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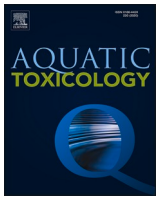

Assessing habitat selection in the prawn *Macrobrachium rosenbergii* using the model toxicant copper and colonization as a test endpoint: Does prior exposure determine biochemical and behavioral responses?

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ABSTRACT

Habitat selection by aquatic organisms is dependent on the availability of adequate conditions to support life and the benefits that the habitat provides. Contaminated environments tend to be less attractive to organisms because reduced habitat quality leads to increased maintenance costs. Consequently, reduced colonization of such disturbed habitats is an expected response. However, colonization has been understudied as an ecotoxicological test endpoint, despite its proven ability to assess habitat selection by populations across various taxa. The aim of the present study was to investigate whether previous exposure to copper could alter the colonization behavior of the freshwater prawn *Macrobrachium rosenbergii* along a non-forced exposure gradient of interconnected copper-contaminated habitats (0 to 500 μg/L) due to physiological and behavioral impairments. To assess this, post-larvae of *M. rosenbergii* were pre-exposed to 0, 50, 250 and 500 μg/L copper for a maximum period of 48 h. The physiological status and motility of the organisms after pre-exposure to copper were evaluated using behavioral endpoints (swimming activity by video tracking) and biochemical biomarkers (biotransformation, oxidative stress and neurotoxicity). The results indicated that pre-exposure to copper (at concentrations of 0, 50 and 500 μg/L) significantly influenced the median colonization concentration (CC50), which decreased from 270 μg/L to 109 μg/L. None of the assessed swimming parameters (speed, motility rate, exploration rate, and total distance) were affected by the pre-exposure to copper (0, 50 and 250 μg/L). Biochemically, cholinesterase levels were only affected in the prawn population exposed to 250 μg/L of copper. The present study provides a better understanding of the relevance of colonization as an ecotoxicological endpoint for assessing the spatial distribution of populations, including both new inhabitants and previously exposed organisms, in recovering habitats.

1. Introduction

The impact of contamination on the colonization of new or recovering habitats by aquatic organisms is an area that has been underexplored in aquatic ecotoxicological assessments (Araújo et al., 2018; Ribeiro et al., 2018). The distribution of contaminants in spatially extended aquatic systems, such as rivers, estuaries, coastal areas, and large lakes, can be heterogeneous, resulting in spatial gradients of contamination. This chemical heterogeneity promotes the formation of patches or areas that are less attractive to organisms due to differences in contamination levels, which influence the habitat's ability to support the establishment of a population and, consequently, affect the dispersal patterns of the organisms. Thus, biodiversity in some areas (habitats) may be reduced, not necessarily because of the direct physiological toxicity of contaminants, but because some organisms can detect contaminants and move away (by active swimming or passive drift) to select more favorable habitats (Knillman et al., 2018: Brittain and Eikeland, 1988; Humphries and Ruxton, 2003; Araújo et al., 2016). Given the above scenarios, adopting a contamination-driven habitat selection approach is essential for evaluating the relevant ecotoxicological effects

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of aquatic contamination in disturbed habitats.

The use of systems with non-forced exposure conditions has allowed for the study of how contamination affects a population's colonization of an ecosystem (Araújo et al., 2017; Moreira-Santos et al. 2019). The conceptual basis of the non-forced exposure approach is based on two conditions: i) the environment must be chemically heterogeneous so that organisms have access to areas with different levels of contamination, and ii) the organisms can move freely through these areas so that exposure to the contaminant is not forced or mandatory. Non-forced exposure systems consist of many interconnected compartments, each containing different concentrations of the contaminant, thereby creating a gradient of contamination. Although the first study that used a non-forced multi-compartment exposure system was published two decades ago (Lopes et al., 2004), the colonization response of aquatic organisms in these exposure scenarios has only recently begun to be investigated (Araújo et al., 2018; Islam et al., 2019; Salvatierra et al., 2022; Vera-Herrera et al., 2022; Stremmel et al., 2023). Initially, Araújo et al. (2018) showed an association between the avoidance and colonization responses of *Danio rerio* to copper, known as the avoidance-recolonization hypothesis. They found a negative correlation between the concentrations that caused avoidance and those that allowed for colonization, resulting in equal median avoidance (AC50) and median colonization (CC50) concentrations (AC50 = CC50). Later, Islam et al. (2019) found that copper strongly inhibited the colonization of new areas by zebrafish, even when these areas offered attractive features such as high food availability. A similar pattern was observed in the estuarine shrimp *Palaemon varians*, also exposed to copper. Although food was a highly attractive factor, the colonization response was primarily determined by predation pressure and contamination levels (Salvatierra et al. 2022). This was also observed in the freshwater shrimp *Atyaephyra desmarestii*, which preferred a moderately contaminated area when predation pressure was higher in the uncontaminated zone (Araújo et al., 2020). Finally, Stremmel et al. (2023) observed that the antidepressant fluoxetine did not prevent colonization by *D. magna*, even at highly toxic concentrations. As a result, there is an increased risk associated with this compound due to its tendency to attract, rather than repel, the organisms. The above studies highlight that, due to the presence of multiple (stress) stimuli in natural environments, a direct relationship between colonization and avoidance is not always expected. Contamination may prevent habitat colonization, but different patterns can emerge if contaminated areas provide other benefits, such as attractive chemicals, abundant food, and shelter, or if uncontaminated areas have stressors such as high predation pressure.

Although the chosen habitat should ultimately provide more benefits than costs (Hamilton 2019), contaminants can impair the organisms' ability to perceive the environment and/or move, thus affecting their capacity to select the most favorable habitat. It is well-documented that contaminants can affect the swimming patterns of many aquatic organisms (Barboza et al., 2018; Bringer et al., 2020; Cormier et al., 2022). Copper was found to alter the swimming behavior of the estuarine shrimp *Penaeus vannamei* and *P. varians*, reducing the movement of the former and stimulating the movement of the latter, although both species detected and avoided the highest copper concentrations (Redondo-López et al., 2023). If changes in motility can affect the ability of organisms to colonize an environment, then biochemical changes can impact this ability in the same way, as behavior is an integrative expression of the biochemical and physiological state of an organism (Scott and Sloman, 2004; Zala and Penn, 2004). With respect to copper, biochemical disruptions have been demonstrated in several decapods, such as *Pachygrapsus marmoratus* (Oliva et al., 2019) and *Eriocheir sinensis* (Sun et al., 2014). In these and other species, copper induced responses in biomarkers of neurotoxicity (inhibition of cholinesterase activity - ChE), biotransformation (glutathione-S-tranferase - GST, and ethoxyresorufin-O-deethylase - EROD) and oxidative stress (catalase activity - CAT, and lipid peroxidation - LPO) (Frías-Espericueta et al., 2022). A correlation between biochemical disruption (ChE activity) and the ability to detect and avoid contamination was demonstrated in *D. magna* exposed to a pesticide mixture of chlorpyrifos and terbuthylazine (Vera-Herrera et al., 2022). Although a correlation between AChE inhibition and avoidance was observed in *D. rerio* exposed to fipronil, this correlation was not observed in the fish *Hyphessobrycon eques* when exposed to either fipronil or 2,4-D (Moreira et al., 2021). Therefore, the behavioral and biochemical impairments that organisms may experience due to exposure to contaminants appear to strongly influence their ability to colonize new or recovering habitats.

Given the evidence discussed above, the main motivation of the present study was to investigate how pre-exposure to copper affects the colonization response of the prawn *M. rosenbergii* to environments contaminated with this metal. To explore possible behavioral and physiological mechanisms underlying the process of habitat selection, we also evaluated swimming patterns (behavioral impairment) and biomarkers (biochemical impairment: ChE, CAT, EROD, GST, LPO) in control and pre-exposed organisms prior to their use in colonization tests. Thus, the current study aimed to apply an integrative approach to assess and understand habitat selection by *M. rosenbergii* in a copper contamination scenario by measuring responses across three levels of biological organization: colonization, behavior, and biomarkers.

2. Materials and methods

2.1. Test organism: Macrobrachium rosenbergii

Post-larvae of *M. rosenbergii* were purchased from a local producer (Langostinos del Río, San Mateo, Alajuela, Costa Rica). Upon arrival at the laboratory, the animals were placed in a 150 L aquarium filled with UV water (tap water purified through a 1 μm filter with granular activated carbon and treated with ultraviolet light; Millipore®) and maintained at 22–24 °C under a 10 h light:14 h dark photoperiod with white-cool fluorescent light (680 lux). The aquarium was equipped with a biological filter and a quarter of the water was renewed weekly. Food (Nicovita Katal Camarón 35 % - 2.5 HO) was provided *at libitum* once a day and the animals used in the tests were not fed for 24 h prior to testing. The animals were acclimated to the laboratory conditions for at least 6 days before testing. Cumulative mortality during the acclimation period was <1 %. Animals used in the tests ($n = 390$) had a mean weight of 0.57 ± 0.26 g and a mean carapace length of 0.99 ± 0.16 cm.

A second batch of organisms was subjected to the same acclimation conditions as the first batch; however, they unexpectedly showed much higher sensitivity to copper (mortality during pre-exposure) and higher activity levels during the assessment of swimming parameters. For this reason, the results obtained for the two batches of organisms are not directly comparable. However, due to their relevance and potential usefulness in designing future studies, the results from the second batch of organisms are included in the Supplementary Materials.

2.2. Non-forced exposure system

Avoidance and colonization tests (see Sections 2.4 and 2.8) were carried out using a non-forced exposure system consisting of five interconnected plastic compartments to create an unrestricted gradient, as shown in Fig. 1. Before each test, the connections between the compartments were sealed with plastic foam plugs, the compartments were filled with their respective copper concentrations (0, 5, 50, 250 and 500 μg/L), and the animals were placed in their designated compartments. To initiate the test, the plugs were removed, allowing the animals to move freely between the compartments. All tests were carried out in the dark at 23 °C with three simultaneous replicates. The position of the animals was recorded every 20 min during the 3 h exposure period, using a red light to avoid any disturbance during the counting. Samples for chemical analysis were collected at the beginning of the test before the removal of the plugs and at the end of the test after the plugs were reinserted.

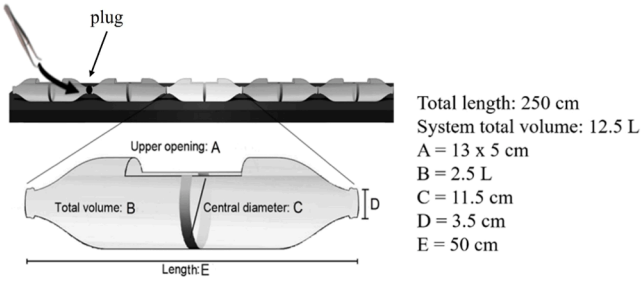

Fig. 1. Multi-compartment exposure system used in the non-forced exposure avoidance and colonization tests. In each test, plugs were inserted into the connections between compartments using tweezers to fill each container with its corresponding copper solution, and were then removed at the time 0 to allow the animals to move freely.

2.3. Test substance: copper

Copper was used as a test substance because of its well-established role as a contaminant in aquatic ecosystems and its proven capacity to cause physiological and behavioral effects in crustaceans (as discussed above). Additionally, numerous studies have demonstrated copper's ability to trigger avoidance responses in aquatic organisms (Moreira-Santos et al., 2019; Alcívar et al., 2021), including freshwater and estuarine decapods (Redondo-López et al., 2023; Vera-Vera et al., 2019). The copper solutions used for testing were prepared by adding aliquots of a pre-made Cu stock solution (150 mg Cu/ml, prepared with $CuSO_4$ in culture water) to the water used for prawn culture.

The actual copper concentrations during the colonization test were verified (see Section 2.8) by inductively coupled plasma mass spectrometry (ICP-MS) following the ISO 17294-2 method (ISO, 2016). At the beginning of the test, samples from each copper compartment were collected from the three test replicates and pooled for each concentration (five in total). At the end of the 3-h exposure period, final samples were collected from each replicate and tested individually for each concentration. The results of the measured copper concentrations during the colonization test are presented in Table 1 and demonstrate that the gradient in the system was maintained throughout the 3 h exposure period. The concentrations between adjacent compartments never overlapped and a low variability was observed among replicates at the end of the test. Therefore, all results of the present study are presented in terms of nominal copper concentrations.

2.4. Avoidance test

An avoidance test was conducted to determine the range of copper concentrations that elicited an avoidance response in *M. rosenbergii*. The median avoidance concentration (AC50) and the AC20, which represents the lowest copper threshold that elicits avoidance in prawns, were calculated. It is expected that the defined concentration range can be used to measure a colonization response, which is considered the inverse of avoidance. The range of copper concentrations for the avoidance test was selected based on levels found in freshwater ecosystems affected by human activities (Kouasssi et al., 2022; Quirós-Bustos et al., 2022). Consequently, a gradient of 0, 5, 50, 250 and 500 μg/L of copper was established in the multi-compartment system and 60 prawns were randomly distributed among the replicates of the open gradient system (20 individuals/replicate; 4 individuals/compartment), following the procedures and conditions described in Section 2.2. The 3 h avoidance concentrations (ACx) estimated from this test (see Results Section 3.1) confirmed that the same copper gradient (0 to 500 μg/L) would be adequate for conducting the colonization tests.

Table 1
Nominal (N) and actual (A) copper concentrations (in μg/L) quantified for the five compartments of the non-forced exposure system at the start ($n = 1$) and at the end (mean ± standard deviation, $n = 3$, range of minimum and maximum values) of the 3 h colonization test.

N concentration	A initial concentration	A final concentration
0	3.18	15.1 (± 1.64) (13.7–16.9)
5	9.33	35.0 (± 1.81) (33.1–36.7)
50	56.1	88.5 (± 11.2) (77.7–100)
250	273	258 (± 7.77) (252–267)
500	506	467 (± 20.6) (454–491)

2.5. Pre-exposure to copper

To investigate whether pre-exposure to copper would influence the colonization response and induce behavioral or physiological effects on *M. rosengergii*, pre-exposure concentrations were set within the range that elicits an avoidance response. Accordingly, four concentrations were used during the pre-exposure period: control (no copper), 50 μg/L, 250 μg/L and 500 μg/L. However, due to a shortage of organisms (as explained below), only those pre-exposed to 0, 50 and 500 μg/L copper were used for the colonization test, while prawns pre-exposed to 0, 50 and 250 μg/L copper were used for the motility and biochemical endpoints.

Pre-exposure was performed using plastic containers filled with 4 L of the corresponding copper concentration, prepared as described in Section 2.3. For each pre-exposure concentration, 10 replicate containers were set up with seven organisms/container and an aeration tube for continuous oxygenation. The pre-exposure period was defined as 48 h, a duration considered sufficient to induce measurable physiological and behavioral changes in decapods due to copper and other contaminants (Frías-Espericueta et al., 2022; Jiang et al., 2021; Redondo-López et al., 2023; Thatipaka et al., 2020). However, if mortality occurred during pre-exposure (at the highest concentrations), exposure to copper was terminated by transferring the organisms to clean water when mortality was close to 20 %. Therefore, the organisms pre-exposed to 50 μg/L remained in the medium for the full 48 h, but organisms pre-exposed to 250 and 500 μg/L copper were transferred to clean water after 24 h and 6 h, respectively, and remained there until the end of the 48 h period. Dead organisms were immediately removed from the containers throughout the pre-exposure period.

2.6. Behavioral endpoints: swimming patterns

After the pre-exposure period, 10 prawns from each treatment were individually placed in a circular recipient (15 cm diameter) with clean (no copper) water (3 cm deep). The area surrounding the recipient was isolated from external stimuli using a cardboard box. A small hole was made in the top of the box and a camera was inserted. The organisms were kept in the recipient for 30 s and then recorded for 5 min. During the recording, the recipient was illuminated from below with a LED light. After this period, the videos were analyzed using the ToxTrac software v.2.96 (https://sourceforge. net/projects/toxtrac/) according to Rodriguez et al. (2018), ensuring that the visibility rate of the prawns in the videos was higher than 0.9. The variables selected for analysis were average speed, motility rate, exploration rate, and total distance. Additionally, parallel tests were conducted with copper-treated water at the pre-exposure concentrations to determine whether transferring the organisms to clean water affected their swimming behavior. However, no differences were observed between the two conditions ($p>0.05$). Therefore, only results from videos recorded in clean water are presented.

2.7. Biochemical endpoints

Biomarkers were measured in prawns that were pre-exposed to copper for 48 h (0 and 50 μg/L treatments) or for 24 h exposure followed by 24 h in clean water (250 μg/L treatment). Biochemical analyses were

not performed on individuals pre-exposed to the highest copper concentration (500 μg/L) because the 6 h exposure to copper before transfer to clean water, implemented to avoid excessive mortality (see Section 2.5), was considered too short to induce biomarker responses.

Animals were weighed and measured (carapace length) after the pre-exposure and before sacrifice by decapitation. Muscle and hepatopancreas samples were collected and immediately preserved in plastic microtubes at −80 °C until biochemical analyses were performed. Cholinesterase activity (ChE) was assessed as a neurotoxicity biomarker; glutathione S-transferase (GST) and ethoxyresorufin-O-deethylase (EROD) were measured as biotransformation responses; lipid peroxidation (LPO) and catalase activity (CAT) were measured as oxidative stress-related markers.

Muscle samples were homogenized with a 100 mM phosphate (K_2HPO_4 / KH_2PO_4) buffer (pH 7.2). The homogenates were then centrifuged (10,600 g, 4 °C, 5 min), and ChE activity was measured in the supernatant using the methodology of Ellman et al. (1961), adapted to microplates by Guilhermino et al. (1996). The assay used 1 mM acetylthiocholine iodide as the substrate and 0.1 mM 5,5′-dithiobis (2-nitrobenzoic acid) (DTNB) as the conjugate. The reaction mixture was prepared in 100 mM phosphate buffer (pH 7.2). Reaction kinetics were measured at 415 nm for 10 min and expressed as nanomoles of substrate metabolized per minute per milligram of protein. Hepatopancreas samples were homogenized in a 100 mM phosphate buffer (pH 7.4). An aliquot of the homogenate was immediately separated and mixed with 0.2 mM butylated hydroxytoluene (BHT) for LPO analysis. The remaining homogenate was centrifuged (15,300 g, 4 °C, 20 min), and the supernatant was used for EROD, GST and CAT analyses. All biomarkers were normalized to the protein concentration in the sample according to the Bradford method (1976) with a BioRad® kit and bovine serum albumin as the protein standard. GST activity was measured in a microplate according to the method of Habig et al. (1974) by assessing the conjugation of 1 mM reduced glutathione (GSH) to 1 mM 1-chloro-2, 4-dinitrobenzene (CDNB). The reaction mixture was prepared in 200 mM phosphate buffer (pH 6.5). The kinetics were recorded at 340 nm for 3 min and the activity was expressed as nanomoles per minute per milligram of protein. LPO was measured by the thiobarbituric acid reactive species (TBARS) assay (Oakes and Van der Kraak 2003). The assay was performed by mixing the sample with 12 % trichloroacetic acid and 60 mM Tris-HCl (pH 7.4), supplemented with 0.1 mM diethylenetriaminopentaacetic acid and 0.73 % thiobarbituric acid. The reaction mixture was placed in a bath at 100 °C for 1 h. The reaction product was then centrifuged (10,600 g, 25 °C, 5 min), and the absorbance of the supernatant was measured at 535 nm. LPO levels were expressed as nanomoles of TBARS per milligram of protein. CAT activity was measured according to Aebi (1974) as the decrease in absorbance at 240 nm due to H_2O_2 consumption. The reaction was prepared in 50 mM phosphate buffer (pH 7.0) and the kinetics were recorded in a UV-transparent microplate for 20 s. The activity was expressed as micromoles of substrate metabolized per minute per milligram of protein.

2.8. Colonization tests

All colonization tests were carried out under the same conditions as the avoidance test in terms of abiotic factors (light and temperature), number of replicates, copper gradient, and exposure time. In these tests, exposure began with all the prawns placed in the uncontaminated compartment, as the purpose was to study their potential to colonize a copper-contaminated environment. Prior to performing the colonization tests, a control test was performed with all compartments filled with culture water to verify that a 3 h exposure was sufficient time for the prawns to colonize the entire system when under optimal conditions. A copper colonization test was then conducted with control test organisms, i.e., organisms that were never exposed to copper. Then, colonization tests were performed with the organisms pre-exposed to 50 and 500 μg/L of copper. Most tests included 20 organisms per replicate; however, due to a shortage of organisms and mortality in the highest pre-exposure treatment, the colonization test without pre-exposure (control) and the test following pre-exposure to 500 μg/L copper were performed with 15 individuals/replicate.

2.9. Data analyses

All analyses were performed in R, version 4.3.0 (R core Team 2023). For the avoidance test and all colonization tests, the data distribution was assessed for normality using the Shapiro-Wilk test, outliers were identified with the identify_outliers function, and differences in the distribution of the organisms among compartments during the 3 h exposure were analyzed with a mixed-design analysis of variance (ANOVA). This analysis included time as a within-subjects (repeated measures) factor and concentration (compartment) as a between-subjects factor. These functions are included in the rstatix package (Kassambara 2023). Sphericity of the repeated measures was evaluated using Mauchly's test, and if sphericity was violated, the Greenhouse-Geisser correction was applied to adjust the degrees of freedom. When statistically significant differences were observed for time or compartment, a pairwise *t*-test with a Bonferroni correction was used.

Avoidance and colonization percentages were calculated using the formulas described by Moreira-Santos et al. (2008) and Araújo et al. (2018), respectively. Effect concentrations (avoidance or colonization; ACx or CCx) and their 95 % confidence intervals (CI) were estimated using probit regression with the Ecotox package (Hlina et al., 2021). CC50 values at different times were compared between treatments using a paired *t*-test. Differences in biomarker responses and swimming pattern-related parameters were tested using ANOVA, with a post-hoc Tukey test applied when significant differences among treatments were detected.

For all comparisons, differences were considered significant at $p<0.05$.

3. Results

3.1. Avoidance test

In the avoidance test, prawns were distributed evenly along the copper gradient, except for the compartment with the highest copper concentration (500 μg/L), where the percentage of organisms was significantly reduced to 13 % compared to compartments with copper concentrations of 50 μg/L or lower, which had 21–23 % of the organisms (Fig. 2A). This result is consistent with the significantly higher avoidance response (35.6 %) observed at the highest copper concentration, compared to all concentrations of 50 μg/L or lower, where the maximum avoidance response was 8 % (Fig. 2B). Copper AC50 values could be calculated for two of the nine exposure periods, namely, 80 and 100 min, which were 62.9 μg/L (CI: 39.9 to 96.3) and 210 μg/L (CI; 167 to 269), respectively. The 3 h AC20, which indicates the threshold copper concentration that can elicit an avoidance response and is calculated by integrating all observation periods, was 183 μg/L (CI: 107 to 329).

3.2. Swimming pattern responses

To assess swimming behavior, four motility-related parameters (average speed, total distance, motility rate, and exploration rate) were evaluated for non-pre-exposed (i.e., exposed to control medium) and copper pre-exposed prawn populations . None of the four parameters showed significant differences among the control, 50 and 250 μg/L copper pre-exposed prawn populations (Fig. 3).

3.3. Biochemical responses

Biomarkers were assessed in prawns after pre-exposure to the control

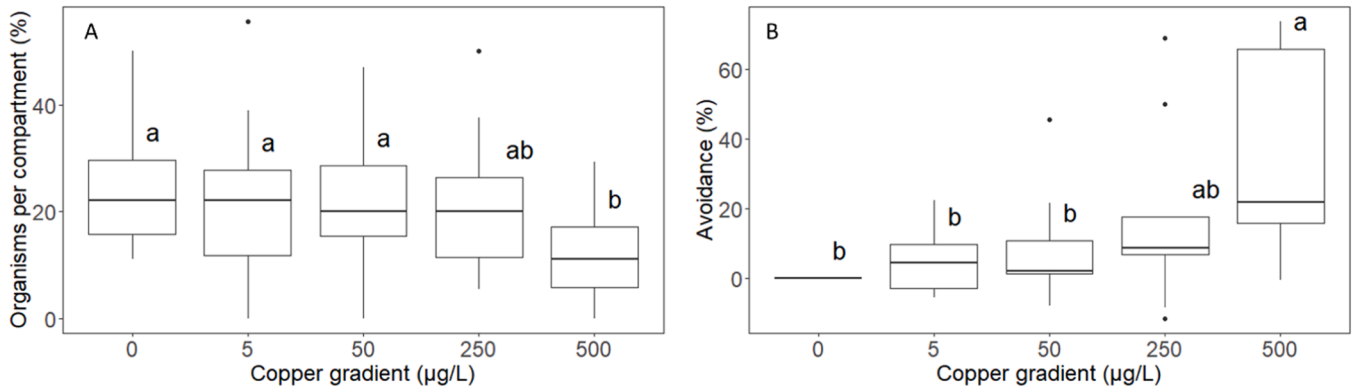

Fig. 2. Avoidance behavior of post-larvae of *M. rosenbergii* with non-forced exposure to a copper gradient for 3 h. A: percentage of organisms per compartment; B: percentage of avoidance relative to the expected number of organisms in the absence of copper. $N = 9$ observation times. Different letters indicate statistically significant differences (p<0.05) among compartments.

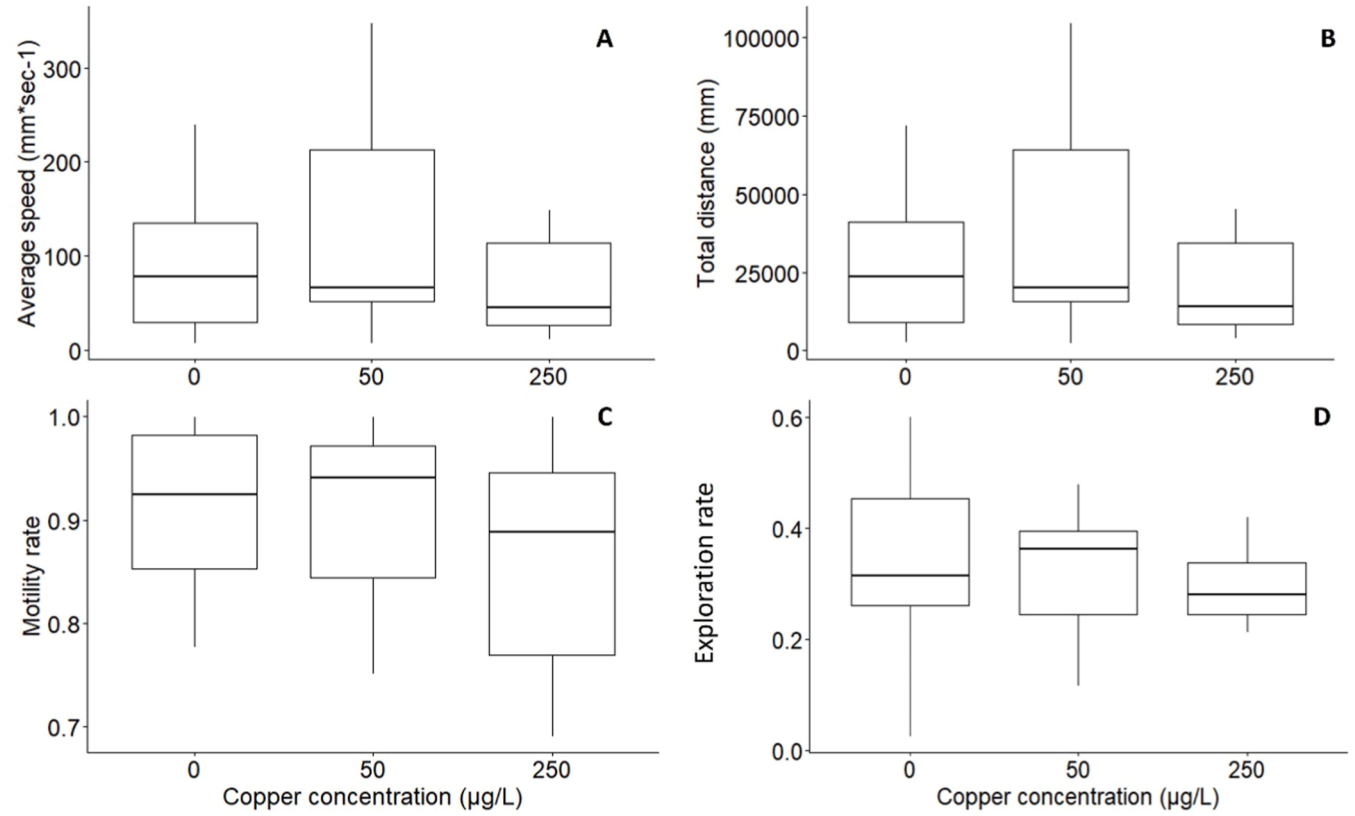

Fig. 3. Swimming behavior parameters (A = average speed, B = total distance, C = motility rate, D = exploration rate) assessed in populations of post-larvae *M. rosembergii* after pre-exposure to 0, 50 and 250 µg/L copper for 48 h. N = 9–10 organisms.

medium, 50, and 250 µg/L copper. EROD activity was not observed in the hepatopancreas of the organisms (activity = 0 units) and therefore no data is presented for this biomarker. None of the remaining responses related to biotransformation and oxidative stress (GST, CAT and LPO) showed significant changes after 48 h copper exposure compared to the controls ($p > 0.05$), likely due to the high replicate variability among the means (Fig. 4A, B, C). Indeed, an apparent decrease in GST and CAT was observed in prawns exposed to 250 µg/L copper (Fig. 4A, C). Regarding neurotoxicity, a significant decrease in ChE activity was observed in organisms exposed to 250 µg/L copper compared to controls (Fig. 4D). However, this possible neurotoxic effect should be interpreted with caution, as we observed a negative correlation between ChE activity and animal size. We also statistically compared prawn size among populations and found that organisms pre-exposed to 250 µg/L copper were significantly larger than control organisms. This information can be found in the Supplementary Materials (Fig. S2).

3.4. Colonization tests

In the colonization control tests, where all compartments were filled with the control medium, the distribution of organisms among the five interconnected compartments was similar ($F = 1.52, p > 0.05$). After the 3 h exposure, the mean percentages of organisms in compartments 1 (where all organisms were present at the start of the test) to 5 were similar, i.e., 15.1 (7.5), 19.6 (4.8), 26.4 (6.6), 22.6 (6.6) and 16.4 (3.2)%, respectively, ($n = 9$; ± standard deviation). This indicates that

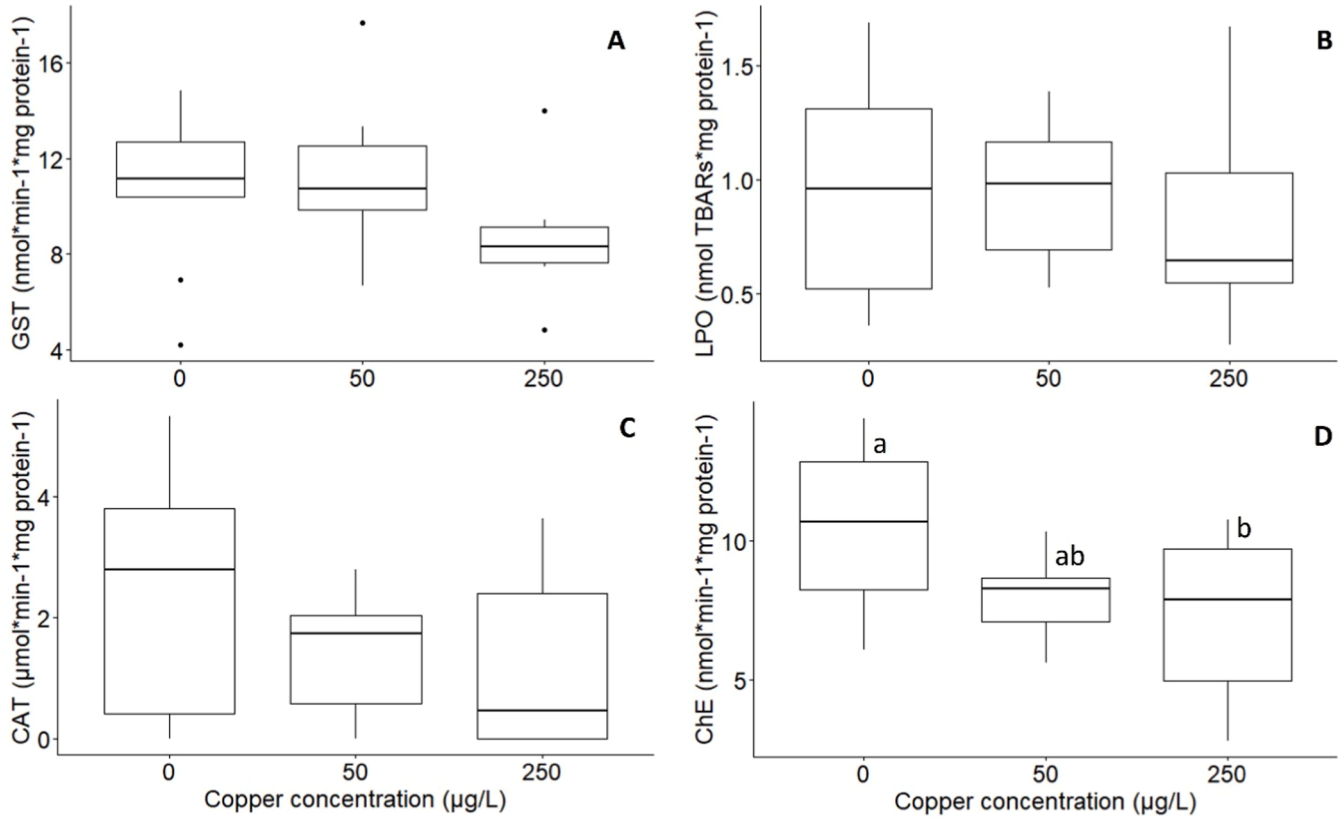

Fig. 4. Biomarkers (A = glutathione S-transferase activity, B = lipid peroxidation, C = catalase activity, D = cholinesterase activity) assessed in post-larvae of *M. rosembergii* pre-exposed to 0, 50 and 250 µg/L copper for 48 h. N = 9–10 organisms. Different letters indicate statistically significant differences ($p < 0.05$) among treatments.

the organisms were able to colonize all compartments and showed no preference for the ends of the system. These distribution percentages correspond to the following colonization percentages along the system: 100, 104, 106, 94.6 and 84.8 %.

The colonization response of the different prawn populations to the copper gradient varied based on their pre-exposure (or lack of exposure) to copper (Fig. 5). All prawn populations showed significantly reduced colonization of copper concentrations of 50 µg/L or higher, (equal to or lower than 80 % (non-pre-exposed), 82 % (pre-exposed to 50 µg/L), and 65 % (pre-exposed to 500 mg/L)) compared to 5 µg/L (99–101 %). In addition, prawns pre-exposed to 500 µg/L copper showed a significantly lower colonization of the most contaminated part of the copper gradient (500 µg/L).

The 3 h CC50 values, estimated from the mean colonization percentages of all observation periods, were 270 µg/L for non-pre-exposed prawns, 252 µg/L for prawns pre-exposed to 50 µg/L copper, and 109 µg/L for prawns pre-exposed to 500 µg/L copper for 6 h. Because the CI could not be estimated, these values could not be compared statistically. However, the CC50 value for the population pre-exposed to 500 µg/L copper was 2.3 to 2.5 times lower than that of the non-pre-exposed and 50 µg/L copper pre-exposed populations, respectively. Considering the exposure times for which a CC50 value could be calculated for the three prawn populations (20, 120, 160 and 180 min), the CC50 values of the 500 µg/L pre-exposed population were significantly lower than those of the non-pre-exposed population ($p < 0.05$), whereas the CC50 values of the 50 µg/L pre-exposed population were similar to those of the other two populations ($p > 0.05$).

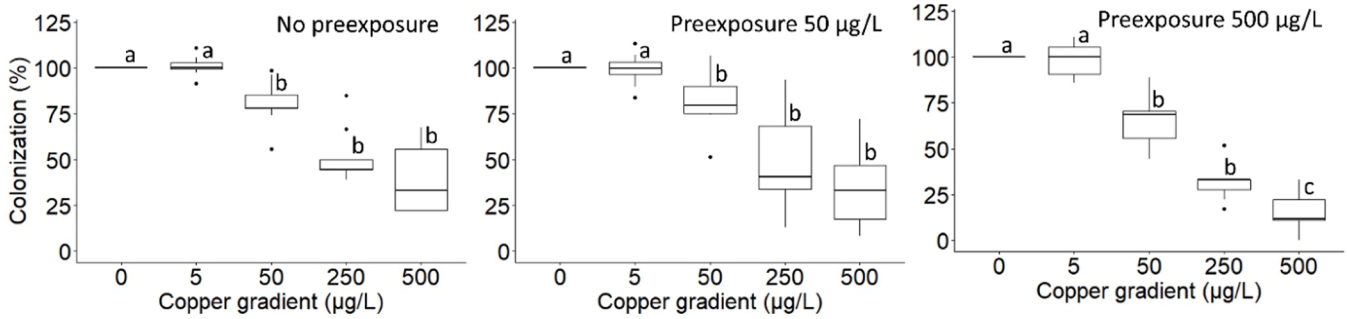

Fig. 5. Colonization of a copper gradient by post-larvae populations of *M. rosenbergii* after pre-exposure to different copper concentrations (no pre-exposure, or pre-exposure to 50 or 500 µg/L). Data represents the percentage of colonization along a non-forced 3 h exposure to the copper gradient. N = 9 observation times. Different letters indicate statistically significant differences ($p<0.05$) among compartments.

4. Discussion

Historically, the ecological risk assessment frameworks for chemical contamination have focused on specific individual- or population-based measurement endpoints that are assumed to represent risk to the ecosystem. More recently, approaches have been promoted that integrate responses at lower levels of organization, such as the adverse outcome pathway (Rohr et al., 2016) or the combination of measurements across different levels of organization (Sandoval-Herrera et al., 2019; El-Gendy et al., 2021). These approaches aim to link events at lower levels of organization, such as biomarkers at the cellular level, to more ecologically relevant events, such as population changes. In this context, our study integrated traditional biomarkers to assess sub-individual physiological stress with swimming activity as an integrative behavioral measure, and used colonization as an endpoint to measure habitat selection and its direct effects at the ecosystem level. All of these responses were assessed using the prawn *M. rosembergii* as the test organism and copper as the model contaminant. Overall, our results showed that pre-exposure to high concentrations of copper (500 μg/L) reduced the prawns' ability to colonize more contaminated areas, with CC50 values significantly reduced from 270 to 109 μg/L. However, exposure to lower levels of the metal only induced apparent signs of physiological effects without impairing the prawns' swimming activity (up to 250 μg/L).

Although high mortality occurred in prawns pre-exposed to 250 μg/L copper (leaving no organisms available for colonization tests), no significant responses were observed in general biotransformation and oxidative stress-related biomarkers. The acute toxicity of copper to freshwater crustaceans has been attributed to the blockage of the Na^+/K^+-ATPase pump, which leads to an imbalance of these ions (Bianchini et al., 2004). We should assume that this mechanism is not reflected in the measured markers; however, the apparent reduction in GST activity is consistent with findings reported by Quintaneiro et al. (2015). They observed a significant decrease in GST in *Atyaephyra desmarestii* after exposure to 200 μg/L copper for 48 h. Similarly, Frías-Espericueta et al. (2022) reviewed the effect of copper on GST in different decapods, including *M. rosenbergii*, and reported a reduction in activity in animals exposed to concentrations similar to those we tested, albeit over a longer exposure period (7 d). This suggests that copper may interfere with phase II of biotransformation.

Regarding the decrease in muscle ChE activity in organisms exposed to 250 μg/L copper, similar inhibitory effects have been reported in fish species such as *Clarias gariepinus* (Padrilah et al., 2017) and *Cyprinus carpio* (Nemcsók et al., 1984), which were exposed to copper concentrations comparable to those used in our study. However, the study by Quintaneiro et al. (2014) found no effect of copper on ChE activity in the crustaceans *Echinogammarus meridionalis* and *A. desmarestii*. Therefore, the response of this biomarker should be further evaluated to confirm whether it is affected by copper in these taxa. It should be emphasized that we also found that an effect of size may confound the occurrence of a neurotoxic effect (results included in Supplementary Materials), and Indeed, a negative correlation between organism size and ChE activity in muscle has already been observed in fish (Mena et al., 2023).

As suggested by the modest responses at the cellular level, no significant effects were observed in the motility (i.e., swimming activity) of the animals following exposure to copper. In a previous study with estuarine shrimp, adult *P. varians* showed a significant decrease in motility after exposure to 50 μg/L copper, while post-larvae of *P. vannamei* showed an increased response in motility after exposure to 250 μg/L of the metal (Redondo-López et al., 2023). In the model crustacean *Daphnia magna*, copper exposure was associated with reduced swimming speed and shorter displacements (Bownik 2017). Conversely, in another cladoceran, *Simocephalus vetulus*, exposure to copper caused hyperactivity and erratic swimming (Mishra et al., 2018). These findings suggest that the sensitivity and response to copper varies widely across different species. Given the clear evidence of copper's impact on crustacean motility, we suggest further research using improved approaches to assess swimming activity in crustaceans. Specifically, studies should be conducted in more natural environment by providing a larger area for the animals to explore. Additionally, the video tracking software should be adapted to eliminate the use of a strong light source, which may temporarily alter the organisms' behavior.

The observed decrease in colonization ability of prawns exposed to the highest copper concentration (500 μg/L) may be related to a reduction in their overall fitness. Unfortunately, due to the number of deaths during pre-exposure, no organisms were available to assess their physiological status. This was demonstrated by Lahman et al. (2015), who reported that the orientation and speed of the crayfish *Orconectes rusticus* were significantly reduced after continuous exposure to copper, even at concentrations as low as 4.5 μg/L. Another factor that may have contributed to the reduced colonization response is the impairment of environmental sensing, as copper is known to affect the development and integrity of chemosensory organs in decapods (Blinova and Cherkashin 2012). This phenomenon of sensory impairment can be related to the damage caused by the contaminant, as would be the case here, but it could also result from differences in the repulsiveness or attractiveness of chemical cues in the environment (Araújo et al., 2020). In this scenario, exposure to contaminants that interfere with perception may induce relevant changes in the spatial distribution of organisms.

Our results indicate that subtle physiological changes can occur in organisms exposed to copper contamination (> 250 μg/L) for short periods (48 h) without apparent impairment of their swimming activity. However, the ability of organisms to colonize a copper-contaminated environment may be affected by previous exposure to copper at around 500 μg/L. Given that chemical contamination can be a habitat fragmentation agent that affects the spatial distribution of organisms through various mechanisms (Araújo et al., 2016, 2020), our study highlights the importance of considering prior exposure when establishing ecotoxicological thresholds. This is especially relevant for water bodies with frequent or permanent contamination inputs. Furthermore, the consequences of habitat fragmentation in conjunction with other stressors in aquatic ecosystems can lead to biodiversity loss (Fuller et al., 2015; Echeverría-Sáenz et al., 2022). In this regard, our results underscore the importance of understanding how various individual and sub-individual responses influence habitat selection during chemical contamination events.

4. Conclusions

Post-larvae of *M. rosenbergii* tolerated exposure to copper concentrations that induced subtle signs of physiological stress without causing obvious impairment of swimming activity. Nevertheless, the ability of pre-exposed organisms to colonize an area contaminated by a copper gradient was reduced following pre-exposure to the highest copper concentration. These results suggest that the presence of a contaminant in the environment can both contribute to habitat fragmentation and modulate the organisms' ability to colonize recovering or degraded habitats.

CRediT authorship contribution statement

Freylan Mena: Writing – original draft, Resources, Methodology, Investigation, Formal analysis, Conceptualization. **Cristiano V.M. Araújo:** Writing – original draft, Resources, Project administration, Methodology, Investigation, Funding acquisition, Formal analysis, Conceptualization. **Silvia Echeverría-Sáenz:** Writing – original draft, Methodology, Investigation, Formal analysis. **Gabriel Brenes-Bravo:** Writing – original draft, Investigation, Formal analysis. **Matilde Moreira-Santos:** Writing – original draft, Supervision, Resources, Methodology, Investigation, Funding acquisition, Formal analysis, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.aquatox.2024.107073.

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