



Structural and functional alterations under stress conditions by contamination: A multi-species study in a non-forced multi-compartmented mesocosm

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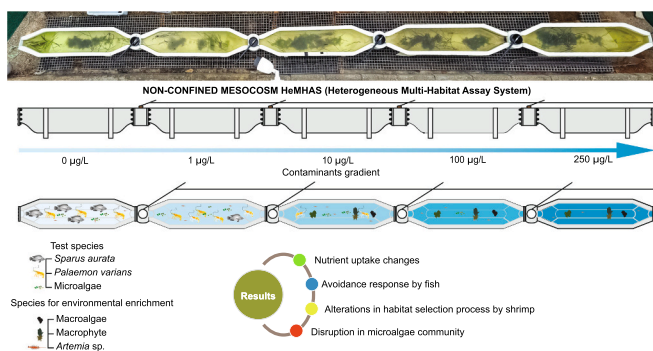
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HIGHLIGHTS

- Fish and shrimp avoided copper contamination.
- Avoidance by organisms may jeopardize populations and the habitat selection process.
- Shrimps' lower avoidance was linked to predation risk and contaminant exposure.
- Contamination affected the system structure.
- The HeMHAS mesocosm approach is a useful tool for risk assessment studies.

GRAPHICAL ABSTRACT



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ABSTRACT

Despite the existing connectivity and heterogeneity of aquatic habitats, the concept of interconnected landscapes has been frequently overlooked in ecotoxicological risk assessment studies. In this study, a novel mesocosm system, the HeMHAS (Heterogeneous Multi-Habitat Assay System), was constructed with the potential to assess structural and functional changes in a community resulting from exposure to contaminants, while also considering the complex ecological scenarios. Fish (*Sparus aurata*), shrimp (*Palaemon varians*) and three species of marine microalgae (*Isochrysis galbana*, *Nannochloropsis gaditana* and *Tetraselmis chuii*) were used as test organisms. Other species, such as *Artemia* sp. and macroalgae were also introduced into the system as environmental enrichment. All the species were distributed in five interconnected mesocosm compartments containing a copper gradient (0, 1, 10, 100 and 250 µg/L). The mobile fish avoided the copper contaminants from 1 µg/L (24 h-AC₅₀: 4.88 µg/L), while the shrimp avoided from 50 µg/L (24 h-AC₅₀: 136.58 µg/L). This finding suggests interspecies interactions influence habitat selection in contaminated environments, potentially jeopardizing population persistence. Among the non-motile organisms, the growth and chlorophyll content of the microalgae were

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concentration dependent. The growth of *I. galbana* was more sensitive (growth inhibition of 50 % at the highest concentration) in contrast to *N. gaditana* (30 % inhibition at the highest concentration) and *T. chuii* (25 % inhibition at the last two highest concentrations). In summary, the mesocosm HeMHAS showed how contamination-driven responses can be studied at landscape scales, enhancing the ecological relevance of ecotoxicological research.

1. Introduction

Ecotoxicity tests have been used for a long time to assess how contaminants affect organisms. In these tests, organisms are experimentally exposed to contaminants in a controlled area and the link between concentration and response is used to figure out what might happen to the organisms in the environment. The significance of this technique cannot be understated, as the data acquired has demonstrated its importance for the effective management of contamination for many stakeholders and decision-makers (Rudén et al., 2016; Hall et al., 2017). However, the main issues of this approach include its poor ecological relevance when attempting to extend the findings to the broader spatial level, mainly regarding the organisms' forced exposure to contaminants, given that landscape habitats are generally interconnected in some way (Ware, 2000; Loreau et al., 2003) and mobile organisms can flee from stressful environments (Lopes et al., 2004; Moreira-Santos et al., 2019). To take this into account, the mesocosm approach provides a greater ecological realism since it considers the interactions of multiple species that are typical in communities rather than what happens when testing only a single species (Brink and Daam, 2014). The word mesocosm refers to an experimental ecosystem created by enclosing portions of an actual ecosystem in the field or by bringing together pieces of several natural ecosystems to attempt to fill the gap between the laboratory and the real world in environmental science (Odum, 1984). Due to the characteristic of this approach that evaluates more than just one species, it is possible to reveal both the direct and indirect effects of contaminants at the same time, as well as to study reactions at many different levels of biological organization under controlled exposure conditions (Caquet et al., 2000).

The mesocosm approach is presently used in ecotoxicological assays and plays a key role in evaluating the fate and effects of chemicals in the aquatic environment on many species (Caquet et al., 2000; Medina et al., 2004; Roussel et al., 2007; Abelho et al., 2015; Sharma et al., 2020). However, despite the mesocosm approach making it possible to study the effects of chemicals at organism, population and community levels, those systems were designed as enclosed areas and the impact of the contaminants on the organisms has been based exclusively on the toxic effects in a confined environment (Caquet et al., 2000; Clements and Rohr, 2009). The organisms of mesocosm communities are not generally considered to move throughout heterogeneous landscapes and select their suitable habitats by applying mandatory exposure to contamination, even though this forced exposure scenario does not frequently occur in landscape ecology (Caquet et al., 2000; Féraud and Blaise, 2013; Carboni et al., 2021). Multiple studies have demonstrated that using non-forced multi-compartmented system exposures (developed by considering the interconnected nature of aquatic ecosystems), organisms can detect chemicals and escape from contaminated or stressful habitats, providing a new perspective for ERA (Ecological Risk Assessment) studies based on the contamination-driven organism habitat selection (Odum, 1984; Araújo et al., 2016a, 2020a; Araújo and Blasco, 2018). The decision of organisms to either flee from or remain in a particular habitat is influenced by several ecological factors beyond contamination alone. Residing in a contaminated area may result in a loss of the ability to encounter prey (Ares, 2003; Fleeger et al., 2003) and increased susceptibility to predation (DeMille et al., 2016). It can also lead to a depletion of motivation to respond and a modification in aggressive behavior, in addition to the direct toxic effects on physiology, which incurs a significant energy cost for homeostasis (Tierney et al., 2010; Saaristo et al., 2018). In such cases, the additional cost of avoiding

predators may become unsustainable for extended periods (Forester et al., 2019). Weis and Candelmo (2012) reviewed and reported that mummichog (*Fundulus heteroclitus*) from polluted estuarine sites were less active, less adept at capturing prey (*Palaemonetes paludosus*), smaller, and had a shorter lifespan than fish at cleaner sites. The authors attributed these observations to poor nutrition and predator avoidance during stays at polluted sites. Further, the presence of predators, food, or shelter can significantly influence an organism's exploratory behavior, impacting on the arrangement, spread of the population, and even its ability to select clean areas (Forester et al., 2019; Davis et al., 2003; Araújo et al., 2016b, 2020b; Islam et al., 2019; Salvatierra et al., 2022). Therefore, contamination may play a pivotal role in altering the biological interactions and dynamics among organisms (Ares, 2003; Fleeger et al., 2003; Weis and Candelmo, 2012; Preston, 2002; Mirza and Pyle, 2009).

Among environmental contaminants, copper is a particular environmental hazard because of its widespread use in mining, agriculture, industry, commerce, and household chores (Rehman et al., 2019). They enter the aquatic environment through a wide variety of natural and man-made processes, including rock weathering, volcanic eruptions, air deposition, sewage, industrial effluents, agricultural runoff, mining, coastal development, dredging, and many more (Comber et al., 2022; da Acosta et al., 2016). Copper is known to increase reactive oxygen species and affect brain extracellular enzymes such as nucleotidases, decreasing their expression and activity (da Acosta et al., 2016). It inhibits cholinesterase activity, impairs various physiological and neurochemical functions and causes behavioral changes at environmentally relevant concentrations (Scott and Sloman, 2004; Malhotra et al., 2020). Araújo et al. (2019) noted distinct avoidance patterns during a multi-species test with copper gradients. When the fish *Danio rerio* and shrimp *Atyaephyra desmarestii* were exposed to copper contamination together, the fish displayed a more pronounced avoidance response to copper compared to the shrimp. This alteration in the shrimp's avoidance behavior during multispecies tests may be attributed to factors such as heightened territorial behavior of the fish or a delay in the shrimp's detection of the contamination risk. In another study, Silva et al. (2018) exposed *Poecilia reticulata* and *D. rerio* to a copper gradient. They observed changes in the avoidance behavior to contamination: *P. reticulata* displaced *D. rerio* to concentrations that had been avoided by this latter species when it was tested alone. However, this behavioral plasticity of organisms when exposed to contamination has only been studied incipiently, even though it might help to improve ecological hazard and risk assessments (Clements and Rohr, 2009; Saaristo et al., 2018).

It has been hypothesized that contamination in aquatic environments may interfere with the organisms' habitat selection process and alter the natural response of organisms to a stressor. However, it is difficult to simulate chemically heterogeneous habitats, which are connected and that can be selected by organisms. Therefore, a multi-compartmented mesocosm system (HeMHAS – Heterogeneous Multi-Habitat Assay System) was built to provide organisms with the necessary conditions to test the effect of contamination considering the ecological interactions in a spatially broader and connected landscape. To prove our hypothesis, we aimed to evaluate the functional and structural responses of many species to copper in a mesocosm multi-species approach using the HeMHAS as a test system. Fish (*Sparus aurata*), shrimp (*Palaemon varians*) and pelagic microalgae of different classes (*Nannochloropsis gaditana*, *Isochrysis galbana*, and *Tetraselmis*

chuii) were used as the test species. Microalgae were used to provide information about the toxicity, while mobile organisms such as fish and shrimp highlighted the effects of contamination on their habitat selection response. Additionally, other species (macroalgae: *Ulva* sp. and *Caulerpa prolifera*; macrophytes *Zostera noltii* and *Spartina densiflora*; and the crustacean *Artemia* sp.) were used for environmental enrichment. The abiotic factors, including measurements of the water's parameter and nutrient concentrations, were regarded as functional changes. Structural responses were assessed through the organisms' avoidance response, chlorophyll fluorescence and population growth. Copper was chosen as a reference chemical due to its widespread contamination of the environment and its ability to trigger avoidance in organisms (Lopes et al., 2004; Moreira-Santos et al., 2008; Islam et al., 2019).

2. Materials and methods

2.1. HeMHAS mesocosm: design

The HeMHAS, a multi-compartmented non-forced system (Araújo

et al., 2018), was constructed at the mesocosm scale using glass fiber. The system is comprised of five compartments, with a total length of 9.4 m and a total volume of 500 L. Each of the compartments was $168.5 \times 41 \times 35.5$ cm in length, width, and height, respectively, and had a capacity of 100 L (Fig. 1). All the compartments were connected to the adjacent compartments and the closing and opening of the connections between the compartments were controlled by rotary gates at the connections. The compartments were partially covered by a Perspex sheet to prevent the organisms from jumping out, from rainwater and bird droppings getting in. The bottom of the compartments was concave with a smooth ascending slope terminating at the connections. The system stands 15 cm above the ground, which allows any water leakage from the system (Fig. 1) to be seen. The system was set up in the outdoor field of ICMAN-CSIC (Instituto de Ciencias Marinas de Andalucía - Consejo Superior de Investigaciones Científicas), Río San Pedro Campus, Spain.

2.2. Calibrating the gradient of contamination

The mesocosm system was calibrated to verify the stability of the

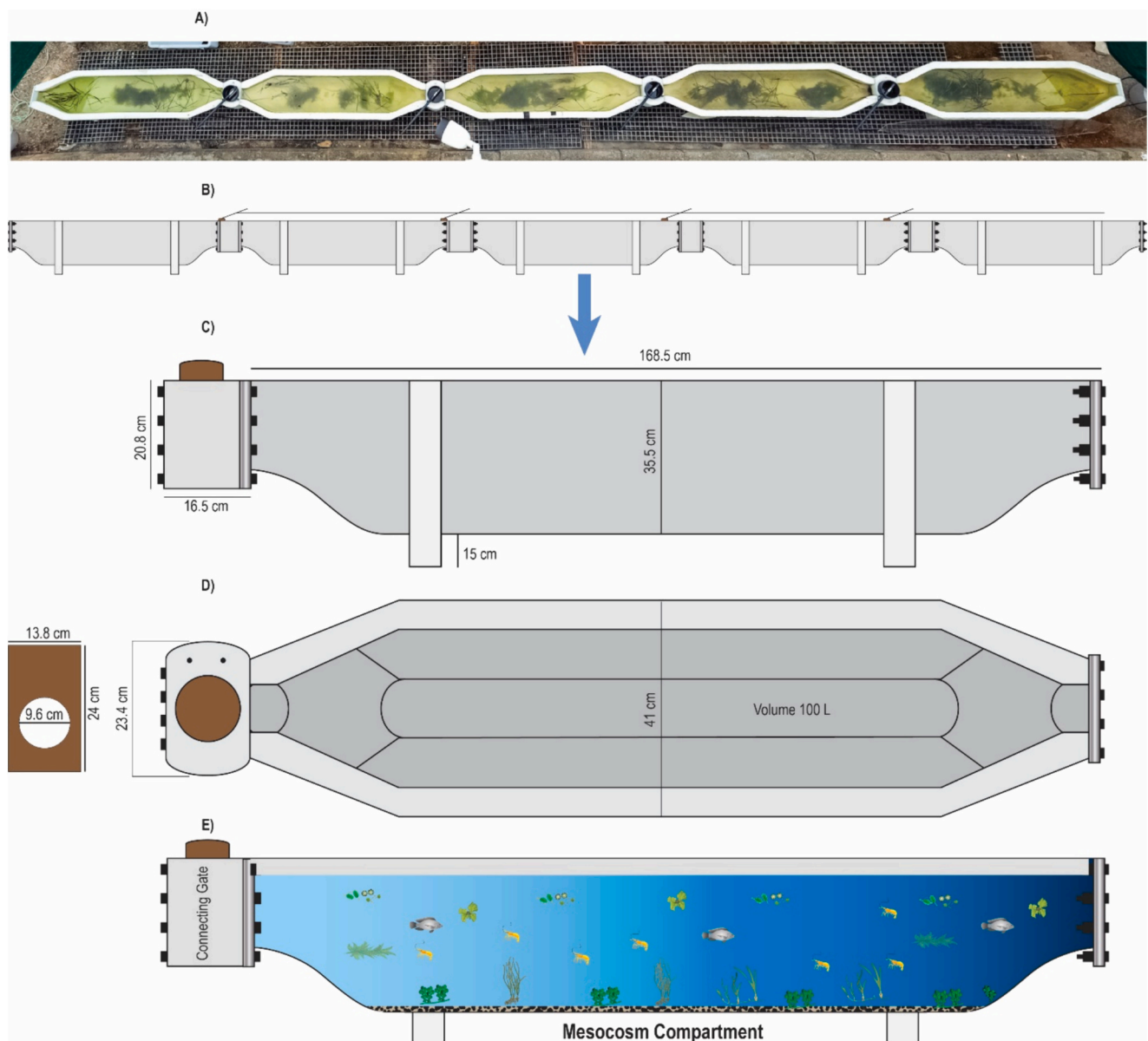


Fig. 1. Schematic diagram of the non-confined mesocosm HeMHAS. A = a real photo of mesocosm HeMHAS; B = the five connected compartments; C = Side view; D = Top view; E = Mesocosm compartment with community. Details of the rotatory gates are also provided.

contamination gradient. Sodium chloride (NaCl) solutions were used for this purpose as an accurate relationship with conductivity values could be easily obtained (41). NaCl concentrations [12, 25, 50, and 100 mg/L and control (0 %)] were prepared in tap water used to simulate the contamination gradient. At the beginning of the calibration, the connecting gates between the compartments were closed. Subsequently, each of the four NaCl solutions and the control treatment were carefully put into their respective compartments. The gates were then opened to establish a linear concentration gradient. The calibration procedure lasted 15 days (periods in which the changes in conductivity values were close to 2 %), the conductivity values were checked twice every day in the center of each compartment. The calibration of the system was performed without the presence of organisms in order to assess the gradient's stability with no interference due to the swimming of organisms. Conductivity data concerning the NaCl concentrations (initial, before introducing the dilutions into the compartments, and final values, measured directly inside each compartment) are presented in Table S1. The values showed statistically significant differences in conductivity across every compartment (Tukey multiple comparison tests: $F_{4, 55} = 2456.33$; $p < 0.001$; see Table S2).

2.3. Species and samples: test organisms, species for environmental enrichment, water and sediment

Species from different biological groups were used in the mesocosm experiments. Some species were used as test organisms, from which some responses were measured to assess the effects of contamination: three marine microalgae, a shrimp and a fish were monitored in this experiment. The use of fish was approved by the Ethics Committee: *Junta de Andalucía*, Spain; Ref. #05/11/2020/126). Other organisms were added as environmental enrichment: a brine shrimp (*Artemia* sp.); two green macroalgae (*Ulva* sp. and *C. prolifera*), and the two marine macrophytes (*Z. noltii* and *S. densiflora*).

The marine microalgae selected were, *N. gaditana* Lubián 1982, strain CCMM (Marine Microalgae Culture Collection) 04/0201 (Eustigmatophyceae); *I. aff. galbana* Parke 1949, strain CCMM 05/0401 (clone T-ISO) (Prymnesiophyceae) and *T. chuii* Butcher 1959, strain CCMM 03/0201 (Prasinophyceae). They were obtained from the Marine Microalgae Culture Collection (CCMM) at ICMAN. These species were chosen as they are sensitive, available and ecologically relevant to marine ecosystems (Walsh, 1997; Debelius et al., 2009; Araújo et al., 2010). They were cultured individually in filtered (0.2 μm) f/2 marine medium (Guillard and Ryther, 1962; Fábregas et al., 2000) in sterile conditions under continuous white light (around 30 $\mu\text{mol m}^{-2} \text{s}^{-1}$, salinity of 35, 20 ± 1 °C temperature and 8.0 ± 0.5 pH value).

The shrimp *P. varians* Leach 1814 (size: 2.8 ± 0.5 cm) were sampled from the salt pond Salina La Esperanza (Puerto Real, Spain). After sampling, the shrimps were acclimatized in three 250-L aquaria containing non-chlorinated seawater (salinity of 35 and pH of 7.5) with continuous aeration and partial renewal every two days by replacing one-third of the total volume with marine water.

Juvenile seabreams (*S. aurata* Linnaeus 1758), of 10 ± 1 cm mean size were obtained from the Marine Culture Laboratory, Faculty of Marine and Environmental Sciences, University of Cadiz, Spain. They were acclimatized similarly to the shrimps at the ICMAN-CSIC laboratory in four 250-L aquaria.

The laboratory conditions for both species (shrimps and fish) were: 24 °C temperature and 12:12 h light/dark photoperiod. The shrimps were fed with either *Artemia salina* or ground TetraMin® flakes and the fish were fed with commercial pellets (D-2 Optibream, 2.5 mm, Skretting, Spain), twice a day, measuring 1 % of their body mass. *P. varians* and *S. aurata* were chosen for their wide geographical location, easy maintenance and sensitivity to toxic compounds (*P. varians*: Smaldon et al., 1993; Araújo et al., 2020c; Salvatierra et al., 2022; *S. aurata*: Kissil, 1991; Minghetti et al., 2008; Rodrigues et al., 2018; Stankus, 2021).

Regarding the environmental enrichment species, the brine shrimp

Artemia sp. was cultured at ICMAN Marine Microalgae Culture Collection, IMMCC [at 24 °C, a salinity of 35 and fed with the microalgae *Phaeodactylum tricornutum* (every two days)]. The macroalgae *Ulva* sp., *C. prolifera*, the macrophytes *Z. noltii*, *S. densiflora* and around 500 L of seawater were collected from the nearby Gulf of Cadiz, southwest (SW), Spain. The macroalgae and macrophytes species were maintained in three 100-L of aquaria at the ICMAN-CSIC wet laboratory with continuous aeration (salinity of 35, 20 ± 1 °C temperature and 8.0 pH value). Natural sediment samples containing communities (around 50 kg) were also collected from the salt marsh tidal creek Río San Pedro (Bay of Cádiz, SW, Spain) to enrich the mesocosm environment.

2.4. Calibration to stabilise the mesocosm: monitoring physical-chemical parameters

Prior to the copper experiments, a second calibration to monitor the environmental conditions was performed. The mesocosms were allowed to stabilise for one week to ensure the main environmental parameters (temperature, pH, salinity and dissolved oxygen-DO) were maintained at acceptable values for conducting the tests with the microalgae, shrimps, and fish.

The bottom of all the compartments was covered with 3 cm of natural sediments [samples from the salt marsh tidal creek Río San Pedro (Bay of Cádiz, SW, Spain)] and 100 L of nearby ocean water (Gulf of Cadiz, SW, Spain). Each mesocosm compartment was equally distributed with rinsed submerged macrophytes (10 cm $\times$ 10 cm): *Z. noltii* and *S. densiflora*; macroalgae (10 cm $\times$ 10 cm): *Ulva* sp. and *C. prolifera*; microalgae (10⁴ cells/mL of each): *N. gaditana*, *I. galbana*, *T. chuii*; shrimp (10 individuals of *P. varians*) and *Artemia* sp. (2 g, wet weight). The fish were not introduced to the mesocosms during this calibration phase to reduce the use of vertebrates only to the essential experiments. The whole facility was fenced so that larger mammals, amphibians or birds did not have access (see photos in Supplementary material: Fig. S1). The average values ($\pm$ standard deviation) of temperature, pH, salinity and DO after seven days are presented in Table S3 for all the mesocosm compartments.

2.5. Copper concentrations

The copper (standard solution from Merck; 1000 mg/L) gradient prepared to use in the tests was: 0 (control), 1, 10, 100, and 250 $\mu\text{g/L}$ (nominal concentrations). All the dilutions were prepared in the same seawater used for the culture of the organisms. During the sample collection from the mesocosm compartments, the connection gates were kept closed to avoid the mixing of the contaminant between each compartment. The concentrations were checked at the end of each day by Inductively Coupled Plasma Mass Spectrometry, ICP-MS (ThermoFisher ICAP-Q). Before analysis, the samples were acidified to pH < 2 with ultrapure grade HCl (Merck) in a class-100 High-Efficiency Particulate Air (HEPA) laminar flow hood and stored for 1 month at 4 °C. The actual final copper concentrations measured in the experiments are presented in Table S4 ($R^2 = 0.9940$; Background Equivalent Concentration (BEC) = 0.026 ppb; Limit of Detection (LoD) = 0.0013 ppb).

2.6. Control and avoidance test: avoidance response by shrimps and fish

Before the experiment with copper, a control test was performed in order to verify any preference or avoidance behavior by the organisms for mesocosm compartments of the test system. The test was conducted followed by the end of the one-week stabilization process. The conditions of the experiments were similar to the physical-chemical calibration test. Initially, each compartment was filled with 100 L of the same seawater used in the culture of the organisms. Then, the organisms (microalgae, macroalgae, macrophytes and *Artemia* sp.) were introduced as described in Section 2.4. The physicochemical parameters were maintained as indicated in Table S3. Then the connections between the

mesocosm compartments were shut and nine shrimps (*P. varians*) were introduced in each compartment. Thereafter, the connection gates were opened to allow the shrimps to move throughout the system. The records of the position of the shrimps were made at 30-minute intervals for the initial 8 h and at the end of each 24-h period for three days (where each day was treated as a separate replication) to ensure no preference or avoidance response for any compartment without contamination. When recording this data, the connecting gates were closed to prevent the shrimp from fleeing from that compartment. The system was maintained under control conditions for seven days to allow the community to acclimate to the environmental conditions before the copper contamination. Fish were not used in this test to meet the concept of reduction of the 3Rs with regard to the use of vertebrates in experimentation. At the end of the control test, the gates were closed, the parameters were measured, samples were taken for analysis and all the shrimps were taken out. The remaining organisms were maintained in the mesocosm for the copper contamination test.

Following the acclimation period, the avoidance test with the copper gradient was initiated, lasting for three days (72 h) with each day (24 h) considered as one replication. For the first replication, initially, the compartments were closed and 50 % of the water was exchanged with seawater (aerated for 1 week and filtered through 0.4 µm prior to adding) in each compartment without removing the settled bacterial communities formed during the control test. Subsequently, additional *Artemia* sp. (1 g, wet weight) and microalgae were added to maintain the similar cell density of the first day of the control test (10^4 cells/mL). The water was then gently homogenized with a sterile glass rod to distribute the organisms evenly. Water parameters and samples were measured and collected for analysis from each compartment, which represents the initial (0 h) water quality of the first replication. Three fish and seven shrimps were then introduced in each compartment and five concentrations of copper (nominal concentrations: 0, 1, 10, 100 and 250 µg/L) were spiked into the respective compartments (from the lowest to the highest concentrations), creating a contamination gradient. The connecting gates were then opened to allow the movements of the organisms throughout the system. The positions of the organisms were recorded following the pattern of the control test. At the end of 24 h, the connecting gates were closed followed by measuring the water's parameters and taking the samples for analysis, which indicated the condition of the water at the end of the first replication (24 h). After this (at the end of the first replication), fish and shrimp were taken out. The system was then prepared and adjusted for the second replication. The second replication was performed in the same way as the first replication: close the gates, renew the water (50 %), add the microalgae (for 50 %), inoculate *Artemia* sp. (1 g), homogenize a bit, take initial samples (0 h) for analysis, introduce the organisms (three fish, seven shrimps), spike with the relevant copper concentration, open the gates to allow movement, close during data recording, take the final samples (24 h) and remove the organisms. The third replication (after being adjusted and prepared) was also repeated in exactly the same way as the first two replications. During all the tests, the system was partially covered by the Perspex sheet to prevent the organisms from jumping out. The average water quality parameters during the experiment with copper are presented in Table S5.

2.7. Microalgal test (cell growth and chlorophyll fluorescence)

Samples from each compartment (10 mL) were taken for analysis at 0 h and 24 h from each replicate for use in flow cytometry (BD Accuri C6, Becton Dickinson). The sample representing 24 h was taken just at the end of the experiment and the samples representing 0 h were taken just after the addition of microalgae (10^4 cells/mL) at the beginning of the next replicate. All the samples were measured immediately after sampling. Autofluorescence was measured using an FL3 detector at 670 nm and with forward light scatter (FSC). The flow cytometry parameter settings were: flow rate 66 µL/min⁻¹, core size 22 µm and sample

volume 25 µL. For the chlorophyll fluorescence, four aliquots of 300 µL from each compartment were placed in a microplate and analyzed by spectrophotometry (TECAN-infinite F200). All the samples were performed in triplicate. The typical settings for both the flow cytometry and chlorophyll fluorescence tests were as described by Kholssi et al. (2023).

2.8. Nutrient analysis

Samples (10 mL) of inorganic nutrients were taken from each compartment at the beginning and end of each replicate of the avoidance test and stored in a freezer at -20 °C until analysis. The concentrations of dissolved ammonium (NH₄⁺), nitrate (NO₃⁻), nitrite (NO₂⁻), phosphate (PO₄³⁻) and silicate (SiO₄⁴⁻) were determined with an auto-analyzer (Alliance Futura) at the ICMAN-CSIC nutrient analysis laboratory using colorimetric techniques (Grasshoff et al., 1983) [(see also more details in Arjonilla and Blasco (2003)]. The Limit of Quantification (LOQ) for ammonium, nitrate, nitrite, phosphate and silicate was 0.2, 0.05, 0.01, 0.05 and 0.10 µM, respectively.

2.9. Statistical analysis

A mixed-design ANOVA was employed to evaluate the distributions (in %) of the organisms at each concentration and time for the avoidance test with the fish and shrimps. The normality was verified using the Shapiro-Wilk test and the homoscedasticity of the variances was checked by performing the Brown-Forsythe test. If the data did not meet ANOVA assumptions, the non-parametric Kruskal-Wallis test was used. Data were subjected to a Tukey post-hoc test to discriminate any statistically significant differences ($p < 0.05$) between time and compartment (concentrations). Time was treated as a within-subjects factor (repeated measure) and compartments (concentrations) were considered as a between-subjects factor. The avoidance percentage in the case of simulating a copper gradient was calculated according to Moreira-Santos et al. (2008): Avoiding organisms = NE - NO, where NE represents the expected number of organisms and NO represents the observed number of organisms [see also more details in Islam et al., 2019]. To determine the copper concentrations triggering avoidance in 50 % (AC50, ± confidence interval) of the fish and shrimp populations exposed, the PriProbit program (Sakuma, 1998) was used.

Microalgae data (cell growth and chlorophyll fluorescence) were analyzed using one-way analysis of variance to verify statistically significant differences among the treatments (concentrations and control) for each endpoint of each exposure period (24 h). All the graphs and statistical analyses were performed using the Sigma Plot 14.0 software package (SysStat, San Jose, California, USA) considering a 0.05 level of significance.

3. Results

3.1. Control and the copper avoidance test

The spatial distribution of the shrimps *P. varians* in the control experiments with no copper did not vary in time ($F = 0.26$ and $p > 0.05$) or the mesocosm compartments ($F = 0.47$ and $p > 0.05$) after 24 h exposure period (see Fig. 2A and Table S6), evidencing no preference for any compartment.

For the avoidance tests with copper, the distribution of the fish *S. aurata* was concentration and time-dependent (for concentration $F = 46.47$ and $p < 0.001$; for time $F = 5.07$ and $p < 0.001$; see Table S7). The fish were able to avoid the copper concentrations from the lowest given concentration of 1 µg/L (Fig. 2B). Nearly half (48 %) of the total population had moved toward the control concentration by the end of the exposure period; there were only 6 % of the total population in the highest concentration. The fish distribution remained relatively stable after 6 h of exposure to both the control concentration and the highest concentration. At 6 h, the mean percentage of fish was 55.6 % for the

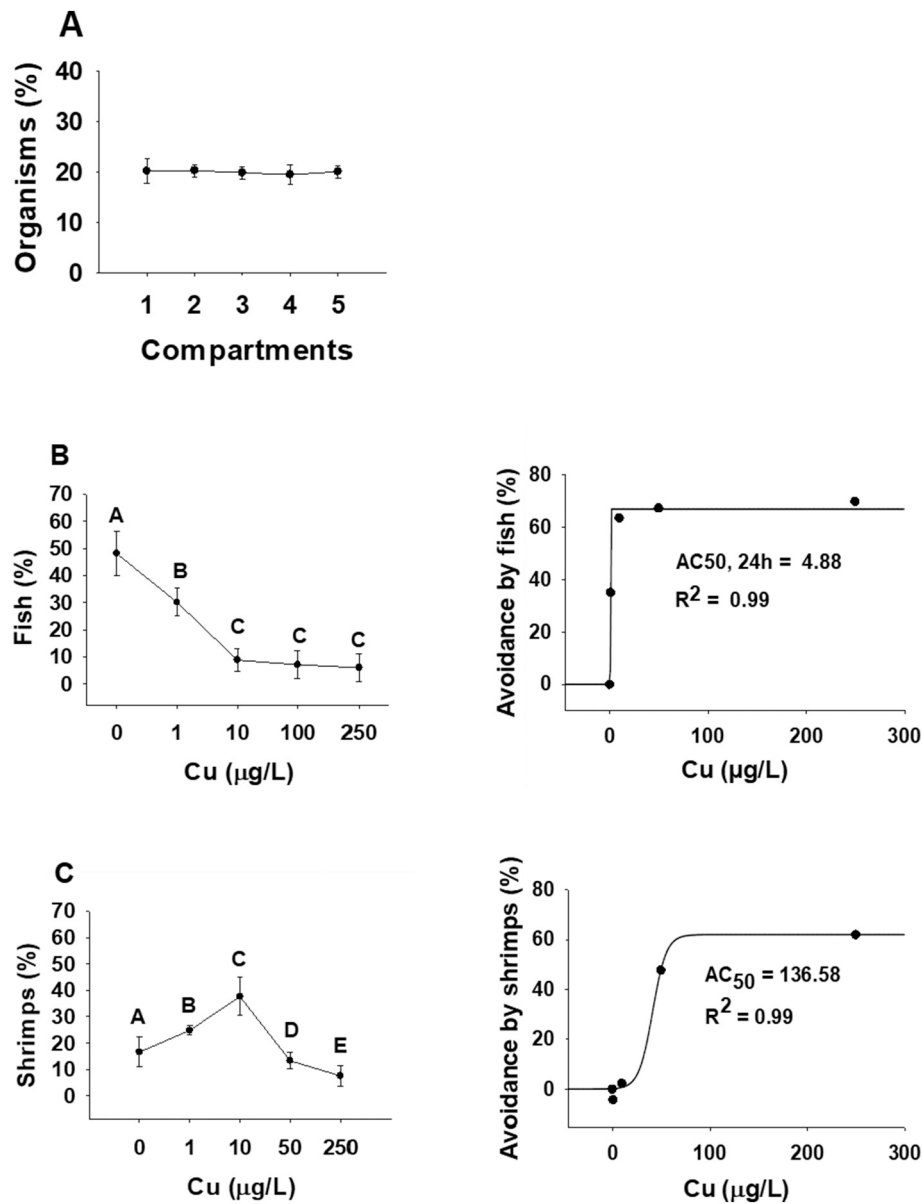


Fig. 2. Distribution of shrimps (mean percentage from seventeen observations; $n = 3$) in the control test (A); percentage of distribution and avoidance response of fish (B) and shrimps (C) throughout the copper gradients in the HeMHAS at the mesocosm scale after a 24 h exposure. Statistical differences among the means are indicated by different letters ($p < 0.05$; Tukey test) and error bars indicate standard error.

control concentration and 4.4 % for the highest concentration. Between 6 and 24 h, the percentage of fish varied between 60 % to 64 % for the control concentration and 2 % to 4 % for the highest concentration. At the end of the 24 h exposure, the AC_{50} was 4.88 $\mu\text{g/L}$ and the percentage of avoidance increased (ranging from 35 % at 1 $\mu\text{g/L}$ to 70 % at 250 $\mu\text{g/L}$) with the increasing concentration of copper.

On the other hand, although the distribution of the shrimp *P. varians* was concentration-dependent ($F = 317.45$ and $p < 0.001$; see Table S8), they only significantly avoided the two highest concentrations of copper (Fig. 2C). Approximately only 20 % of the total shrimp population was located in the compartments with the two highest concentrations, while the rest (approximately 80 %) of the shrimp population inhabited the first three compartments with concentrations of up to 10 $\mu\text{g/L}$. Interestingly, their preferred choice was to stay in the compartment with 10 $\mu\text{g/L}$ (approximately 40 %). The movement of the shrimps did not vary upon observation time ($F = 1.13$ and $p = 0.34$; see Table S8). At the end of 24 h exposure, the AC_{50} for the shrimps was 136.6 $\mu\text{g/L}$ (which was comparatively 30 times higher than for the fish AC_{50}) and the avoidance

percentage was approximately 50 % and 60 % at the two highest copper concentrations.

3.2. Microalgal test (growth, chlorophyll fluorescence and nutrient consumption)

3.2.1. Growth

Microalgal growth was concentration-dependent; when the concentration increased, the growth was inhibited in different ways, depending on the species (Fig. 3; see Tables S9–S26). *I. galbana* was more sensitive and the number of cells significantly decreased from 1 $\mu\text{g/L}$. After the final day of exposure, the growth was inhibited by nearly 50 % at the highest concentration. For *N. gaditana*, a significant reduction was observed from 10 $\mu\text{g/L}$ and, after the three-day exposure period, it was reduced by approximately 30 % at the final concentration. The growth of *T. chuii*, however, was significantly inhibited only in the two highest concentrations, with about a 25 % inhibition at the highest concentrations after the final days of exposure. Despite the partial renewal of the

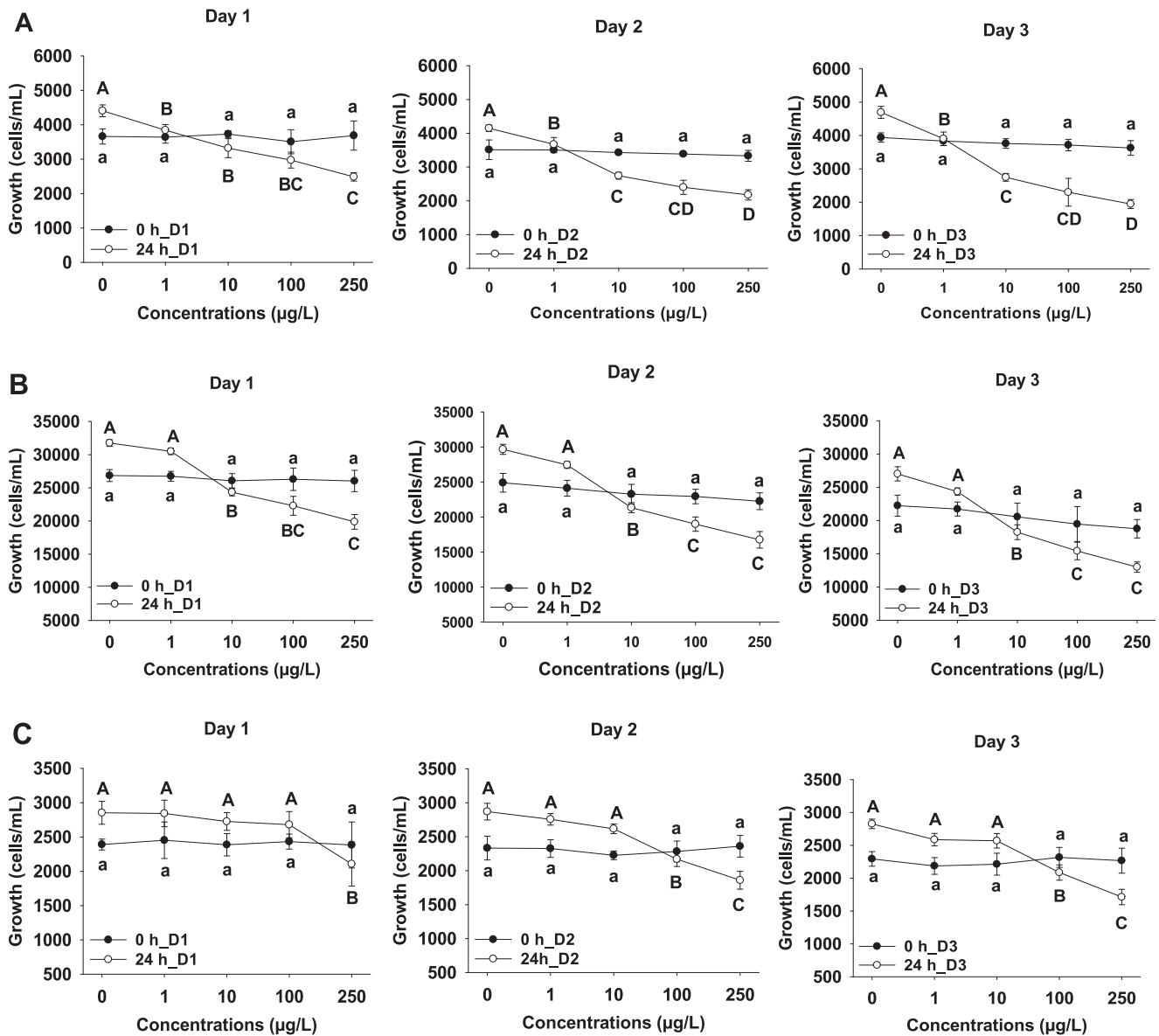


Fig. 3. Growth response (cell density) of the three marine microalgae (A: *Isochrysis galbana*; B: *Nannochloropsis gaditana*; C: *Tetraselmis chuii*) exposed to different copper concentrations for three days in the HeMHAS at the mesocosm scale. Different uppercases represent significant differences ($p < 0.05$; Tukey's post hoc test; $n = 3$) among the concentrations measured on each day (24 h) and the lower cases represent the significant differences among the initial values (0 h).

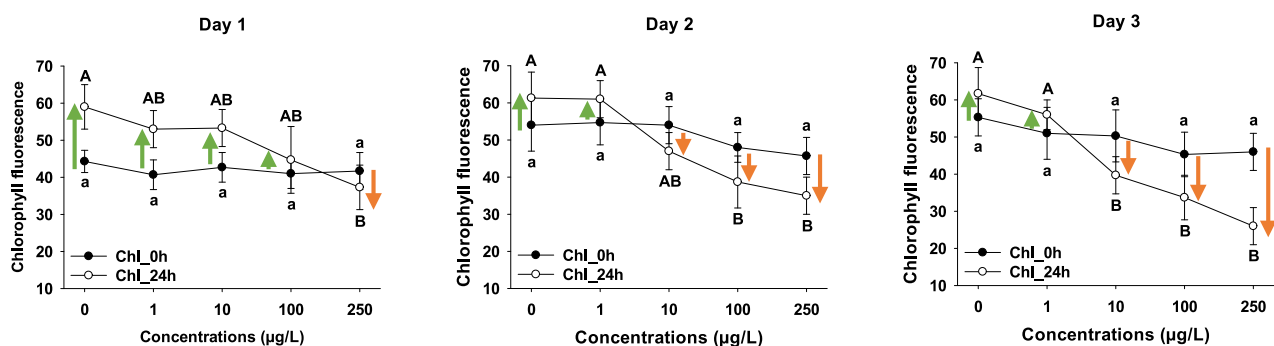


Fig. 4. Chlorophyll fluorescence of the microalgal community (*Nannochloropsis gaditana*, *Isochrysis galbana* and *Tetraselmis chuii*) exposed to a Cu gradient for three days in the HeMHAS at the mesocosm scale. Different upper-case letters represent the significant differences ($p < 0.05$; Tukey post hoc test) of chlorophyll fluorescence among the concentrations tested on each day (24 h) and the lower cases represent the significant differences among the initial values (0 h). The colors indicate changes in chlorophyll fluorescence levels: green arrows indicate an increase and red arrows indicate a decrease of chlorophyll fluorescence relative to the initial levels.

mesocosm water for replicates 2 and 3, the number of cells at the beginning of those replicates was statistically similar for all the compartments (Fig. 3).

3.2.2. Chlorophyll fluorescence

Significant changes were observed in the chlorophyll fluorescence levels in the microalgae community exposed to the Cu gradients (Fig. 4). On the first day of copper exposure (0 h), there were no statistically significant differences in chlorophyll fluorescence among all the compartments and it was homogeneously distributed ($F = 0.43$ and $p = 0.8$; see Table S27). However, 24 h after the first day of exposure, the chlorophyll fluorescence gradually decreased between all the concentrations tested and it reduced significantly at the highest concentration ($F = 5.38$ and $p < 0.05$; see Table S28). This trend of reduction gradually increased over the following two days. On the second day of exposure, the initial chlorophyll fluorescence (0 h) was similar between all the compartments ($F = 1.71$ and $p = 0.2$; see Table S29) and after 24 h the values significantly declined at the two highest concentrations ($F = 12.09$; $p < 0.001$; see Table S30). On the final day, the initial value (0 h) was also not statistically different among all the compartments ($F = 1.3$ and $p = 0.3$; see Table S31), but after 24 h the chlorophyll levels reduced significantly from 10 µg/L ($F = 22.75$ and $p < 0.001$; see Table S32); the level of reduction was 21 %, 26 % and 33 % at 10, 100 and 250 µg/L, respectively. However, the chlorophyll fluorescence level exhibited an increase of approximately 10 % compared to the initial value for the first two concentrations.

3.2.3. Nutrient consumption

During the 24-hour test, among the five analyzed nutrient contents (ammonium, nitrate, nitrite, phosphate and silicate), the levels of ammonium gradually decreased compared to their initial measurements (see Table 1). The ammonium concentration decreased by approximately 70 %, while the levels of other nutrients remained relatively stable and were consistent with the initial measurements after the 24 h

Table 1
Average ($\pm$ standard deviation; $n = 3$) nutrient uptake values by algal communities at different time intervals (0 h, 8 h and 24 h) in the HeMHAS at the mesocosm scale.

Nutrient (μM)	Time	C1	C2	C3	C4	C5	
Ammonium (NH ₄ ⁺)	0 h	60 ± 44	60 ± 51	58 ± 43	59 ± 40	60 ± 51	
		30 ± 41					
		18 ± 59					
	8 h	41	30 ± 40	31 ± 52	31 ± 34	31 ± 37	
		24 h	59	19 ± 36	19 ± 30	19 ± 33	20 ± 40
		99 ± 51	102 ± 50		103 ± 40	102 ± 44	
Nitrate (NO ₃ ⁻)	0 h	93 ± 41		96 ± 58			
		8 h	41	99 ± 30	88 ± 57	98 ± 52	99 ± 49
		98 ± 53	103 ± 55		101 ± 42		
	24 h	53	55	95 ± 43	42	97 ± 57	
		13 ± 24	12 ± 40	13 ± 22	12 ± 35	13 ± 20	
		12 ± 36	12.8 ± 34	12.5 ± 21	13.1 ± 40	12.9 ± 29	
Nitrite (NO ₂ ⁻)	0 h	36	34	21	40	29	
		13 ± 22	11 ± 39	13 ± 30	13 ± 20	13 ± 31	
		0.8 ± 0.7	0.7 ± 0.6	0.7 ± 0.8	0.6 ± 0.9	0.7 ± 0.8	
	8 h	0.03	0.05	0.06	0.05	0.09	
		24 h	0.7 ± 0.8	0.6 ± 0.7	0.8 ± 0.8	0.9 ± 0.8	0.8 ± 0.8
		0.02	0.06	0.07	0.08	0.06	
Phosphate (PO ₄ ³⁻)	0 h	0.8 ± 0.7	0.7 ± 0.6	0.8 ± 0.8	0.8 ± 0.9	0.8 ± 0.8	
		24 h	0.05	0.06	0.07	0.03	0.06
		175 ± 20	173 ± 55	176 ± 40	175 ± 48	172 ± 59	
	8 h	173 ± 33	178 ± 41	175 ± 39	173 ± 40	168 ± 49	
		24 h	176 ± 30	173 ± 28	175 ± 31	173 ± 23	176 ± 41
		Silicate (SiO ₄ ⁴⁻)	30	28	31	23	41

exposure period.

4. Discussion

4.1. Avoidance response

Although no study of avoidance with *S. aurata* in a non-forced multi-compartmented system was found in the literature, previous studies have shown that fish are able to detect environmentally relevant concentrations of copper (Moreira-Santos et al., 2019; Hansen et al., 1999). Moreira-Santos et al. (2008) found similar results of 24 to 96-h-AC₅₀ values from 16 to 23 µg/L for *Danio rerio* using a non-forced linear exposure approach. The results from Silva et al. (2018) are also comparable with the present study. They reported 3-h-AC₅₀ values of 15 µg/L for *D. rerio* using the multi-habitat linear assay system. Araújo et al. (2018) found an approximately 70 % avoidance response for *D. rerio* at 221 µg/L copper concentration with a 2-h-AC₅₀ of 60 µg/L using HeM-HAS. The differences in relation to the avoidance response could be the result of the different approaches adopted as well as the range of concentrations used in the gradient and species (Redondo-López et al., 2023). The distribution of the fish in the present study was also time-dependent, which can be compared with the results of Moreira-Santos et al. (2008). They found similar AC₅₀ results from 24 h to 96 h of exposure. In general, the avoidance response to copper observed for *S. aurata* in the current study was sensitive, as it occurred at very low concentrations.

Comparing our results with other sub-lethal effects of copper, it seems obvious that the fish's ability to avoid copper is advantageous for them. Drummond et al. (1973) found that exposure to copper at a concentration of 15 µg/L for 24 h resulted in changes in the locomotor activity and feeding behavior of *Salvelinus fontinalis*. Winberg et al. (1992) demonstrated that the Atlantic salmon, *Salmo salar*, when exposed to a copper concentration of 12.7–616.4 µg/L for 65 min, the electro-olfactogram response was significantly suppressed. Vieira et al. (2009) found that exposing *Pomatoschistus microps* to 50 µg/L of copper for 96 h led to detrimental effects including an inhibition of the cholinergic function (AChE), reduced detoxification capacity (EROD), increased energy requirements to mitigate chemical damage and oxidative stress, which collectively impaired the fish's swimming. In addition, Driessnack et al. (2017) found that exposing *Pimephales promelas* to 45 µg/L of copper for 21 days led to decreased egg quantity and size. Therefore, this very quick avoidance behavior might prevent organisms from being exposed to contaminants over the long term, thereby reducing the probability of suffering toxic effects.

In the case of *P. varians*, the shrimp only significantly avoided the highest two copper concentrations (100–250 µg/L) and the 24 h AC₅₀ was 136.6 µg/L. They preferred to stay in compartments of a 10 µg/L copper concentration. This could be explained by the fact that the majority of the fish *S. aurata* stayed in the first two compartments (total was approximately 80 %). As *S. aurata* is an aggressive carnivore that can prey on shrimp (Ferrari and Chierigato, 1981; Pita et al., 2002; Arechavala-Lopez et al., 2019), the shrimp *P. varians* preferred to seek refuge in the compartments with a copper concentration of 10 µg/L (where the percentage of *S. aurata* was lower than 10 %). The fish did not move into the more contaminated compartments and prey on the shrimps as they were sensitive to the existing levels of copper and had alternative sources of food, like the *Artemia* sp., microalgae and possibly other small organisms present in the macroalgae and macrophytes. There are many studies documenting that contaminants such as metals or pesticides can alter behavioral responses such as locomotion, aggression, schooling, and feeding by damaging the nervous system at the biochemical level (Scott and Sloman, 2004; Winberg et al., 1992; Weis et al., 2001; Jacquin et al., 2020). Regarding the non-forced exposure approach, some authors have observed changes in habitat selection patterns when motile organisms are exposed to contaminants. Silva et al. (2018) studied the avoidance response of two fish species,

D. rerio and *P. reticulata*, in a copper gradient and observed that *P. reticulata* became more aggressive when tested simultaneously with *D. rerio*, displacing them to contaminated areas, which had previously been avoided by *D. rerio* when tested alone. Araújo et al. (2019) showed that the avoidance response to copper altered when *D. rerio* and the freshwater shrimp *A. desmarestii* were tested together: the shrimps were more sensitive than the fish in the monospecies tests, but the shrimps' avoidance response was less intense in the multispecies tests. They reported that contamination seems to have increased the fish's territorialism and aggressive behavior and reduced the shrimps' ability to select a favorable habitat in the contaminated environment. Saaristo et al. (2018) reported that interactions among species could reduce the ability of organisms to respond to stimuli by acting on the sensory system. There are studies showing that the contaminants can have an impact on the ecological relationship between organisms. For example, Forester et al. (2019) reported that shrimp behavior might be different in a zone of chronic predation risk due to the energy cost of avoiding predators. This was clearly observed by Araújo et al. (2020b) in a study with the freshwater shrimp *A. desmarestii*. The shrimps avoided the copper contamination, moving to the compartments with lower levels of copper; however, when a chemical cue of a potential predator was introduced in the first two cleanest compartments, the shrimps moved to an intermediate area. The chemical cues that shrimp might receive from fish as predators typically include metabolites, kairomones, or alarm substances, which indicate the presence and activity of predators (Ferrari et al., 2010; Ocasio-Torres et al., 2021). Thus, they demonstrated a balanced habitat selection process by reducing the risk of predation (by avoiding the kairomones in the first compartments) and of contamination (by avoiding high copper levels in the last two compartments).

4.2. Microalgal responses

The growth of the microalgae was concentration-dependent, as their cell growth was inhibited to the increasing levels of copper. Among the species tested, *I. galbana* was more sensitive to copper than *N. gaditana* and *T. chuii*. Similar responses to copper were also observed by Debelius et al. (2009) based on the 72 h-EC₅₀ values of cellular densities, which were for *I. galbana*, *N. gaditana* and *T. chuii*: 58 µg/L, 137 µg/L and 330 µg/L, respectively. In another study, Levy et al. (2007) found 72 h-IC₅₀ (growth inhibition by copper) values of 4 µg/L and 47 µg/L for *I. galbana* and *T. chuii*, respectively. Satoh et al. (2005) demonstrated the sensitivity of nine marine microalgal strains to the heavy metals (Cu, As, Sb, Pb and Cd) and found the 72 h-IC₅₀ value for *I. galbana* and *T. chuii* for Cu was, respectively, 1.6 mg/L and 3.2 mg/L (*I. galbana* was 2 times more sensitive than *T. chuii*). The differences in the sensitivity observed in the current experiments was expected, as microalgal susceptibility to contaminants can be influenced by cell size, toxicokinetic and toxicodynamic processes and uptake rates across the plasma membrane, in internal binding mechanisms and/or detoxification mechanisms among species (Levy et al., 2007; Johnson et al., 2007).

In the present study, the effect of copper on the chlorophyll fluorescence of the three combined species was concentration-dependent. Although on the initial day, differences were only at the highest concentration (250 µg/L), the levels of chlorophyll fluorescence were significantly lower from 10 µg/L on the final day of exposure. Reduction in chlorophyll fluorescence levels due to exposure to copper at a similar concentration range has been recorded for many strains (*Chlorococcum littorale*, *Chlorococcum* sp., *Tetraselmis tetraele*, *I. galbana*, *Synechococcus* sp., *Prasinococcus* sp., *Heterocapsa* sp., *Cylindrotheca* sp.). For example, Satoh et al. (2005) reported the chlorophyll fluorescence of *I. galbana* reduced from a 12.5 mg/L copper concentration, while for *Tetraselmis* sp. the chlorophyll fluorescence levels reduced from 25 mg/L. They also observed a linear relationship between the chlorophyll-a concentration and intensity of chlorophyll fluorescence. Barón et al. (1995) outlined the direct effects of copper on photosynthesis and

reported that while copper is an essential component of the primary electron donor in PSI (the Cu-protein plastocyanin), elevated copper concentrations can inhibit the photosynthetic electron transport rate, particularly in PSII. Both copper deficiency and copper toxicity disrupt pigment and lipid biosynthesis, consequently affecting the chloroplast ultrastructure, which ultimately impairs photosynthetic efficiency. An increase in endogenous Cu²⁺ concentrations even only slightly beyond the optimum range has been shown to disrupt various metabolic pathways, resulting in photosynthetic suppression and a variety of deleterious effects at the physiological, biochemical, and structural levels (Kumar et al., 2009; Zhang et al., 2014). According to Griffiths and Harrison (2009), the growth status of algal cells can be reflected by photosynthesis and chlorophyll content. Suresh Kumar et al. (2014) summarized that the heavy metal copper alters the photosynthetic responses of algae (using the chlorophyll-a fluorescence value) at sublethal and lethal concentrations.

Microalgae and macroalgae have distinct methods of nutrient assimilation. They prefer ammonium nutrients and can rapidly absorb and utilize them compared to other sources (Giordano and Raven, 2014; Nayar et al., 2018; Kumar and Bera, 2020). In our study, we observed a gradual decline in ammonium concentrations over the 24 h exposure period, while the levels of other nutrients remained consistent regarding their initial values. Luo et al. (2012) conducted a study of nutrient uptake by two algal species, *Ulva prolifera* and *U. linza* and found that both species exhibited higher affinity for NH₄⁺ than for NO₃⁻ and PO₄³⁻. The preference for ammonium is often attributed to the lower energy demand associated with enzymatic reduction for assimilation (Bloom et al., 1992; Abreu et al., 2011). Nitrate utilization demands more energy due to the numerous electrons (up to 8 electrons) needed for its reduction to ammonia (Losada et al., 1970; Serra et al., 1978). According to Hellebust and Ahmad (1989) ammonium can also inhibit the uptake and assimilation of other nitrogen sources. In another study, Hii et al. (2011) found that *Nannochloropsis* sp. preferred ammonium uptake and the nitrate was utilized only during the absence of ammonium. In general, and in spite of the differences in cell growth and chlorophyll fluorescence, the level of nutrients was not affected by the level of copper. There is limited data on the potential impact of copper on nutrient uptake; hence, it is recommended to conduct more studies, and at wider exposure time, in order to fully understand how copper may influence nutrient absorption.

5. Conclusion

The species of fish and shrimp studied in this experiment were able to avoid copper gradients in different ways. The shrimps' lower avoidance was attributed to a cost-benefit balance between the risk of predation and exposure to the contaminants. The study suggested that the avoidance pattern of both species could jeopardize population persistence and species' habitat selection thereby indicating that contaminants may strongly affect interspecies relations. The study also shows how contamination by copper in diverse ecological conditions may affect microalgal growth and chlorophyll concentration, but not nutrient consumption.

The novel HeMHAS mesocosm proved to be a suitable system to simulate landscapes with interconnected and chemically heterogeneous habitats. The HeMHAS mesocosm brings an integrative methodological and conceptual tool for ecotoxicology, allowing measuring toxic effects on non-mobile populations that cannot escape from contamination, changes in the habitat selection process in mobile species able to move and avoid contamination and functional alterations like nutrient uptake. The approach applied in the current study using the HeMHAS mesocosm should be considered as a complementary tool in ERA studies. This is because it can model the complex scenarios of disturbed interconnected habitats in such a way that it reaches a higher level of ecological relevance and may be used to assess the stressor's effects from a broader biological and spatial perspective.

CRediT authorship contribution statement

Mohammed Ariful Islam: Writing – original draft, Visualization, Methodology, Investigation, Formal analysis, Conceptualization. **David Salvatierra:** Writing – review & editing, Methodology, Investigation, Conceptualization. **María Pilar González:** Writing – review & editing, Methodology, Investigation, Conceptualization. **Andrea Cordero-de-Castro:** Writing – review & editing, Methodology, Investigation, Conceptualization. **Rajaa Kholssi:** Writing – review & editing, Methodology, Investigation, Conceptualization. **Ignacio Moreno-Garrido:** Writing – review & editing, Resources, Methodology, Investigation, Conceptualization. **Julián Blasco:** Writing – review & editing, Writing – original draft, Validation, Supervision, Resources, Methodology, Investigation, Conceptualization. **Cristiano V.M. Araújo:** Writing – review & editing, Writing – original draft, Validation, Supervision, Resources, Project administration, Methodology, Investigation, Funding acquisition, 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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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.scitotenv.2024.175849>.

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