

Behavioral effects of acute exposure of zebrafish (*Danio rerio*) to arsenic and ampicillin, and the protective role of quercetin

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Abstract: Water contamination with heavy metals and pharmaceutical residues is a major threat to aquatic ecosystems, affecting the health and behavior of non-target organisms. In this study, we investigated the potential protective role of quercetin (QUE), a natural antioxidant, against behavioral deficits induced in zebrafish (*Danio rerio*) by combined exposure to arsenic (As) and ampicillin (AMP). The experiment included four groups: a control group, a group exposed to QUE (1000 µg L⁻¹), a group exposed to As (50 µg L⁻¹) and AMP (100 mg L⁻¹), and a group administered with a combination of As, AMP, and QUE. The fish were subjected to acute exposure (96 hours) and, at the end of this period, social behavior parameters were evaluated. The results showed that concomitant exposure to As and AMP significantly affected social behavior parameters. In addition, co-administration of QUE partially ameliorated these behavioral changes, although complete normalization was not achieved. These findings highlight the behavioral toxicity of chemical mixtures in aquatic organisms and suggest that antioxidant supplementation may provide a partial neuroprotective strategy.

1. Introduction

Pollution of aquatic environments with heavy metals and pharmaceutical residues represents a major ecotoxicological challenge, in the context of increasing industrial, agricultural, and medical activities. Heavy metals such as arsenic, and antibiotics such as AMP, frequently reach surface waters through industrial or agricultural effluents, contributing to the degradation of aquatic ecosystems and affecting the behavior of non-target species (Kümmerer, 2009; Shaji *et al.*, 2021). Arsenic, especially in inorganic form, it is known for its high toxicity and ability to induce oxidative stress, epigenetic alterations, and neurological dysfunctions, being classified by International Agency for Research on Cancer as a group 1 carcinogen (International Agency for Research on Cancer, 2012).

Antibiotics, even in low concentrations, can significantly influence the physiology and behavior of aquatic organisms, affecting the microbiota-brain-behavior axis (González-Pleiter *et al.*, 2013). For example, ampicillin, a β-lactam antibiotic of the penicillin class, is frequently used in the treatment of bacterial infections and also as an

experimental agent in microbiome, toxicology, and immunomodulation studies. Animal models such as zebrafish and laboratory rats are widely used to evaluate the pharmacological and toxic effects of this compound (Uddin *et al.*, 2021). Notably, some of the behavioral impairments associated with streptococcal antigen exposure can be partially alleviated by antibiotic treatment, supporting the potential prophylactic efficacy of agents such as ampicillin in post-streptococcal neuropsychiatric disorders (Lotan *et al.*, 2014). At the same time, scientific interest in natural compounds with antioxidant potential has increased, with QUE being intensively studied for its neuroprotective and anti-inflammatory effects (Boots *et al.*, 2008; Li *et al.*, 2016). By reducing levels of reactive oxygen species (ROS) and stabilizing cellular signaling pathways, QUE may play a key role in combating oxidative stress induced by contaminants. Also, in adult zebrafish, amoxicillin administration causes a decrease in locomotor activity and social interactions, concomitant with an increase of oxidative stress in the brain, surprisingly reflecting some clinical symptoms associated with autism (Gonçalves *et al.*, 2020).

The toxic mechanism of As involves, in the initial phase of exposure, an intensification of the production of ROS and reactive nitrogen species (RNS). These induce structural and functional changes in intracellular macromolecules, such as nucleic acids, proteins, and lipids, thus affecting cellular homeostasis and favoring the occurrence of pathological processes. Normally, the body has an endogenous antioxidant system, composed of protective enzymes such as glutathione peroxidase, superoxide dismutase, and catalase, designed to counteract oxidative stress (Valko *et al.*, 2006; Flora, 2011; Chakraborty *et al.*, 2014; Hu *et al.*, 2020). Similarly, behavioral impairments such as anxiety, cognitive dysfunction, and reduced social interaction have been observed in zebrafish and rodents following exposure to As, highlighting the vulnerability of the neurobehavioral axis to both environmental toxicants and pharmacological agents (Sánchez-Santed *et al.*, 2016; Mandal *et al.*, 2022). Exposure to As can trigger persistent inflammation in the body (Duan *et al.*, 2022), and ampicillin, also having an immunomodulatory effect, can alter this reaction, having an impact on the immune system, which could lead to a reduction in the effectiveness of antibiotic treatment or an overload of the immune system (Li *et al.*, 2023). Moreover, low concentrations of arsenite (As^{III}) have been shown to significantly reduce zebrafish larval survival and activate apoptotic pathways, particularly through upregulation of Caspase-3, Caspase-9, and the Bax/Bcl-2 ratio, indicating that As can also compromise immune integrity via apoptosis (Zhao *et al.*, 2021). Complementary to these findings, chronic As exposure, even at ecologically relevant levels, has been associated with neurological dysfunction, increased oxidative stress in tissues of organs such as the liver and eye, as well as transgenerational effects, suggesting that the apoptotic mechanisms activated by As^{III} may have long-term implications on cellular homeostasis and organismal development (Hallauer *et al.*, 2016). Therefore, evaluating the combined effects of these substances is particularly relevant, given the possibility of synergistic or antagonistic interactions, which could amplify or attenuate individual toxic effects.

Zebrafish is an ideal model organism in behavioral ecotoxicology studies, due to its small size, and ease of handling, but especially its behavioral sensitivity to chemical stressors (Steenbergen *et al.*, 2011). Alterations of behaviors such as motor activity or social interactions are sensitive indicators of neurological toxicity and allow rapid assessment of the impact of contaminants (Kalueff *et al.*, 2013).

In this context, the present study aimed to evaluate the behavioral effects of acute (96 h) exposure to As and AMP, as well as to determine whether co-administration of QUE can have a protective effect on the behavior of zebrafish. By using standardized swimming and sociability tests, the study contributes to the understanding of the mechanisms involved in the neurotoxicity of chemical contaminants and explores potential strategies to mitigate adverse effects through natural compounds.

2. Materials and Methods

2.1. Chemicals

Three chemicals were used in this study: As, AMP and QUE. As was administered as a certified standard solution (1000 mg L⁻¹ As in 0.5 M HNO₃, Certipur®), and AMP and QUE were purchased from a local pharmacy; to avoid any conflict of interest, the trade names of the products used have been omitted. The choice of these commercial forms reflects the intention to simulate a realistic exposure scenario, as such substances can enter aquatic ecosystems through common human uses.

2.2. Model organism and maintenance conditions

Adult zebrafish (*Danio rerio*), aged 6–8 months, were purchased from a licensed breeder in Iasi and acclimatized for two weeks in 100 L glass aquariums at the Ecotoxicology Laboratory. During this period, the fish were maintained under controlled environmental conditions: constant water temperature of 21 °C, day/night cycle lighting regime (12:12 h), with lights on at 07:00 and off at 19:00, light intensity being 307.5 lux.

Feeding was performed twice daily with standardized portions of 2 grams of TetraMin Tropical Flakes per experimental group, according to species-specific protocols. Water quality was monitored daily using a Hanna HI 9828 portable multiparameter, which allows the analysis of up to 13 water parameters (6 directly measured and 7 calculated). Parameters monitored included pH, temperature, salinity, total dissolved solids (TDS), electrical conductivity, and oxidation-reduction potential (ORP). The equipment was calibrated daily for measurement accuracy, especially for standardization of pH, conductivity, and dissolved oxygen.

2.3. Experimental design

The experiment was conducted on four groups: Group 1 (Control): no substance administration; Group 2 exposed to QUE; Group 3 exposed to As and AMP, and Group 4 exposed to the mixture of the three substances.

All substances were diluted in dechlorinated water at concentrations established based on the specialized literature. The exposure was acute, for 96 hours. After this period, the individuals were euthanized by rapid immersion in water cooled to 2–4 °C, as recommended in the AVMA Guidelines (2020).

2.4. Behavioral Assessments

Zebrafish were subjected to a standardized social preference test (Figure 1a) to assess the neurobehavioral effects of exposure, by using a T-maze. Behavioral recordings were performed daily throughout the 96-hour exposure period. Each test session lasted 4 minutes per individual. Videos were analyzed using the EthoVision XT 11.5 tracking system (Noldus Information Technology, Wageningen, The Netherlands) (Figure 1b), a widely used automated software for quantitative analysis of animal behavior.

The following parameters were recorded and analyzed: total distance moved (cm), velocity (cm/s), time spent in the left arm (s), time spent in the right arm (s), time spent in the start arm (s), cumulative duration of mobility state (s), cumulative duration of immobility state (s). These behavioral metrics allowed for sensitive detection of locomotor and social interaction changes, providing insight into the neurotoxic effects, and potential neuroprotective effects of the tested substances. A schematic representation of the full experimental protocol is provided in Figure 2.

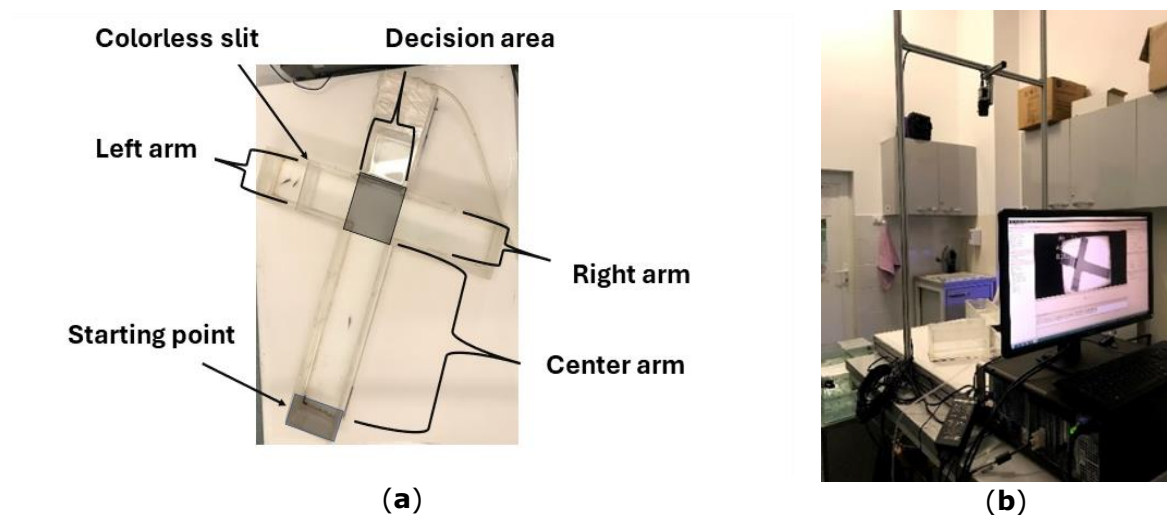


Figure 1. Experimental setup and behavioral tracking system for the social preference test in zebrafish. (a) T-maze used for assessing sociability in adult zebrafish, composed of a center arm, a starting point, two lateral arms (left arm associated with the social stimulus, and right arm without stimulus), and a decision area at the intersection. A transparent slit was placed to separate the conspecific from the test fish. (b) Overhead view of the experimental setup showing the EthoVision XT 11.5 video-tracking system used to monitor and quantify zebrafish behavior.

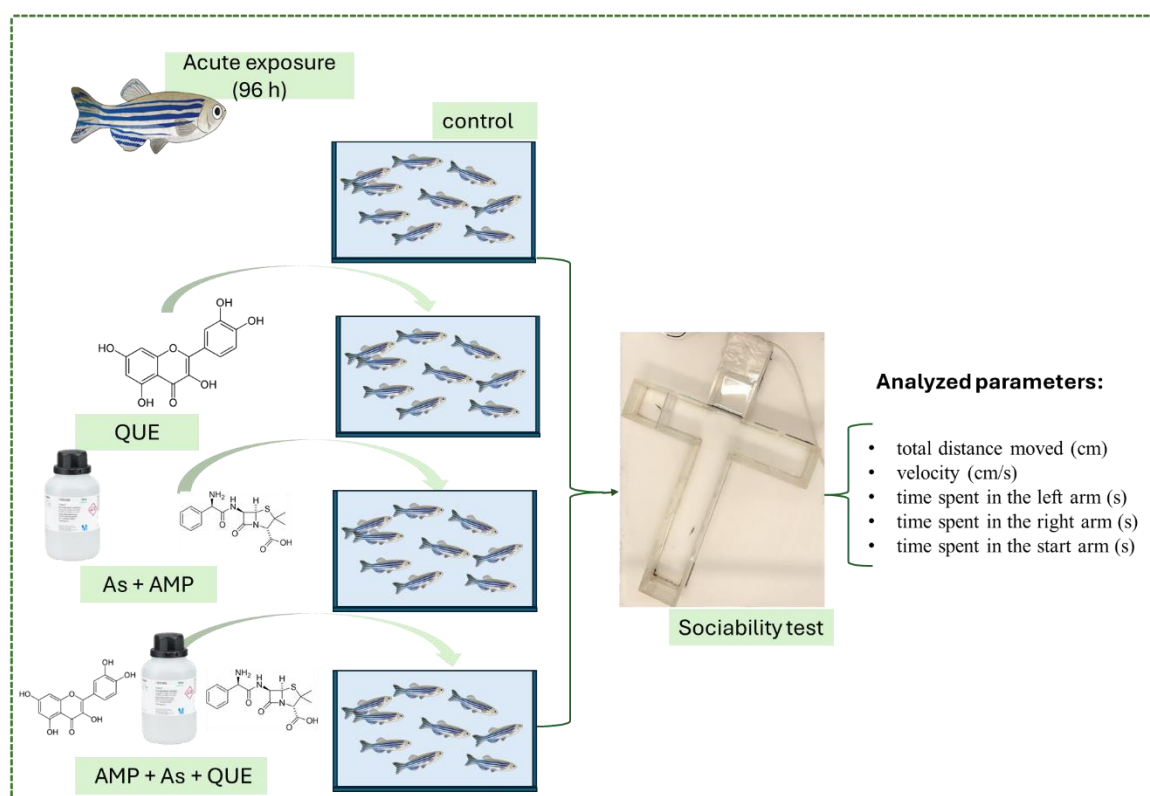


Figure 2. Experimental Protocol: Acute Exposure to As, AMP, and QUE, and Sociability test in Zebrafish

3. Results

We measured the total distance traveled by zebrafish in a T-shaped maze to assess the effects of substance administration on locomotor behavior (Figure 3a). Concomitant administration of As+AMP led to a significant reduction in total distance traveled ($p < 0.001$), compared to the control group. Similarly, the group receiving the combined administration of As+AMP+QUE exhibited a significant decrease in distance moved relative to the Control group ($p = 0.010$). The distance moved by the As+AMP group was significantly less than that observed in the group administered with QUE alone ($p = 0.021$). No significant differences were observed between the Control and the QUE groups, indicating that QUE administration alone does not substantially affect locomotor behavior. These variations in distance moved reflect the cumulative effects and potential pharmacological interactions between As, ampicillin, and QUE in terms of locomotor behavior, potentially extending to parameters of social behavior in zebrafish.

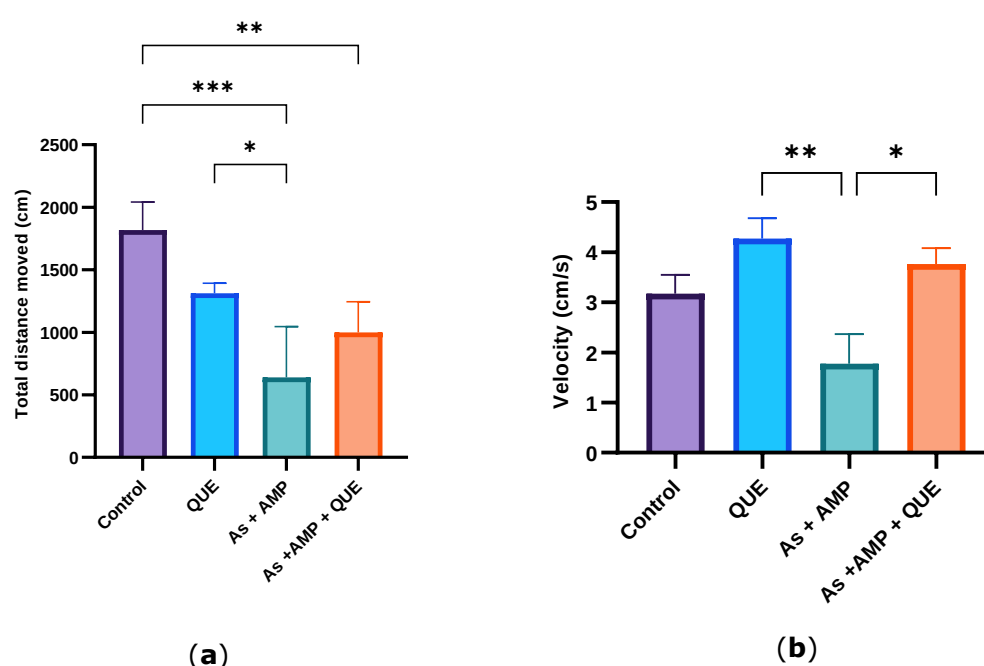


Figure 3. Effects of arsenic (As), ampicillin (AMP), and quercetin (QUE) on zebrafish sociability test in the T-maze. (a) Total distance moved (cm). (b) Velocity (cm s^{-1}). Data are presented as mean $\pm$ standard deviation (SD) ($n=5$ per group). Statistical analysis was performed using one-way ANOVA, followed by Tukey's multiple comparison test. Asterisks indicate statistical significance: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

Average velocity (Figure 3b) followed a similar pattern. The Control group exhibited a mean velocity of approximately 3 cm s^{-1} , whereas fish exposed to As+AMP displayed a pronounced reduction. QUE administration partially improved locomotor performance in the As+AMP+QUE group, with statistically significant differences compared to the As+AMP group ($p = 0.005$ and $p = 0.023$, respectively).

In the social preference test, the time spent in the left arm (associated with the social stimulus - a conspecific) (Figure 4) was significantly influenced by exposure to the substance. Administration of the As+AMP mixture resulted in a significant reduction in the time spent in the social arm compared to the Control group ($p = 0.034$) (Figure 4a). Although the QUE and As+AMP+QUE groups also demonstrated reduced occupancy in the social arm compared to the control group, these differences did not reach a statistical significance.

Similarly, the time spent in the right arm (lacking social stimulus) was significantly affected (Figure 4b). The As+AMP group spent a significantly longer period on the right

arm compared to both the Control group ($p=0.011$) and the QUE group ($p=0.036$). The As+AMP+QUE group also showed a significantly increased duration in the right arm compared to the Control ($p=0.022$) and QUE groups ($p=0.019$).

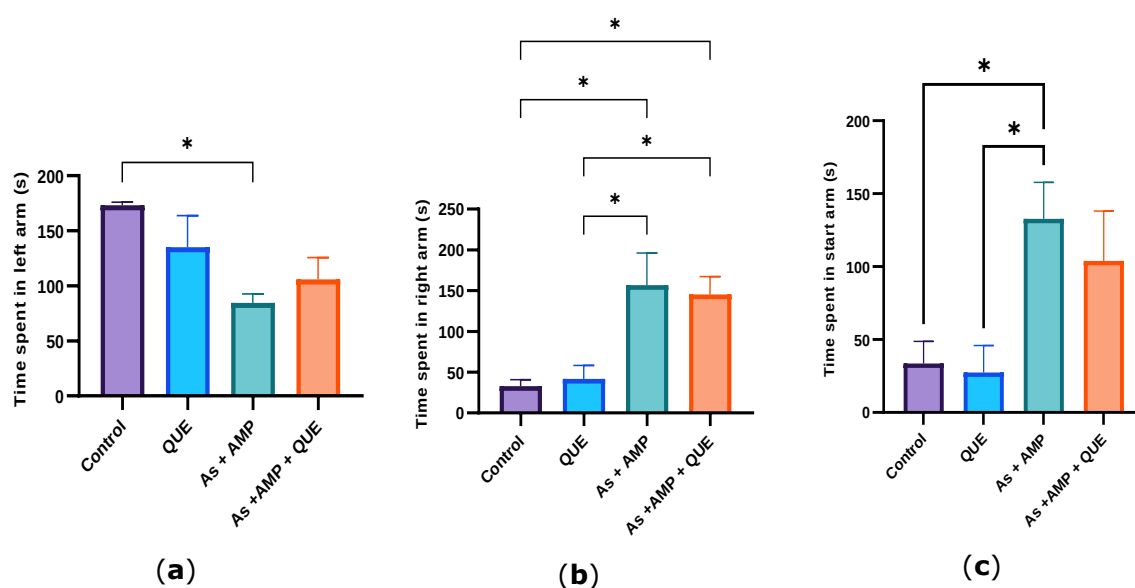


Figure 4. Effects of arsenic, ampicillin, and quercetin on zebrafish social preference in the T-maze test. (a) Time spent in the left arm (s). (b) Time spent in the right arm (s). (c) Time spent in the start arm (s). Data are presented as mean $\pm$ standard deviation (SD) ($n=5$ per group). Statistical analysis was performed using one-way ANOVA, followed by Tukey's multiple comparison test. Asterisks indicate statistical significance: * $p < 0.05$.

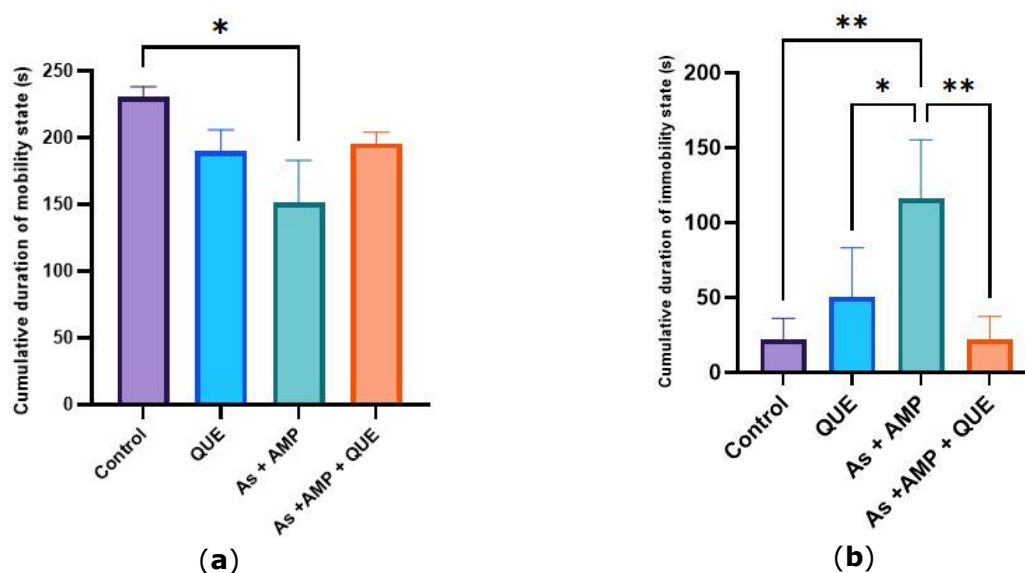


Figure 5. Effects of arsenic, ampicillin, and quercetin on cumulative movement behavior in zebrafish during the T-maze test. (a) Cumulative duration of mobility state (s). (b) Cumulative duration of immobility state (s). Data are expressed as mean $\pm$ standard deviation (SD) ($n=5$ per group). Statistical significance was assessed by one-way ANOVA, followed by Tukey's post hoc test. Asterisks indicate statistical significance: * $p < 0.05$, ** $p < 0.01$.

Comparable trends were observed for time spent in the start arm (Figure 4c). The As+AMP group showed a significant increase in duration compared to both the Control group ($p=0.0321$) and the QUE group ($p=0.0221$). Although co-administration with QUE slightly attenuated this parameter, the difference did not reach a statistical significance.

The cumulative duration of mobility state (Figure 5a), as an index of general activity, was significantly reduced in the As+AMP group compared to the Control group ($p=0.0471$). Furthermore, the cumulative duration of the immobility state (Figure 5b) was significantly increased in the As+AMP group compared to the Control ($p = 0.0320$) and QUE groups ($p = 0.0031$). Co-administration of QUE resulted in a significant reduction of immobility compared to the As+AMP group ($p = 0.0031$), suggesting a partial compensatory effect on the toxin-induced motor deficits.

4. Discussion

The results obtained highlight the synergistic neurotoxic effects of combined exposure to As and AMP on the locomotor behavior of zebrafish, with potential extension also on social behavior. The significant reduction in the distance traveled and the average speed in the As+AMP group suggests a severe impairment of motor functions, possibly mediated by damages in the central and peripheral nervous system. These data are consistent with previous studies that have shown that As can induce peripheral neuropathies and alterations in dopaminergic neurotransmission, which is reflected in deficient locomotor behaviors (Flora, 2011). As is known to induce oxidative stress and apoptosis in neural tissue, even at sublethal doses, by activating caspases 3 and 9, as well as by unbalancing the Bax/Bcl-2 ratio (Zhang *et al.*, 2021). These processes can compromise the cellular integrity of motor neurons, affecting the ability to move and respond to stimuli. In addition, sodium arsenite (As^{III}) has been associated with reduced survival rates in zebrafish larvae and persistent behavioral dysfunction (Hallauer *et al.*, 2016). Concomitant administration of AMP appears to potentiate the toxic effects of As, possibly through induced changes in the gut microbiota or through pharmacodynamic interactions that amplify systemic oxidative stress. Although the literature on the combined effects of heavy metals and antibiotics on animal behavior is still limited, current data support the hypothesis that simultaneous exposure to multiple contaminants can generate complex biological responses, often nonlinear and more pronounced than those elicited by each substance alone (Martin, 2023). Notably, adjuvant treatment with quercetin (QUE), a flavonoid with antioxidant properties, partially reduced the impairment of locomotor performance caused by combined As and AMP exposure. This protective effect supports the hypothesis of a central involvement of oxidative stress in the observed pathogenesis. Quercetin is known for its ability to reduce ROS and RNS levels, stabilize cell membranes, and modulate key redox pathways such as Nrf2/ARE, while inhibiting apoptosis by suppressing caspase activation (Khan *et al.*, 2016; Li *et al.*, 2020). Therefore, the improvement in behavioral parameters in the As+AMP+QUE group may reflect a partial restoration of neuronal function affected by oxidative stress and apoptotic imbalance.

In addition, the administration of As+AMP significantly increased time spent in the right arm, suggesting impairments in social preference behavior. These findings are consistent with hypotheses that propose that environmental contaminants disrupt neuronal circuits involved in the processing of social stimuli, particularly dopaminergic and serotonergic pathways (Rajeshkumar *et al.*, 2017; Hama Aziz *et al.*, 2023). Furthermore, chronic exposure to As has been shown to induce anxiety-like behaviors in zebrafish, as evidenced by increased latency to explore new environments and reduced time spent in upper tank zones, behaviors indicative of heightened anxiety (Wang *et al.*, 2023). Such changes may underlie the shift in preferences towards non-social areas observed in our study.

Administration of QUE, a flavonoid recognized for its antioxidant and neuroprotective properties (Boots *et al.*, 2008; Li *et al.*, 2016), exerted a partial protective effect.

Co-administration of As+AMP+QUE in zebrafish displayed significant improvements in locomotor parameters compared to the As+AMP group, although a full return to control levels was not achieved. Notably, QUE administered alone induced a slight reduction in locomotor activity, suggesting that, in certain contexts, QUE may independently influence motor and social behaviors, even in the absence of toxicological stress.

Regarding time spent in the arms of the T-maze, we found that combined exposure to As and AMP causes a significant reduction in social preference in zebrafish. This behavioral change is manifested by a decrease in the time spent in the arm associated with the social stimulus and an increase in the time spent in the opposite arm, devoid of the social stimulus (Peng *et al.*, 2025; Pinho *et al.*, 2023). These observations suggest an impairment in the processing of social stimuli, possibly due to dysfunction of neural circuits involved in social behavior (Hosaka *et al.*, 2024). Regarding the time spent in the social stimulation arm, its reduction in the treated groups suggests a compromised motivation for conspecific interaction, potentially related to hypothalamic-pituitary-adrenal axis dysfunction and increased oxidative stress (Rajeshkumar *et al.*, 2017). The inability of combined As+AMP+QUE administration to completely normalize social behavior highlights the limitations of antioxidant interventions in the context of severe neurotoxicity.

Overall, these results support the hypothesis that chemical stressors and pharmacological interventions can profoundly modulate fundamental behaviors such as exploration and sociability in aquatic model organisms. These findings have important implications for understanding the ecological impact of pollution and for developing neurobehavioral protective strategies.

5. Conclusions

The present study demonstrates that acute combined exposure to As and AMP induces significant neurobehavioral disorders in zebrafish, reflected by reductions in social preference parameters. The pronounced decrease in total distance moved, velocity, and time spent in the three arms of the T-maze, as well as the decrease in time spent in the social stimulation arm, confirms the neurotoxic potential of these contaminants, probably mediated by oxidative stress and disruption of neurotransmitter systems. Co-administration of QUE partially ameliorated locomotor deficits and improved social behavior, highlighting its antioxidant and neuroprotective potential. However, the failure of QUE to completely reverse behavioral impairments suggests that antioxidant supplementation alone may not be sufficient to counteract the severe neurotoxicity induced by combined chemical exposures. These findings highlight the complex interplay between environmental pollutants and biological defense mechanisms, providing valuable insights into the risks associated with aquatic contamination and the potential role of natural compounds in neurobehavioral protection strategies.

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