxide is presented in Figure 1 below. The regression equation (y=a+bx) derived from the mortality of *Clarias gariepinus* exposed to sublethal concentrations of arsenic was found to be:

$$y = 1{,}574x + 1{,}9328$$

The correlation coefficient (R^2) for *Clarias gariepinus* juveniles exposed to sublethal concentrations of arsenic was found to be 0.6108. The median lethal concentration (LC_{50}) of arsenic was calculated to be 77.16mg/L by taking the antilogarithm of the x-value at y=5. The estimated lethal concentration (LC) and Effect concentration (EC) and their respective confidence interval are presented in Table 1 below. The LC_{50}/EC_{50} value for *Clarias gariepinus* juveniles was estimated as 77.16 mg/L of arsenic.

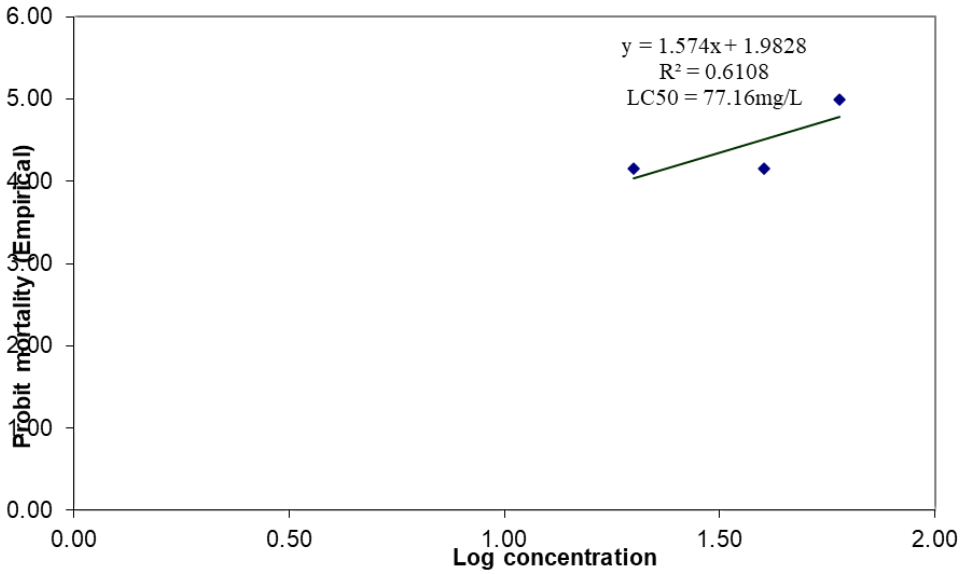

Figure 1: The linear relationship between probit mortality and Log of concentration in *Clarias gariepinus* juveniles exposed to sublethal concentrations of arsenic for 96hrs. R^2= coefficient of determination.

Table 1: Estimated LC/EC values and the associated 95% confidence limits of arsenic for *Clarias gariepinus* juveniles

Points	Exposure (mg/L)	95% Confidence interval	
		Lower	Upper
$LC/EC_{1.0}$	3.28	0.07	144.63
$LC/EC_{5.0}$	8.27	0.73	93.22
LC/EC_{10}	13.54	2.43	75.41
LC/EC_{15}	18.89	5.32	67.09
LC/EC_{50}	77.16	22.48	264.86
LC/EC_{85}	315.26	12.38	8025.47
LC/EC_{90}	439.77	10.54	18343.43
LC/EC_{95}	720.26	8.27	62692
LC/EC_{99}	1817.21	5.22	632836

Body weakness, sluggish swimming, frequent surfacing, lethargy, loss of mucus, state of inactivity, and jumping was the behavioral responses observed from *Clarias gariepinus* juvenile**s** exposed to sublethal concentrations of arsenic and are presented in Table 2. The severity of abnormal behavior increased with the increasing concentration of arsenic. The fish group exposed to 60 mg/L demonstrated greater disequilibrium from normal activity than the control group. Whereas mild responses to 20mg/L were mildly elicited through mucus loss, jumping and state of inactivity, no lethargy, body weakness, slow swimming, and frequent surfacing resulting from these fish groups. The Fish group exposed to 40mg/L arsenic gave moderate responses in all observed behavior except body weakness which was mild in this group. The result obtained on the impact of sublethal arsenic concentration on juvenile *Clarias gariepinus* survival is in Table 3.

Table 2: Behavioral responses of *Clarias gariepinus* juveniles exposed to sublethal concentrations of arsenic

Signs	0mg/L	20 mg/L	40 mg/L	60 mg/L
Body weakness	–	–	+	+++
Sluggish swimming	–	–	++	+++
Frequent surfacing	–	–	++	+++
Lethargy	–	–	++	+++
Loss of mucus	–	+	++	+++
State of inactivity	–	+	++	+++
Jumping	–	+	++	+++

Notes: no observable response (–), mild response (+), moderate response (++), severe response (+++)

Table 3: The survival rate of *Clarias gariepinus* juveniles exposed to sublethal concentrations of arsenic

Parameters	0mg/L	20 mg/L	40 mg/L	60 mg/L	*p*-value
Number of exposed fish	30	30	30	30	
Number of survivals	30	28	26	25	
Survival rate (%)	100^{a}	93.33^{ab}	86.67^{bc}	83.33^{bc}	0.003

Moreover, there were significant differences in the influence of arsenic on fish survival rate ($p<0.05$). Compared to the control fish group, exposure to arsenic reduced survival, where the arsenic concentration of 60mg/L showed the highest mortality (83%) compared to 100% in the control group. The liver enzyme activities and antioxidant enzyme activities measured are presented in Table 4.

Table 4: Liver enzyme activities and antioxidant enzyme activities of *Clarias gariepinus* juveniles exposed to sublethal concentrations of arsenic

Parameters	0mg/L	20 mg/L	40 mg/L	60 mg/L	*p*-value
ALP (UI/L)	26.35±11.53[a]	26.45±5.16[a]	27.55±2.76[a]	31.45±4.74[a]	0.863
AST (UI/L)	23.65±2.47[b]	45.05±2.47[a]	40.85±7.28[a]	43.80±1.41[a]	0.019
ALT (UI/L)	49.75±0.35[a]	41.05±15.76[a]	40.70±0.14[a]	47.30±1.13[a]	0.620
SOD	104.00±10.61[a]	107.75±25.10[a]	92.85±19.30[a]	88.35±0.21[a]	0.649
CAT	0.81±0.39[a]	0.39±0.23[a]	0.89±0.17[a]	0.50±0.16 [a]	0.287

Note: data presented are means of 3 replicates. mean±standard deviation (SD). ALP=alkaline phosphatase; AST=aspartate aminotransferase; ALT=alanine aminotransferase. SOD=superoxide dismutase (mUnits/L), CAT=catalase (mol H_2O_2 decomposed/min). Means with different superscripts are significantly different.

For alkaline phosphatase (ALP), there was no significant difference (p>0.05) between all the groups, however, the lowest value was recorded in the control group, while the highest value was recorded in the group exposed to 60 mg/L of arsenic. For aspartate aminotransferase (AST), there was a significant difference (p<0.05) between the control group and all other groups, however, there was no significant difference (p>0.05) between the 20 mg/L, 40 mg/L and 60 mg/L groups. For alanine aminotransferase (ALT), there was no significant difference (p>0.05) between all the groups, however, the lowest value was recorded in the 0 mg/L group, while the highest value was recorded in the 40mg/L group. Moreover, the antioxidant enzyme activities are recorded in Table 3. There was no significant difference (p>0.05) in the superoxide dismutase (SOD) level among the exposed groups, however, the lowest value was recorded in fish exposed to 60mg/L of arsenic, while the highest value was recorded in fish exposed to 20mg/L of arsenic. For catalase activity, no significant difference (p>0.05) was recorded across the groups, however, the lowest level was recorded in fish exposed to 20mg/L while the highest level was recorded in fish exposed to 40mg/L of arsenic.

Discussion

The open nature of the aquaculture environment has meant that the aquaculture stocks are inevitably exposed to emerging aquatic environment pollutants. The persistence of heavy metals makes them stand out as pollutants of significant threats. The present study evaluated the effect of sublethal concentration of arsenic (As) on the early life stage of African catfish. The lethal (LC_{50}) arsenic concentration estimated for *C. gariepinus* was higher than that reported for other freshwater species. For example, Kumar *et al.* (2019) reported a lethal arsenic concentration of 28.61 mg/L for *Pangasianodon hypophthalmus*. These varied LC_{50} values depict interspecies differences in the tolerance to arsenic. To examine the responses of juvenile fish to sublethal arsenic concentration, the behavioral response, including

mucus secretion, loss of swimming, body weakness, and lethargy in juvenile African catfish, were observed. The swimming activities of juvenile fish were reduced in the fish group exposed to 40mg/l and 60 mg/L arsenic concentrations. It is, therefore, possible for aquaculture stock to lose activities in the presence of mild water arsenic concentration. In *Clarias batrachus*, similar behavioral response was reported in exposure study assessing the impact of sublethal copper, however, at much lower concentration of 0.50ppm (0.50 mg/L) and 0.75ppm (0.75 mg/L) for longer duration (15 days) (Siddiqui and Arifa, 2011). Again, in a recent study using *Mytilus vittatus*, Verma and Prakash (2019) reported an arsenic-induced stress behavior, including sluggishness and increased surface activities (or jumping). In contrast to our study, this exposure study compared an extended duration of 15 days. From an aquaculture management perspective, swimming alteration may hinder the capacity of aquaculture stock to chase feed and thereby impact on feed intake of fish, ultimately resulting in loss of growth potential. However, it is noteworthy that this observation may not always be the case for other emerging contaminants. For example, previous studies found no differences in the swimming activity of fish from contaminated sites with uranium (Goertzen *et al.,* 2012).

Moreover, the fish group treated with an arsenic concentration of 40 mg/L and 60 mg/L showed excessive depositions of skin mucus. This study confirms previous exposure studies, for instance, in gilthead seabream (*Sparus aurata*) treated with a range of sublethal concentrations of heavy metals (Guardiola *et al.,* 2015). It was reported that sublethal arsenic (As_2O_3), cadmium ($CdCl_2$), and mercury (CH_3HgCl) induced an increment in mucus activities. It is acceptable to believe that increased mucus activities often elicited a response by fish to counter the effects of metal insults. There are claims that the polyanionic nature of mucus allows them to trap and bind metals, subsequently protecting fish and their gill from metal insults. Surprisingly, *C. gariepinus* show little tolerance to arsenic, as reflected in the increasing mortality relative to increasing concentration, despite the increased mucus production. Nevertheless, another possible effect of the increased mucus production may be reduced heavy metal uptake by fish tissue. This is reflected in the insignificant tissue damage reported in our study. Liver enzyme activities, including alkaline phosphatase, aspartate aminotransferase (AST), and alanine transaminase, have been used as important markers for tissue damage in toxicity studies (Javed and Usmani, 2019; Prakash and Verma, 2020). The result obtained from the present study revealed no significant damage to the tissue of juvenile *C. gariepinus* exposed to sublethal arsenic concentration. The lack of tissue damage may be due to subacute exposure (96 hrs.) of *C. gariepinus* to sublethal arsenic. However, there are traces of increased tissue enzyme activities as

reflected by an increment in the activity of one of the hepatic enzymes (aspartate aminotransferase) between the exposed and unexposed groups. This latter observation was similar to what was reported in Mystus vittatus exposed to sublethal arsenic, however, at a much longer duration (30 days) (Prakash and Verma, 2020).

To keep track of the oxidative response of *C. gariepinus* juvenile exposed to sublethal arsenic, we measured catalase and superoxide dismutase (SOD). To guard against the denaturation of polyunsaturated fatty acids (PUFA), fish have developed antioxidant systems employing both enzymatic and non-enzymatic antioxidants to remove reactive oxygen species, a major cause of oxidative stress (Javed and Usmani, 2019). In the present study, it was observed that no oxidative stress occurred throughout the duration of arsenic exposure. Although we observed a dose-dependent decrease in the activity of SOD, this sublethal concentration of arsenic was not strong enough to elicit a noticeable difference (p>0.05). In another study comparing the effect of other heavy metals' sublethal concentration ($CdCl_2$ at 7.02 mg/L or $PbCl_2$ at 69.3 mg/L) on antioxidants enzyme activities in African catfish, SOD activities were significantly decreased (Elarabany and Bahnasawy, 2019).

Overall, sublethal arsenic in the aquaculture environment may not present greater risks to aquaculture stocks. However, the results obtained from this study may help conclude that the presence of arsenic concentration, even at lower concentrations, may alter the normal behavior of African catfish. This behavioral alteration may act as the underlying factor reducing fish feed intake, consequently reducing feed utilization and aquaculture efficiency. Although the current sublethal concentration was exposed for a short duration, there is evidence in the literature that chronic exposure to low arsenic concentration may significantly alter fish functioning. And tissue damage may become much more apparent following continuous exposure to arsenic. Nevertheless, no oxidative stress threat may exist for aquaculture stock situated in mildly arsenic-polluted water.

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