Environmental Science and Pollution Research

Dissimilar behavioral and spatial avoidance responses by shrimps from tropical and temperate environments exposed to copper

--Manuscript Draft--

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	Fundación General CSIC (201830I081)	Mr Enrique González-Ortegón

Abstract:

Behavioral changes associated with exposure to pollutants represent the earliest response for organisms confronted by perceivable chemical signals. This study was carried out with the objective of evaluating behavioral responses associated with different scenarios of exposure to pollutants (non-forced vs forced) in two shrimp species (*Penaeus vannamei* and *Palaemon varians*), representative of different latitudes and using copper as a model contaminant. The effects on locomotion were evaluated by exposing the shrimps to a range of copper concentrations (0, 0.5, 5, 50 and 250 µg/L) in the forced scenario. After exposure, the movement patterns for each shrimp were recorded and used to estimate changes in the shrimps' locomotion. For the non-forced scenario, the avoidance response was assessed by placing shrimps in a multi-compartment system where they were able to move freely along a gradient of copper (0, 0.5, 5, 50 and 250 µg/L). In terms of locomotion, an opposite trend was observed between the species: movements were significantly reduced in *P. varians* with concentrations above 50 µg/L, while hyperactivity was observed for *P. vannamei*. When exposed to a gradient of copper in the multi-compartment system, both species significantly avoided the highest concentrations of copper, although the repellence of copper was stronger for *P. vannamei*. In summary, both species of shrimps were able to recognize and avoid copper; however, in terms of locomotion, they showed an opposite behavioral reaction. These results show that a contamination event can have different behavioral outcomes depending on the species and complementing forced and non-forced exposure with species specific information can be helpful to characterize and predict the effects of contaminants at higher biological

	levels.
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7 - Line 377- 390: I think this part of the discussion should be simplified because this explanation is quite speculative. I suggest discussing on the basis of the results obtained in this study and avoid translocating the results to what could potentially occur in nature. The natural environments are much more complex in terms of biodiversity, interactions and envoironmental charcteristics, which may pomote different responses as observed in the experiments.

Response: That section of the discussion was rewritten and simplified to focus on the results of this study. Lines 381-402.

Reviewer #2

1 - Line 79: This needs further description of the ecosystem implications. After that, start a new paragraph with behavioural responses.

Response: More details were added to describe the ecosystem implications. Lines 75-85

2 - Line 90-94: Under an ecotoxicological risk assessment, which is the worst-case scenario? Both?

Response: This is a very interesting issue. Even though both approaches have advantages and disadvantages, we consider that an important contribution of this work is to combine these approaches to the assessment of behavioral effects. Both outcomes: the avoidance of polluted areas or the reduced fitness of organisms that remain inside them would affect the ecosystem services that those populations provide and, consequently, increase the vulnerability of the ecosystem. More detail was added in the lines 100 -102.

3 - Line 97: Please, use the full genus for the species.

Response: The complete names of both species were given the first time they were mentioned, in lines 55 and 58.

4 - Line 118: Indicate the number of animals that were collected and transported to the laboratory. What water was used? Why mean field salinity? Was it conducted in several studies to get a mean value? How was the container, volume, etc.? What were the physical-chemical properties of water? What was the pond conditions and culture?

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5 - Lines 120-121: Field seawater: please describe this water, properties, etc.

Response: The description of the water was improved.

6 Line 123: country, brand, etc. of the food.

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Response: This species, at the age tested, naturally inhabits estuaries. Therefore, they are exposed to frequent changes in salinity. In the referred study, the osmoregulation was assessed and they performed better at salinity of 10. They also tolerated the gradual change in salinity of five units/day.

8 - Line 132: How was treated the tap water, chloride? How was the physical-chemical consitions of water? natural water? synthetic?

Response: Additional details were included. Tap water was passed through two membrane filters (5 µm and 1 µm), then a filter of activated carbon and treated with a

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9 - Line 134-143: The same information must be included in this paragraph for the second species

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10 - Line 145: What was the salt used to get the copper? Include reference number, etc.

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Response: Details of the water properties were included.

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Response: We agree with the Reviewer and, such as mentioned, if we want to discriminate the effect that salinity produces, either increasing or reducing the toxicity of copper, experiments should be performed at similar salinity values. However, our goal in the current study was to assess the behavioral response of both shrimps' species; then, we decided to use the salinity in which their osmoregulation capability was not altered. That decision was taken considering the results we have obtained in a previous study (Mena et al., 2020 – see reference in the manuscript), in which it was observed that avoidance response changes if organisms are exposed to an additional stress such as changes in salinity.

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In general, time of exposures to copper need further justification. Why these exposure times?

Response: Regarding the exposure period, a phrase and a reference were included to justify it, based on the evidence that behavioral responses in most aquatic organisms can be observed early after the initiation of the exposure.

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Response: in the Reply #12 we have explained why salinity was different for each species. Regarding the pseudoreplication, although during the exposure period

	different groups of organisms tested were exposed in only two times/replicates, the responses were individually measured in ten organisms, being each organism, a replicate of the stress produced by contaminant. # 18 - Line 224-227: This main result can be a consequence of the different salinity and therefore the different toxicity. With the present experimental design authors cannot answer the question. Response: As mentioned in a previous comment, the decreased salinity used for P. vannamei was defined after assessing the physiological status (as osmoregulation) in different salinities. This criterion was used to run the present experiments focusing on behavioral outcomes with a realistic environmental background, eliminating or at least reducing the role of salinity in the behavior of the organisms.
Additional Information:	
Question	**Response**
§Are you submitting to a Special Issue?	No

Universidad Nacional
Facultad Ciencias de la Tierra y el Mar
Instituto Regional de Estudios en Sustancias Tóxicas

Costa Rica, Heredia, June, 2022.

Editorial Department of Environmental Science and Pollution Research.

Dear Editor,

Please find the attached revised manuscript entitled “Dissimilar behavioral and spatial avoidance responses by shrimps from tropical and temperate environments exposed to copper” to be considered for publication in Environmental Science and Pollution Research. The comments and suggestions by the Reviewers were carefully considered and replied to as follows.
To facilitate the review, the comments of the two reviewers have been listed with consecutive numbers and the answer to each comment has been placed immediately afterwards, in blue.

Responses to Reviewers' comments:

Reviewer #1

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Yours respectfully,

Sergei Redondo López
(on behalf of the authors)
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Universidad Nacional
Facultad Ciencias de la Tierra y el Mar
Instituto Regional de Estudios en Sustancias Tóxicas

Cover letter

January 20, 2022

Environmental Science and Pollution Research
Dear Editor,

We are submitting the manuscript entitled "Dissimilar behavioral and spatial avoidance responses by shrimps from tropical and temperate environments exposed to copper" to be considered for publication in Environmental Science and Pollution Research.

In this paper we report the evaluation of behavioural responses in shrimp, applying forced and non-forced scenarios of exposure to copper. The assessment was carried out using two species with different geographical distributions, one from the tropics and one from a temperate region. We found differences in the avoidance response observed under a non-forced exposure. Furthermore, a 24-hour forced exposure caused opposite effects on the locomotion of individuals of each species. We consider that these results are relevant as they demonstrate that the behavioural responses and effects elicited by one contaminant can vary greatly among taxonomically close species. In the context of coastal pollution such differences should be considered when estimating the risk for the ecosystems. For these reasons, we respectfully recommend this study to be considered for your journal.

2021
Año de las Universidades Públicas por la conectividad como derecho humano universal
Bicentenario de la Independencia de Costa Rica

We confirm that this manuscript has not been published elsewhere and is not under consideration by any other journal. All authors have approved the manuscript and agree with its submission to Environmental Science and Pollution Research. We declare that no conflict of interest exists. We declare that the study does not involve human subjects. We declared that we have submitted our manuscript to a preprint server before submitting it to Environmental Science and Pollution Research.

Thank you for your consideration.

Yours Respectfully,

Sergei Redondo-López
(On behalf of the authors)
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86-3000, Heredia
Tel.: +506-22773884
E-mail: sergei.redondo.lopez@est.una.ac.cr

Dissimilar behavioral and spatial avoidance responses by shrimps from tropical and temperate environments exposed to copper

Sergei Redondo-López[a*], Enrique González-Ortegón[b], Freylan Mena[a], Cristiano V.M. Araújo[b]

[a] Instituto Regional de Estudios en Sustancias Tóxicas (IRET), Universidad Nacional, 86-3000, Heredia, Costa Rica.

[b] Department of Ecology and Coastal Management, Institute of Marine Sciences of Andalucía (CSIC), 11510, Puerto Real, Cádiz, Spain.

*****Correspondence: sergei.redondo.lopez@est.una.ac.cr.

Abstract

Behavioral changes associated with exposure to pollutants represent the earliest response for organisms confronted by perceivable chemical signals. This study was carried out with the objective of evaluating behavioral responses associated with different scenarios of exposure to pollutants (non-forced vs forced) in two shrimp species (*Penaeus vannamei* and *Palaemon varians*), representative of different latitudes and using copper as a model contaminant. The effects on locomotion were evaluated by exposing the shrimps to a range of copper concentrations (0, 0.5, 5, 50 and 250 µg/L) in the forced scenario. After exposure, the movement patterns for each shrimp were recorded and used to estimate changes in the shrimps' locomotion. For the non-forced scenario, the avoidance response was assessed by placing shrimps in a multi-compartment system where they were able to move freely along a gradient of copper (0, 0.5, 5, 50 and 250 µg/L). In terms of locomotion, an opposite trend was observed between the species: movements were significantly reduced in *P. varians* with concentrations above 50 µg/L, while hyperactivity was observed for *P. vannamei*. When exposed to a gradient of copper in the multi-compartment system, both species significantly avoided the highest concentrations of copper, although the repellence of copper was stronger for *P. vannamei*. In summary, both species of shrimps were able to recognize and avoid copper; however, in terms of locomotion, they showed an opposite behavioral reaction. These results show that a contamination event can have different behavioral outcomes depending on the species and complementing forced and non-forced exposure with species specific information can be helpful to characterize and predict the effects of contaminants at higher biological levels.

Keywords

Behavior toxicology, avoidance, locomotion, aquatic invertebrates, copper, pollutants.

1. Introduction

Coastal transition areas sustain rich and productive ecosystems (Arbi et al., 2018; Dalu et al., 2020; Wang et al., 2018; Yokoyama et al., 2009). The biota established in such habitats has adapted to the inherent stressful environmental conditions, mainly related to frequent changes in salinity, temperature, and dissolved oxygen (Elliott and Quintino, 2007; González-Ortegón et al. 2006). Apart from this natural stress, coastal ecosystems are exposed to high anthropogenic pressure caused by the dense human population and fast development of these areas (González-Ortegón et al., 2019; Michalec et al., 2013; Peng et al., 2013). Because of this, the contamination in coastal waters has increased in the last few decades, thus representing a risk factor for marine and estuarine organisms (Ali et al., 2019). Although contamination is a worldwide problem, the scenarios and dynamics for ecological risk assessment might differ between tropical and temperate regions due to the differences in physical, chemical, and biological attributes (Lacher Jr. & Goldstein, 2009). The diversity of the communities, the sensitivity of the species and the environmental behavior of contaminants require a better understanding of the particularities in such different habitats (Abele, 1974; Daam & Van Den Brink, 2010; Peterson et al., 2017).

Decapods are representative fauna and play an integral ecological role in coastal ecosystems. Within this group, species with different geographical distributions and life histories have already been used for ecotoxicological studies. For instance, the Peneid *Penaeus* (=*Litopenaeus*) *vannamei* is a shrimp of tropical distribution that migrates into estuaries during its post-larvae stage and develops there until the sub-adult stage, leaving the estuary to spend its adult life in the open ocean (Valles-Jimenez et al, 2005). Meanwhile, the Palaemonid *Palaemon varians* inhabits temperate regions and spends its whole life cycle inside shallow coastal lagoons and salt marshes (González-Ortegón et al 2015). These species are distributed throughout the tropical American area and in temperate European saltmarshes, respectively, with a high overall abundance and are also ecologically and economically important. Several studies have demonstrated the suitability of *P. vannamei* (Betancourt-Lozano et al., 2006; Comoglio et al., 2005; García-de la Parra et al., 2006; Osuna-Flores et al., 2019; Wang et al., 2012) and *P. varians* (Araújo, Gómez et al., 2019; Araújo et al., 2020; Brown and Hauton, 2018; Ehiguese et al., 2019) for ecotoxicological assessments, covering biochemical, physiological and behavioral responses to different environmental pollutants and physico-chemical stressors.

Regarding relevant endpoints to be assessed, behavioral changes associated with exposure to pollutants represent the earliest response and the first line of defense for organisms confronted by perceivable chemical signals (Beitinger & Freeman, 1983). Aquatic crustaceans use olfactory and taste receptors to gather information from their surroundings and assess the presence of hazardous molecules present in the ecosystem (Blinova & Cherkashin, 2012; Lahman et al., 2015; Olsén, 2011; Oulton et al., 2014).

Continuous exposure to pollutants can disrupt receptor function, which alters their ability to process and respond to key environmental information. Such alterations in behavior can lead to ecological consequences at the population, community and ecosystem level and are likely to have other cascading implications within the ecosystem (González-Ortegón et al., 2019; Oulton et al., 2014; Schmidt et al., 2010). For instance, populations with a high rate of organisms evading contamination are likely to have a reduced local abundance in their original area, which could impair the ability of those populations to recover from pollutant stress (Moe et al., 2013). Furthermore, emigration of the organisms to less contaminated zones can trigger (or even increase) competitive interactions between species, modifying the arrangement of the species throughout the surrounding environments (Silva et al. (2018).

Generally, behavioral responses have been measured by keeping organisms in confinement, exposed constantly to a sub-lethal concentration of a contaminant, after which an effect is recorded (Amiard-Triquet et al., 2013). However, when non-forced, multi-compartment exposure systems are used, this allows the avoidance response to be evaluated by replicating the possibility that organisms have of escaping from a polluted area and avoiding exposure in a real scenario, reducing the probability of suffering acute or even physiological transient effects (Moreira-Santos et al., 2019). One of the strengths of this approach is its ability to predict the effect of a pollutant on the spatial distribution of a population (Araújo, Rodríguez et al., 2016; Vera-Vera et al., 2019). By integrating both non-forced and forced approaches it is possible to assess the effects of contamination from two different and complementary perspectives: i) simulation of a heterogeneously dispersed contamination scenario to assess the potential repellence of contaminants by triggering an avoidance response in organisms and ii) assessment of the potential toxicity of contaminants by simulating the conditions in which organisms cannot escape from and, therefore, are susceptible to suffer the damage caused by contamination. This combination of approaches should contribute to characterizing the risk for an ecosystem when either the spatial distribution or the fitness of organisms is affected by pollution.

This study was carried out with the objective of evaluating the behavioral responses associated with two different scenarios of exposure to contaminants (non-forced vs forced exposure scenarios) in two shrimp species (*P. vannamei* and *P. varians*), representative of different latitudes with different life histories regarding the use of coastal ecosystems. As copper has been shown to affect the behavior of different decapod species (Gutierrez et al., 2012; Hansen and Roslev, 2016; Krång and Ekerholm 2006; Lahman et al., 2015; Mishra et al., 2018), it was selected as the test substance. Using this trace metal as a model contaminant, the avoidance response was assessed as a primary endpoint based on perception and escape response, while a forced, short, confined exposure allowed the assessment of the effects on locomotion. This experimental design should contribute to characterizing how the behavior of these organisms can be affected depending on how the exposure occurs in a homogeneous (forced exposure) or heterogeneous (non-forced exposure) contamination scenario. In addition, the use of both approaches makes it possible to integrate both behavioral responses by assessing whether a behavior of overexcitement implies higher avoidance, while lethargic behavior might imply a lower ability to escape from contamination.

2. Material and Methods

2.1. Test organisms

Penaeus vannamei (whiteleg shrimp) is a species widely cultured in American and Asian tropics, although its distribution was originally limited to the Pacific coast of America, from the North of Mexico to Peru (FAO 2006-2020). Juveniles of *P. vannamei* [mean size: 19.2 ± 2.3 mm carapace length (CL)] were collected from a culture pond in Punta Morales, Puntarenas, Costa Rica, during October 2019. Approximately 300 individuals were transported to the laboratory in a plastic 200 L container with aerated field water (salinity of 30, pH = 8.0, conductivity = 49 mS/cm, dissolved oxygen >90%). In the laboratory, the shrimp were placed in a 100 L aquarium, filled with field seawater (with the same conditions of the transport), with continuous aeration and biological filtration. The temperature was maintained at 26 ± 2 °C and the organisms were fed *ad libitum*, daily, with commercial dry pellets (Nicovita 28% protein, VITAPRO, Ecuador). The animals used in the experiments were not fed during the last 24 h. The salinity to which the juveniles of this species are more frequently exposed varies in a wide range; however, in a previous study it was observed that individuals from the same pond showed a better physiological performance at a salinity of 10 (Mena et al., 2020). Although the water salinity was 30 during the sampling of the organisms, and to reach the target salinity of 10, the organisms were gradually acclimated with changes of 5 units of salinity every 24 h. The organisms used in the assays were acclimated to salinity of ten for at least 24 h before any exposure to copper was carried out. The reduction in salinity was achieved by diluting the seawater with filtered (1µm membrane and activated carbon), UV-treated tap water (Millipore). Salinity was measured with a calibrated multi-field meter (WTW Cond 315i; ± 0.1).

Palaemon varians naturally inhabit the North and Baltic seas, as well as the Atlantic and Mediterranean coasts of Africa and Europe (Holthuis 1980). Adult organisms (mean size: 9.03 ± 1.5 mm CL) were captured in a salt marsh at Puerto Real, Cadiz, Spain, during February 2020. Approximately 300 individuals were transported to the laboratory in plastic bags, then immediately placed and maintained in 200 L tanks with a flow-through marine water at the salinity of 30 (field salinity in the salt-marsh) and at room temperature or 15 ºC, with continuous aeration and fed daily, ad libitum, with commercial dry pellets (Ultra Fresh - Shrimp Delight). As this species lives permanently in an environment with marine salinity, the tests were carried out with filtered marine water, with a salinity of 30.

2.2. Reagents (Copper)

Copper solutions for the exposure of the shrimps were prepared using stocks (63.8 mg/L Cu^{2+} at IRET, Costa Rica, prepared from copper chloride (Sigma-Aldrich 221783) in ultrapure water (Millipore) / standard solution from Merck; 1000 mg/L at ICMAN, Spain) solutions. Aliquots of these stock solutions were diluted in water with the appropriate salinity for each species: salinity of 10 and pH of 8.0 for *P. vannamei* and salinity of 30 and pH of 8.2 for *P. varians*. The same range of nominal concentrations of copper (0, 0.5, 5, 50 and 250 µg/L) was used in every experiment for both species.

At the end of the forced and avoidance tests, a sample of every solution used in the experiments were collected to evaluate the final copper concentration by ICP-MS (inductively coupled plasma mass spectrometry). The accuracy (recovery rate) of the standard concentrations was between 90 and 110%. As the concentrations measured at the end of the experiments did not vary to more than 10% regarding the nominal concentrations, these were used in the results.

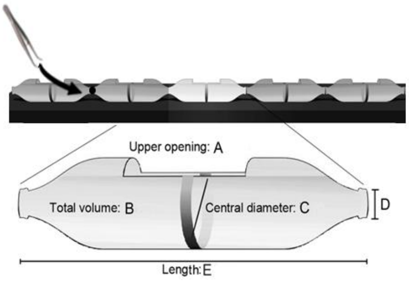
2.3 Locomotion assessment experiments

Shrimps of both species were exposed to the same range of concentrations of copper (0, 0.5, 5, 50 and 250 µg/L) at the experimental salinity indicated previously. Ten organisms (five shrimps per flask) were exposed per treatment, using plastic containers (5L for *P. vannamei* and 1.5L for *P. varians*), and maintaining the density below 1 g of shrimp/L. Each treatment was tested in duplicate during 24 h, this period was considered sufficient as behavioral locomotion responses are expected to occur earlier during the exposure than other toxicological outcomes (Faimali et al., 2016). After exposure, the movement patterns (total, vertical and horizontal displacement) for each shrimp were recorded individually inside an aquarium containing clean water, following a modified form of the methodology proposed by Sandoval-Herrera et al. (2019). The dimensions of the aquariums were adjusted to the size of the species: 40 cm long, 20 cm high and 10 cm wide for *P. vannamei* and 42 cm long, 28 cm high and 21 cm wide for *P. varians*. For the recordings, the tanks were illuminated with a fluorescent light, located 20 cm above the tank and lateral and posterior walls of the aquarium were covered with white adhesive paper. After the transfer, the shrimp were allowed to be in the aquarium for 3 min before the start of the recording, then a 5 min video was recorded per individual.

The videos were analyzed using the background subtraction and optical flow algorithms from the OpenCV Library in Python (Bradski, 2000). Coordinates (X/Y) from individual shrimp were extracted from each frame of the video each 0.07 seconds. These coordinates were used to estimate the shrimp's locomotion based on total, horizontal and vertical displacement; the use of the upper area [calculated as the proportion of time spent in the area above an imaginary line (drawn horizontally in the middle of the water column) divided by the proportion of time spent below that line] and the displacement routes were represented with the data visualization program Paraview (Ayachit & Utkarsh, 2015).

2.4. Avoidance tests

The avoidance tests were carried out using multi-compartment, non-forced exposure systems (Fig. 1, system A for *P. vannamei* and system B for *P. varians*), where the shrimp were able to move freely along a gradient of copper. Previously, control tests without copper were performed with each species in order to prove the random distribution of the organisms in the system. For these tests, the systems were filled with uncontaminated water and shrimps (four *P. vannamei* and five *P. varians*) were placed in each compartment. For the tests with copper, the connections between compartments of the system A and B were blocked with plastic plugs and plasticine, respectively, before the volumes of the test solutions (3 L for system A and 1 L in system B) were added to the compartments and only unblocked after the introduction of the shrimps. Plugs between compartments were removed using tweezers (Fig. 1). In the systems, both species were exposed to the same range of copper: 0, 0.5, 5, 50 and 250 µg/L. All the experiments were carried out in triplicates (three systems in parallel), the organisms were exposed during 4 h and their distribution in the system was recorded every 30 min. The exposure was carried out in the dark to avoid any alteration to the behavior of the shrimps. To reduce interference, a red light was used during the observations of the experiment. No mortalities occurred during these assays.



System A	**System B**
Total length: 250 cm	Total length: 245 cm
Total volume: 15 L	Total volume: 5 L
A = 13 x 5 cm	A = 4 x 28 cm
B = 3 L	B = 1 L
C = 11.5 cm	C = 8 cm
D = 3.5 cm	D = 2.2 cm
E = 50 cm	E = 49 cm

Figure 1. Multi-compartment exposure systems used in the non-forced avoidance tests: system A used with *P. vannamei* and system B used with *P. varians*. Tweezer and the plugs used to block the connections between compartments are also shown.

2.5. Statistical analysis

Locomotion data were compared among treatments using the package FSA (Ogle et al., 2020) in R, v3.6.1 (R Core Team 2020). First, the data were tested for normality using the Shapiro test; as the distribution of the data was not normal, a Kruskal-Wallis test coupled with a Dunn post-hoc pairwise comparison was applied. Data outside two standard deviations from the mean of the treatment group were treated as outliers and excluded from the analyses. For the avoidance experiments, generalized estimating equation models (GEE) in R were used to compare the quantity of shrimps in each compartment during the time spent in the copper gradient. The function geeglm from the package geepack (Højsgaard et al., 2016) with the family ‘poisson’ with a logit link was used. The geepack contains an ANOVA method that allows us to compare models and perform Wald tests (Zuur et al., 2009). The model investigated the main effects; ‘copper concentration’ over ‘time’, including ‘ID’ and an ‘ar1’ correlation structure (continuous-time first-order autoregressive correlation structure) to account for temporal correlation. The grouping structure is provided by the ID option; this specifies which shrimp observations form a block of data. The correlation is applied on each block of data, and auto-correlation structure was used; hence corstr = "ar1".

The avoidance percentages were calculated using the formula described by Moreira-Santos et al. (2008). The R package ecotox (Hlina et al., In Review) was used to estimate the concentrations of copper that caused an *X*% of avoidance by shrimp (AC*x*), with a 95% confidence interval. Graphical representations and linear models were carried out in R.

3. Results

3.1. Locomotion assessment

When the shrimp were placed in an aquarium with clean water after a 24 h exposure to copper, an opposite trend in the pattern of locomotion was recorded between the species: movements were reduced in *P*. *varians*, while hyperactivity was observed in *P*. *vannamei*. In general, in the absence of copper, the *P*. *varians* organisms were more active than *P*. *vannamei*. The total displacement observed in the control organisms was significantly higher ($p<0.05$) in *P*. *varians* compared to *P*. *vannamei*. Forced exposure of *P*. *varians* to increasing concentrations of copper caused a significant decrease in the total and vertical displacement with concentrations above 50 µg/L compared with the controls (Fig. 2-A, 2-C) and a reduced permanence in the superior zone of the water column at those concentrations (Fig. 2-D). Horizontal displacement had no significant reduction, although a tendency towards a decrease was observed (Fig. 2-B). On the other hand, an opposite trend was observed in those responses by *P*. *vannamei*, with a trend to increase locomotion after exposure to copper, especially at the highest concentration (250 µg/L) (Fig. 2).

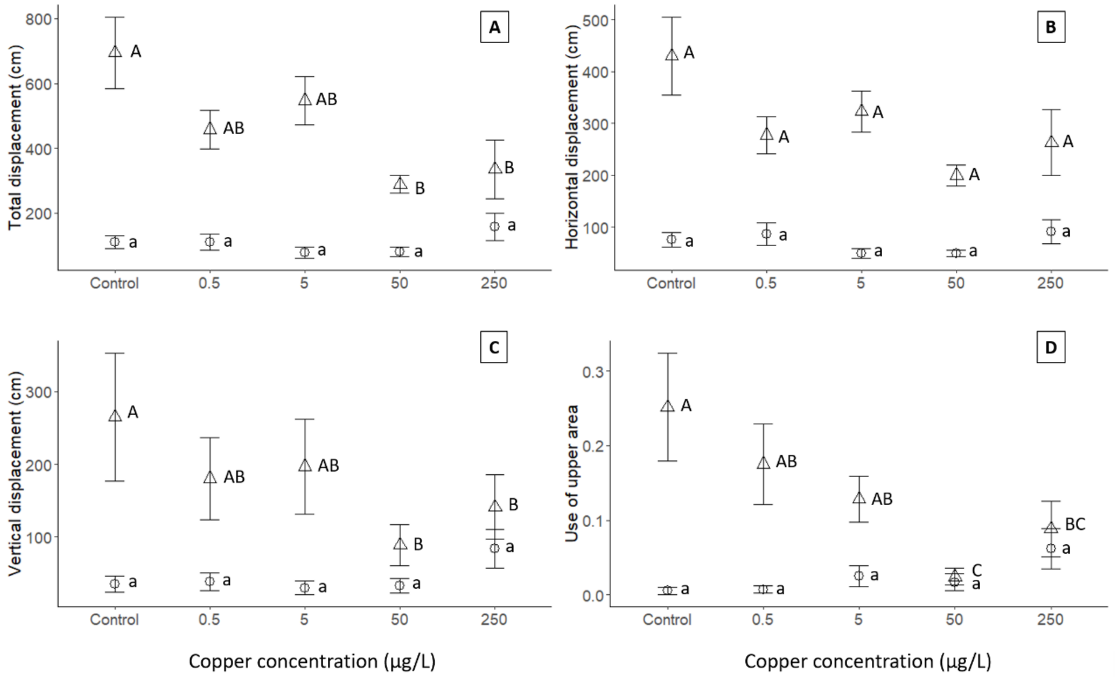


Figure 2. Changes in the locomotion of *P*. *varians* (triangles) and *P*. *vannamei* (circles) after a forced 24-h exposure to a range of concentrations of copper. Different letters

indicate significant differences among the treatments for each species, uppercase for *P. varians* and lowercase for *P. vannamei*.

These results are complemented with the displacement routes shown in Figure 3 where, in the case of *P. vannamei* (Fig 3), the apparent tendency towards increased activity in the organisms exposed to higher concentrations of copper is marked by a more intense movement at the borders of the aquarium compared with the control group. Analyzing the displacement routes by *P. varians* (Fig 4), it is shown the significant loss of movement that these organisms suffered, as they were exposed to the higher concentration of copper.

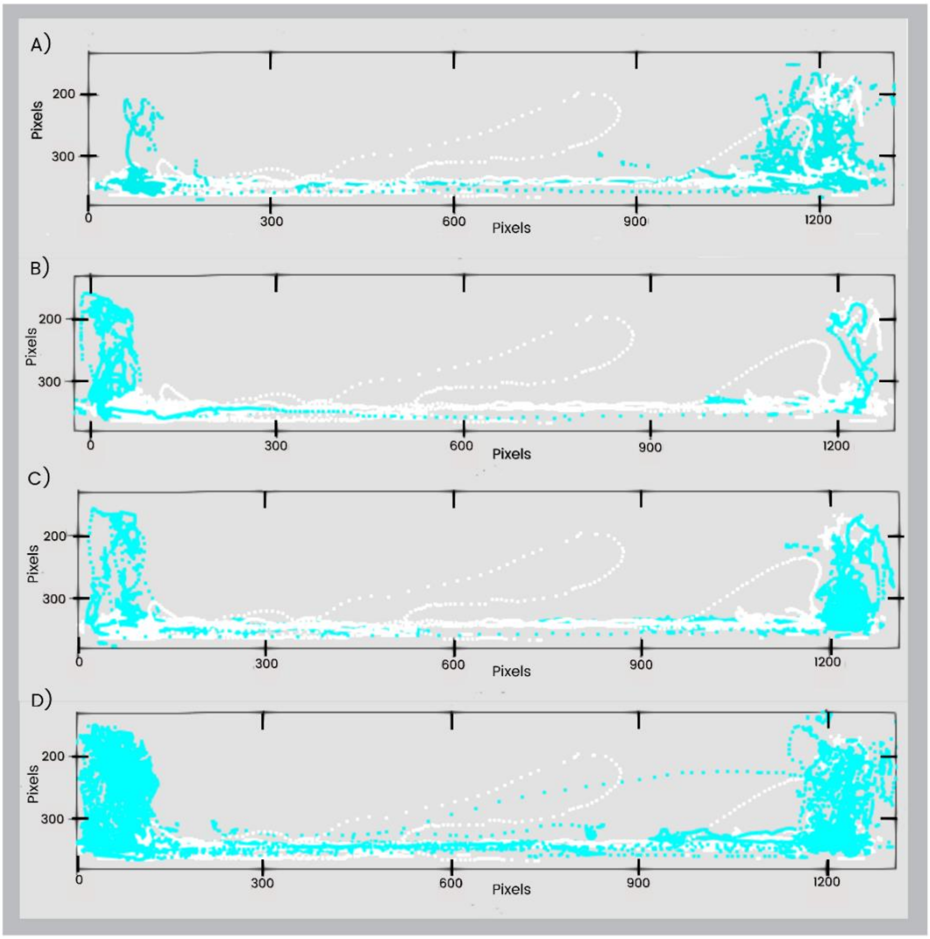


Figure 3. Representation of the displacement routes by individuals of *P. vannamei.* White lines correspond to the control group and light blue to the organisms after exposure to the copper concentrations of 0.5 µg/L (A), 5 µg/L (B), 50 µg/L (C) and 250 µg/L (D).

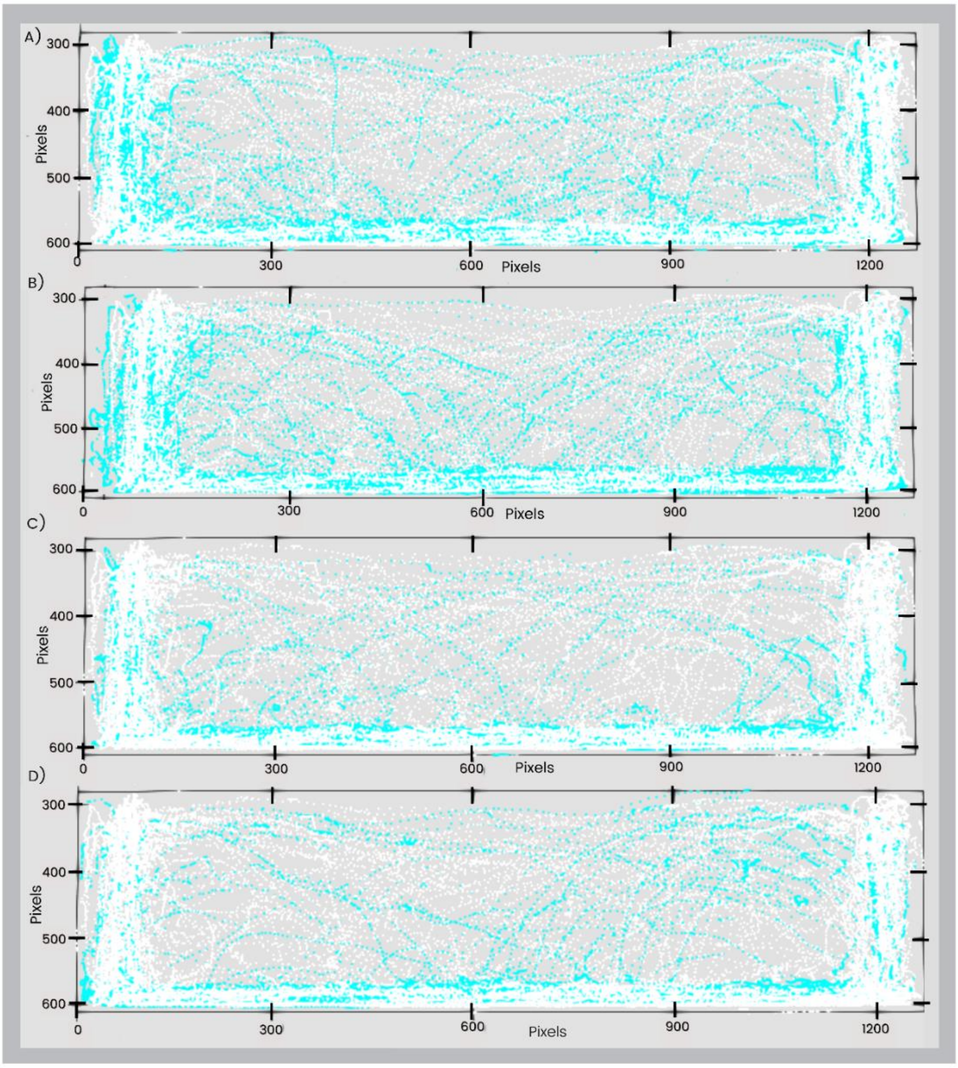


Figure 4. Representation of the displacement routes by individuals of *P. varians*. White lines correspond to the group and light blue to the organisms after exposure to the copper concentrations of 0.5 µg/L (A), 5 µg/L (B), 50 µg/L (C) and 250 µg/L (D).

3.2. Avoidance response

When exposed to a gradient of copper, both species significantly avoided the highest concentrations of copper, although the repellence of copper was stronger for *P. vannamei* (Table 1). The distribution of shrimps throughout the compartments of the exposure system was clearly conditioned by the copper concentration for both species (Fig. 5). At the lowest concentration (0.5 µg/L), the absence of avoidance was observed for *P. vannamei*, while an effect of attraction was observed for *P. varians*. Then, a significant reduction of the number of organisms occurred in *P. vannamei* from the compartment with 5 µg/L, and that reduction continued in the compartments with the two higher concentrations of the metal (Fig. 5, left). Meanwhile, the number of *P. varians* only diminished significantly, compared to the control compartment, at concentrations of 50 and 250 µg/L (Fig. 5, right). This clearer and sharper avoidance by *P. vannamei* lead to lower ACx values (Table 1). AC_{25}, AC_{50} and AC_{75} for *P. vannamei* were about 6.3, 3.8 and 2.3 times lower than those for *P. varians*.

Table 1. Concentrations (in µg/L) of copper (with their respective 95% confidence intervals) that triggered avoidance in 25, 50 and 75 percent (AC_{25}, AC_{50} and AC_{75}, respectively) of the shrimp populations (*Penaeus vannamei* and *Palaemon varians*) after 4 h exposure in a non-forced system.

Species	AC_{25}	AC_{50}	AC_{75}
P. vannamei	0.75 (0.10 - 2.29)	11.30 (4.07 - 30.50)	170.0 (56.8 - 1200)
P. varians	4.70 (0.14 - 18.9)	42.7 (9.78 - 512)	389 (76.8 - 124000)

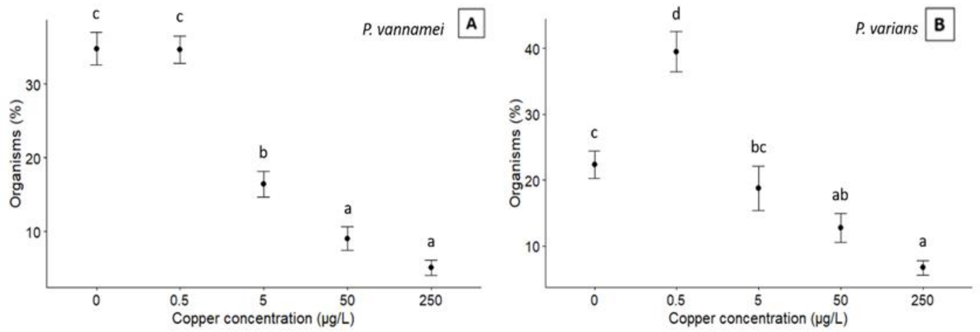


Figure 5. Distribution of the individuals of *P. vannamei* (A) and *P. varians* (B) exposed to a gradient of copper in a multi-compartment system. Different letters indicate statistically ($p<0.05$) significant differences among treatments.

4. Discussion

In this study, we tested two behavioral responses in two shrimp species with different latitudinal distributions and different use of coastal habitats. Both species were able to recognize and avoid copper when they were exposed to the metal in a free-choice multi-compartment system; however, the avoidance response was clearer in *P. vannamei*, while *P. varians* showed some tolerance to lower concentrations of (or inability to recognize the risk of) copper. When locomotion was assessed after a forced exposure, a clear induction of lethargy was observed in *P. varians*, which suggests that this activity was strongly affected by the exposure to copper; though, an opposite reaction was observed in *P. vannamei*, showing an apparent tendency of hyperactivity after exposure to higher concentrations of copper, even though, in the absence of contamination, it moved slower than *P. varians*.

Hyperactivity has been documented for other species of crustaceans exposed to heavy metals such as: *Simocephalus vetulus* (Mishra et al., 2018), *Hippolyte inermis* (Untersteiner et al., 2005), *Macrobrachium lamarrei* (Lodhi et al., 2006), *Neomysis integer* (Verslycke et al., 2003), *M. nipponense* (Gerhardt et al., 2002) and an increase in the startle response in crabs (White & Briffa, 2017). The tendency of *P. vannamei* to increase movement speed at high concentrations could be related to the stress response model proposed by Untersteiner et al. (2005) for the shrimp *H. inermis* exposed to cadmium. According to this model, at low concentrations of the metal, there is an adaptation reaction where metabolic energy is used for osmoregulation, a process that is characterized by a decreased motility due to a decline in spontaneous muscular activity (Knops et al., 2001). Then, as the concentrations of the metal increase, there is an escape reaction characterized by increased motility, ventilation, and powerful beats of the pleon (Untersteiner et al., 2005). This model might be appropriate for the behavior shown by *P. vannamei*, since at low concentrations the organisms' movement was very low and, at higher concentrations, an apparent hyperactivity was shown. This is also coherent with the greater capacity of *P. vannamei* to regulate their internal concentration of trace metals, as shown by Núñez-Nogueira et al. (2012) and Dadar et al. (2014). When the regulation of the internal body concentration of metals makes it possible to maintain the value below the threshold level, no negative effect is developed, but if the capacity for regulation is surpassed, effects like changes in the behavior of the organism start to appear (Núñez-Nogueira et al., 2012).

Further, the loss of locomotion (as shown by *P. varians*) has been documented in other crustaceans. Lahman et al. (2015) reported that organisms of the species *Orconectes rusticos* had significantly lower walking speed towards the odor of food after exposure to copper. Also, Krång and Ekerholm (2006) found delayed reactions and increased time before initiating mating activity in *Carcinus maenas* exposed to the metal. Trace metals influence many physiological processes in crustaceans, including neurological processes. These effects on the nervous system are very important in the regulation and coordination of the locomotory behaviors (Untersteiner et al., 2005). Trace metals affect cellular calcium levels, resulting in low levels of serotonin, acetylcholine, and norepinephrine, which affects the organisms' motivation and, therefore, can restrict locomotion (Tripathi, 2016). Additionally, exposure to copper has been related to cytological and histochemical damages, ion regulation and disruption of protein functions in osmoregulation and respiration, which causes a decrease in oxygen consumption and metabolic rate (Frías-Espericueta et al., 2008; Lahman et al., 2015b; Thatipaka et al., 2020). This can lead to physiological impairment and therefore decreased muscular activity, which might be related to the drop in locomotion showed by *P. varians* (Lahman et al., 2015).

This kind of opposite behavioral reaction has been reported previously. For example, Gutierrez et al. (2012) found hyperactivity and a loss of the ability to escape in copepods and cladocerans. The interaction of metals with the chemoreception system of crustaceans can lead to different responses depending on: the intensity of the exposure (time and concentration), mechanism of action, the developmental stage of the animal, the species and other environmental factors (Blinova & Cherkashin, 2012). For instance, differences in the avoidance response in larvae of *P. vannamei* when confronted with a copper gradient have been related to the age of the organisms (Araújo et al., 2016). This difference might be linked to the development of the sensory organs in younger animals and then an enhanced tolerance at older stages. Pollutants like trace metals have been linked to a reduction of the length of antennular flagellum in crustaceans, which leads to problems in perceiving, interpreting, and responding to a chemical attractant and, finally, changes in the animal's behavior (Blinova & Cherkashin, 2012; Oulton et al., 2014; White & Briffa, 2017). This erroneous processing of olfactory information may lead to opposite responses to a pollutant between species or even between organisms of the same species. If the exposure is above the specific limit of sensitivity, the organisms will not be able to make accurate assessments of the environment due to an inhibition of the sensory and motor systems (Gutierrez et al., 2012). However, there is a lack of information regarding species-specific sensitivity of chemoreceptors to sublethal concentrations of contaminants (Blinova & Cherkashin, 2012). It is remarkable that the forced exposure approach has elicited opposite behaviors (lethargy vs hyperactivity) in the species tested. According to Gerhardt (2007), regarding locomotion, organisms confronted by pollution can either increase movement and actively avoid it, or reduce their movement, and drift away. Whichever kind of behavioral response they display, it could make the organism more vulnerable to predators or other threats they face in the environment (Gutierrez et al., 2012).

Regarding the lack of avoidance response observed in *P. varians* at lower concentrations and the more intense response presented by *P. vannamei*, these differences between species and their decision to stay near or avoid the pollutant could be attributed to two main factors: how repulsive the stimulus caused by the pollutant is and the organism's ability to identify the substance and recognize the risks of exposure (Araújo et al., 2016; Rodríguez et al., 2016; Harper et al ., 2009). Some species such as *P. varians,* are naturally less sensitive to detecting and interpreting the risk associated to copper contamination, as evidenced by the hormesis at the lowest copper concentration tested (the density of shrimps was significantly higher at this concentration than in the other ones; Figure 5, B). In others, such as *P. vannamei,* its hyperactivity favored the avoidance response when confronted by a copper gradient, showing a greater ability to detect and recognize the risk of copper contamination.

From an ecological point of view, the responses based on both exposure approaches (non-forced and forced) may indicate possible changes in the spatial distribution of the species and help to understand the decline of populations at the local scale (Rosa et al., 2012; Araújo & Blasco, 2019). Considering the outcome of our experiment, *P. vannamei* could be better suited to deal with this specific stressor than *P. varians,* due to its ability to escape from contamination. The differences in the responses observed between both shrimp species could help us to understand how the spatial distribution of these species would be affected by contamination. On the one hand, one would be repelled from contaminated sites earlier (with a lower level of contamination) than the other and, on the other hand, the lower ability to escape from the contamination would lead to a higher susceptibility to suffering the toxic effects. This evidence reinforces the hypothesis that contaminants act as habitat disruptors by affecting organisms directly (e.g., lethal and sub-lethal effects if the organisms do not avoid them) and indirectly by triggering the organisms’ avoidance response (Araújo et al., 2014, 2016).

5. Conclusions

Organisms of the species *P. vannamei* and *P. varians* were able to recognize and avoid a copper gradient. However, in terms of locomotion, they showed an opposite reaction, with *P. vannamei* showing an apparent hyperactivity and *P. varians* showing a significant decrease in its movement. This result shows that a contamination event can have different behavioral outcomes depending on the species. Even though behavior and avoidance stand as important endpoints in the evaluation of contaminants present in an ecosystem, there is a need for more information regarding species-specific sensitivity to sublethal concentrations of contaminants. The risk for aquatic organisms being affected by environmental contamination has increased in coastal habitats, that is why complementing forced and non-forced exposure with species specific information can be helpful to characterize and predict the effects of contaminants at higher biological levels.

6. Statements & Declarations

6.1 Funding

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6.2 Competing Interests

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.

6.3 Author Contributions:

Sergei Redondo-López: Conceptualization, Methodology, Investigation, Data curation, Writing—original draft preparation, Writing— review and editing. **Freylan Mena:** Conceptualization, Supervision, Methodology, Investigation, Resources, Data curation, Writing—original draft preparation, Writing— review and editing. **Enrique González-Ortegón**: Methodology, Investigation, Resources, Data curation. **Cristiano V.M. Araújo:** Conceptualization, Methodology, Investigation, Resources, Writing— review and editing.

6.4 Compliance with Ethical Standards:

Under the legislation applicable during the assessment, this project did not require any special permits.

6.5 Consent to Participate:

Not applicable.

6.6 Consent to Publish:

All authors agree to submit this paper for publication, and we all have the consent from our institutional authorities.

6.7 Availability of data and materials.

The authors declare that the results presented in this paper are supported by data, and that such data are available upon request to the authors.

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Graphical Abstract

Titule Page