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Investigation of Tolerance and Avoidance Responses in Meiobenthic Copepod Species to Phenanthrene and Chrysene Exposure

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ABSTRACT

This study investigates the effects of two Polycyclic Aromatic Hydrocarbons (hereafter PAHs), chrysene and phenanthrene, on the vertical movement of meiobenthic copepods in open microcosm environments. PAHs are environmental contaminants of significant concern owing to their prevalence and toxicological effects on aquatic organisms. This investigation highlights the significance of PAHs as ecological stressors affecting the movement behavior of meiobenthic copepods following exposure to chrysene and phenanthrene, both individually (1.16 ng/g Dry Weight) and in combination. Indeed, it was identified that the most vulnerable species to PAHs are *Asellopsis hispida* and *Bulbamphiascus imus*. These species demonstrated a clear aversion to PAHs, probably via remote chemodetection. In contrast, species like *Canuella furcigera* and *Heterolaophonte stromeii stromeii* showed a high tolerance to PAHs by moving into all contaminated regions. Interestingly, the study outcomes supported that the movement patterns of meiobenthic copepods may serve as crucial indicators of the availability and nature of PAHs in benthic ecosystems.

KEYWORDS: Meiofauna; Copepods, Chrysene; Phenanthrene, Vertical migration.

INTRODUCTION

In recent decades, environmental pollution has emerged as one of the most pressing global challenges (Selamoglu et al., 2008, 2009; Klavins et al., 2022). Industrialization and urbanization are primary factors negatively impacting ecological health due to ongoing challenges from both traditional and emerging pollutants (Failler et al., 2015). Regardless of their sources or modes of release, aquatic ecosystems act as ultimate sinks where these substances and their degradation products accumulate. Therefore, it is crucial to evaluate the risks associated with environmental chemical pollutants. Ecotoxicology plays a crucial role in two main areas: (1) evaluating the impacts of pollutants on biota within an ecosystem before implementing preventive strategies, and (2) understanding and modeling the mechanisms of environmental contamination (Lesueur, 2014).

One significant class of organic pollutants found in marine environments is polycyclic aromatic hydrocarbons (hereafter referred to as PAHs), which can have adverse effects on aquatic life. PAHs can disrupt the natural balance of ecosystems, potentially jeopardizing human health, damaging biological resources, and interfering with various legitimate uses of the ocean (Meador, 2003).

Aquatic products and animals are essential sources of nutrients in the human diet and play a significant role in the global aquatic product industry for consumers (Selamoglu, 2021). Therefore, we need to protect our aquatic environment against several pollutants, with confirmed toxicity (Selamoglu et al., 2009; Ozdemir et al., 2006, 2007, 2010). Aquatic ecosystems and living organisms are affected by environmental impacts resulting from the emissions of volatile organic substances and water pollution caused by oil, chemicals, and various hazardous agents. Every trophic level is susceptible to hydrocarbon contamination, ranging from planktonic species to marine mammals. Mollusks and crustaceans (such as mussels, shrimp, and crabs) are particularly prone to bioaccumulating contaminants, even when the pollution level is low or the source is removed. In fact, Molds are unable to stop pollutants from infiltrating. Conversely, crustaceans that consume detritus or suspended particles will experience changes in their reproductive rate (decrease in hatch rate) or their feeding behaviours. Genotoxicity, carcinogenicity, effects on reproduction and development (Arkoosh et al., 1996; Johnson et al., 1998; Rice et al., 2000), and immunotoxicity (Reynaud and Deschaux, 2006) were emphasized to varying extents based on the molecular weight of PAHs and the metabolism processes.

Numerous studies have focused on the impact of hydrocarbons on benthic communities (Lee et al., 1981; Boucher, 1981; Beyrem and Aïssa, 2000; Mahmoudi et al., 2005). For instance, Lotufo (1997) demonstrated the substantial effects of PAHs on copepod reproduction, leading to a significant reduction in the number of eggs laid by females. Environmental monitoring of this specific type of pollutant may rely, among other factors, on its impact on both positive and negative bioindicators, which is necessary for government policymakers to consider.

The topic investigated in this research has emerged as a significant global concern. Thus, this research aims to primarily address the gap concerning PAHs, whose impacts on one of the major meiobenthic groups, copepods, have yet to be investigated based on their migratory behavior. The inquiries explored were: (1) Will copepod species exhibit tolerance or avoidance responses to phenanthrene and/or chrysene? Moreover, (2) if so, do copepods exhibit taxonomic changes after their exposure to chrysene, phenanthrene, and their combination? The applied experimental approach was original and focused on the vertical taxonomic modifications.

MATERIALS AND METHODS

Sediment collection

The initial samples were collected on March 18, 2019 (7 A.M.), at a coastal site near the city of Menzel Jemil, Tunisia, situated in the subtidal zone in Bizerte lagoon (37°13'22''N; 9°55'48''E). The sampling site was chosen for its location in a protected and low-hydrodynamic region (Boufahja et al., 2006). On the day of sampling, sediments were taken during low tide using the method proposed by Boufahja et al. (2016) to a depth of 5 cm. This layer serves as a microhabitat that exhibits the greatest taxonomic richness and abundance of meiofauna (Bin-Jumah, 2024).

Microcosm description

To investigate the potential vertical migration of meiobenthic copepods from a healthy natural environment to an azoic and contaminated medium, twelve microcosms were established. Each microcosm consisted of two sediment compartments: natural sediments on top and azoic-contaminated sediment below (refer to Fig. 1). These sediments were enriched with seawater that had been pre-filtered through a 40 µm sieve to prevent the inclusion of organisms that could skew the results. After fifteen days, all replicates were preserved in 4% neutralized formalin at hexamethylene tetramine and subsequently stained with Bengal Rose (0.2 g/l) to enhance the coloration of the specimens found in the sediment (Waweru et al., 2024). Following preservation in formalin, harpacticoids and cyclopoids were stored in 70% ethanol for subsequent taxonomic identification (Apostolov, 2016).

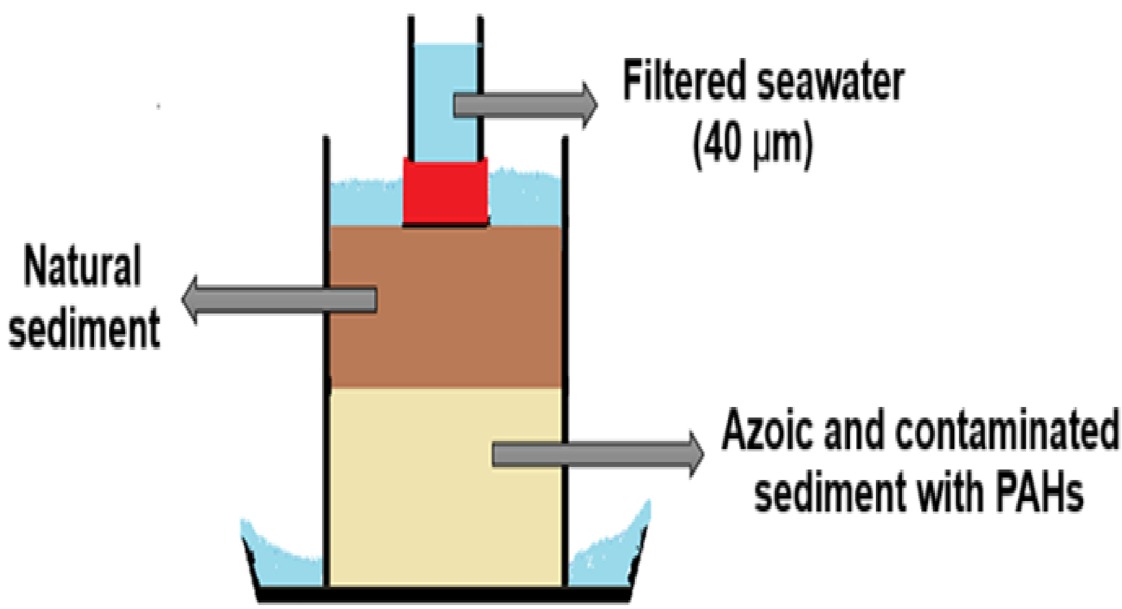

Figure 1. Experimental design used to study the vertical migration of meiobenthic nematodes after exposure to polycyclic aromatic hydrocarbons.

Sediment preparation

To render the sediment azoic, the collected samples underwent three cycles of freezing for 12 hours at -20 °C, followed by 48 hours of thawing at room temperature (Schratzberger et al., 2004). After processing, large particles were removed manually. The retained portion was then separated into two fractions: fine (for later contamination) and coarse, using a sieve with a 300 µm mesh (Boufahja et al., 2016). A 100 g fresh weight (FW) sub-sample was dried for 96 hours at 45 °C to determine sediment water content (Boufahja et al., 2016) and dry weight (DW).

Selection of PAH concentrations and sediment contamination

In this study, a single concentration was utilized for both hydrocarbons examined (chrysene and phenanthrene). This concentration was selected based on findings of Zrafi-Nouira et al. (2010), who reported levels of various PAHs in and around the discharge area of refining industries in Tunisia. The chosen concentration to initiate this bioassay is one-third of the chrysene concentration from the sedimentary layer, located 2,380 m away from the discharge area of the Bizerte refinery. Zrafi-Nouira et al. (2010) reported that at this distance, the sedimentary chrysene concentration is 347.5 ± 0.014 ng/g DW, which is approximately 348 ng/g DW. For our study, we contaminated the sediment with a concentration of 1.16 ng/g DW, a level nearly identical to that measured at 0.59 m from the Bizerte refinery rejection zone. To accomplish the sediment contamination with PAHs, it was necessary first to dissolve these hydrocarbons in acetone. Specifically, we dissolved 1 mg of phenanthrene (99% pure crystal) in 200 µl of acetone (Louati et al., 2014) and 1 mg of chrysene (99% pure crystal) in 625 µl of acetone (Lange et al., 2006).

Meiobenthic descriptors

The extraction of meiofauna from sediment was performed using the levitation-decantation-sieving method as described by Rzeznik-Orignac et al. (2017). This technique is based on the principle that the organisms to be extracted have a lower density than the sediment particles. The sediments were kept in suspension in a water-filled crystallizer through manual rotary motions. After a brief decantation period, the water containing the meiobenthic organisms was filtered twice through two sieves. The first sieve had a mesh size of 1 mm, while the second sieve had a mesh size of 40 µm. These sieves were used to separate macrobenthic organisms and large debris (≥ 1 mm) from the meiobenthos. The material collected by the 40 µm sieve was then retrieved using a gentle spray of 4% neutral formaldehyde (Guo et al., 2001) and transferred into plastic flasks containing a 70% ethanol solution (Apostolov, 2016).

The extracted material was carefully placed into a tiled Dollfus chamber, which contains 200 squares, each measuring 5 mm^2. The squares are bordered by raised edges that limit the movement of copepods as they were positioned on the stage of a stereomicroscope (type Leica zoom 2000) for counting. The magnifier aided in counting harpacticoids and cyclopoids, enabling their collection for both generic and species identification. Copepods were collected using a fine needle under the stereomicroscope. Each specimen was then placed on a slide and immersed in a dissection solution of glycerin (made of equal parts glycerin and distilled water). All appendages were separated and stored in a drop of glycerin. The furca and roster were additionally covered with a cover slip. To prevent the cover, slip from shifting, a thin coat of nail polish was applied to its edges. The taxonomic classification of harpacticoid and cyclopoid copepods was based on two guides: Lang (1948) and Huys et al. (1996).

Data processing

Four univariate indices were considered for every microcosm: abundance, species number, Margalef's species richness, and Shannon-Wiener diversity index. Kolmogorov-Smirnov tests were utilized to assess the normality of the data, whereas the Bartlett test was applied to examine the equality of variances. Log transformations were utilized when required. A one-way analysis of variance (1-ANOVA) was conducted using Statistica version 5.1

software to identify globally significant differences among the treatments tested. If significant differences ($p < 0.05$) were found, the Tukey's HSD *post-hoc* test was applied to identify pairs of treatments that differed significantly ($p < 0.05$). All replicates related to the treatments were consistently projected from a non-parametric multidimensional scaling (nMDS) analysis. A stress factor indicated on the nMDS 2D-plot below 0.2 suggests that the resulting representation is statistically and ecotoxicologically reliable (Clarke, 1993). This author also recommended the SIMPER process (SIMilarity PERcentages) to assess the contribution of each species to the overall dissimilarity among the various treatments. These multivariate analyses were performed using the software PRIMER v. 5.0, developed by the Plymouth Marine Laboratory (Clarke and Gorley, 2001).

RESULTS

Univariate indices

Regarding the average abundance of copepods, the compartments contaminated with the different polycyclic aromatic hydrocarbons (PH, CH, and M) showed significantly lower numbers than those associated with the control treatment (see Fig. 2 and Table 1). Comparisons of these abundances revealed discernible differences ($p < 0.05$) across all contaminated compartments and controls. The sediments contaminated with phenanthrene exhibited a reduced number of significant species compared to the control sediment. In contrast, the chrysene-contaminated replicates were associated with a slightly higher number of species. However, this difference was not statistically significant (see Fig. 2). The one-way ANOVA variance analysis revealed no significant differences among the Shannon indices for the various compartments, and a similar outcome was observed for Margalef's species richness.

Table 1. Comparisons of the average abundance of copepods from different compartments contaminated or not by polycyclic aromatic hydrocarbons using the Tukey-HSD *post-hoc* test. The values indicate the probabilities (p). Bold values indicate significant differences ($p < 0.05$). The data are *log*-transformed. C: Control; PH: phenanthrene; CH: chrysene; M: mixture of phenanthrene and chrysene.

Abundance: one-way ANOVA: $F = 9.1$; $p < 0.05$				
	C	PH	CH	M
C		**0.047**	**0.044**	**0.022**
PH			0.375	0.637
CH				0.955
M				

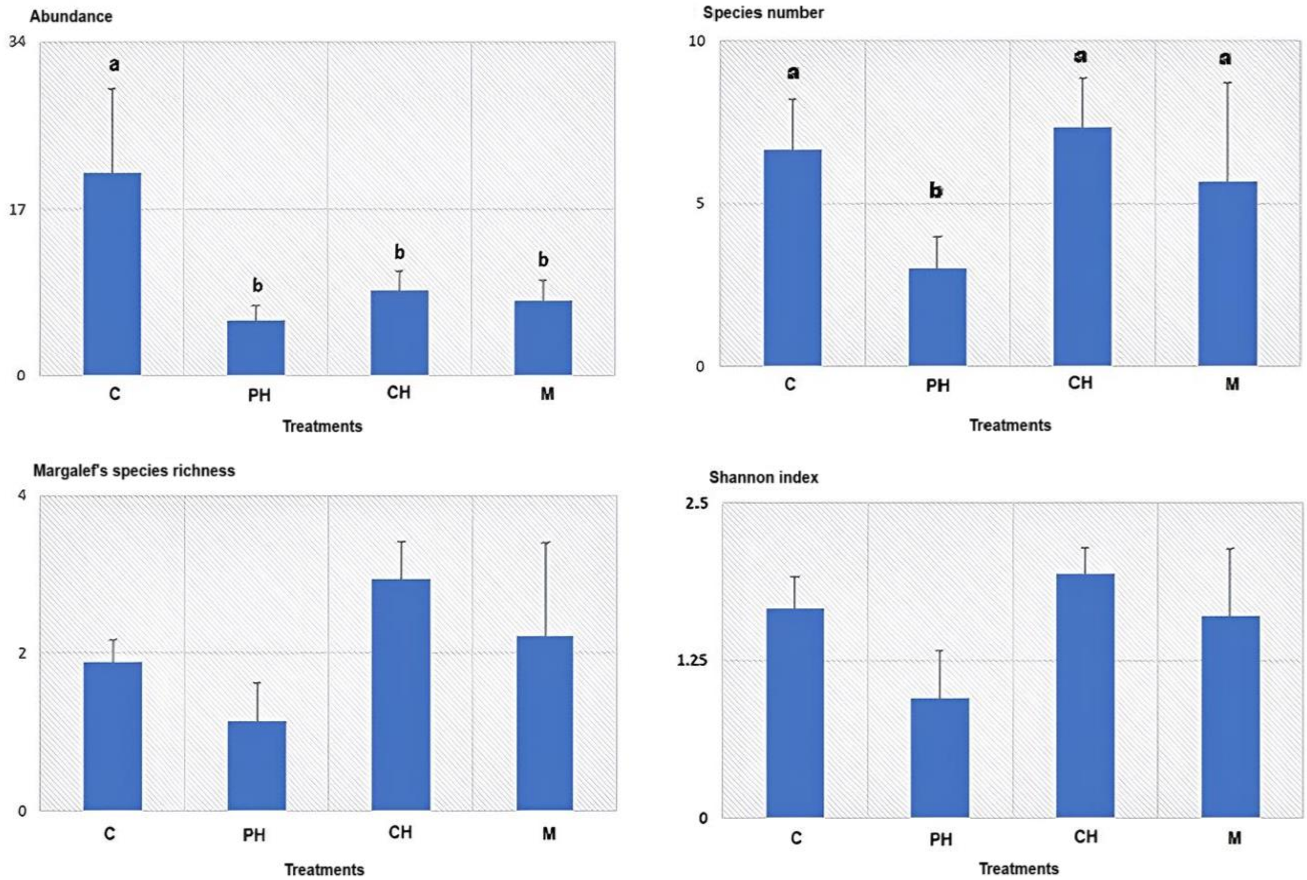

Figure 2. Changes in univariate indices related to copepod assemblages from the various sediment compartments contaminated or not with polycyclic aromatic hydrocarbons. The vertical bars represent the standard deviations. C: Control; PH: phenanthrene; CH: chrysene; M: mixture of phenanthrene and chrysene. Different letters above bars indicate significant differences (Tukey's test: $p < 0.05$).

Multivariate analyses

The results of the replicates' ordination based on the nMDS approach (Fig. 3) demonstrated a notable influence of PAHs on species vertical distribution (stress = 0.01). This effect allowed for the differentiation of replicates based on sediment quality; the controls were located on the left of the factorial plane, while contaminated sediments with phenanthrene, chrysene, and their combination appeared on the right. The results of the SIMPER analysis, detailed in Table 2, identified the cumulative species that contributed approximately 70% to the overall dissimilarity between the control copepod assemblage and those exposed to PAHs. The experiment concluded with the complete removal of two copepod species, *Enhydrosoma* sp. and *Amphiascus parvulus*, from the phenanthrene-contaminated compartment. In contrast, the compartments contaminated with chrysene and the chrysene/phenanthrene mixture experienced only a reduction in the population sizes of these species, rather than their complete elimination.

Table 2. Relative contributions (in %) of *copepod* species participating in around 70 % of the average dissimilarity between the various treatments considered. Decrease in abundance (-); Total elimination (Elim). C: Control; PH: phenanthrene; CH: Chrysene; M: mixture of phenanthrene and chrysene.

C vs. PH	C vs. CH	C vs. M
Enhydrosoma sp.1 (32,25%) <u>Elim</u>	*Enhydrosoma* sp.1 (22,31%) (-)	*Enhydrosoma* sp.1 (23,86%) (-)
Canuella furcigera (46,83%) (-)	*Canuella furcigera* (43,93%) (-)	*Canuella furcigera* (44,21%) (-)
Heterolaophonte stromeii stromei (59,36%) (-)	*Heterolaophonte stromeii stromei* (55,47%) (-)	*Heterolaophonte stromeii stromei* (55,12%) (-)
Amphiascus parvulus (69,93%) <u>Elim</u>	*Amphiascus parvulus* (63,40%) (-)	*Asellopsis hispida*
		Amphiascus parvulus (69,14%) (-)

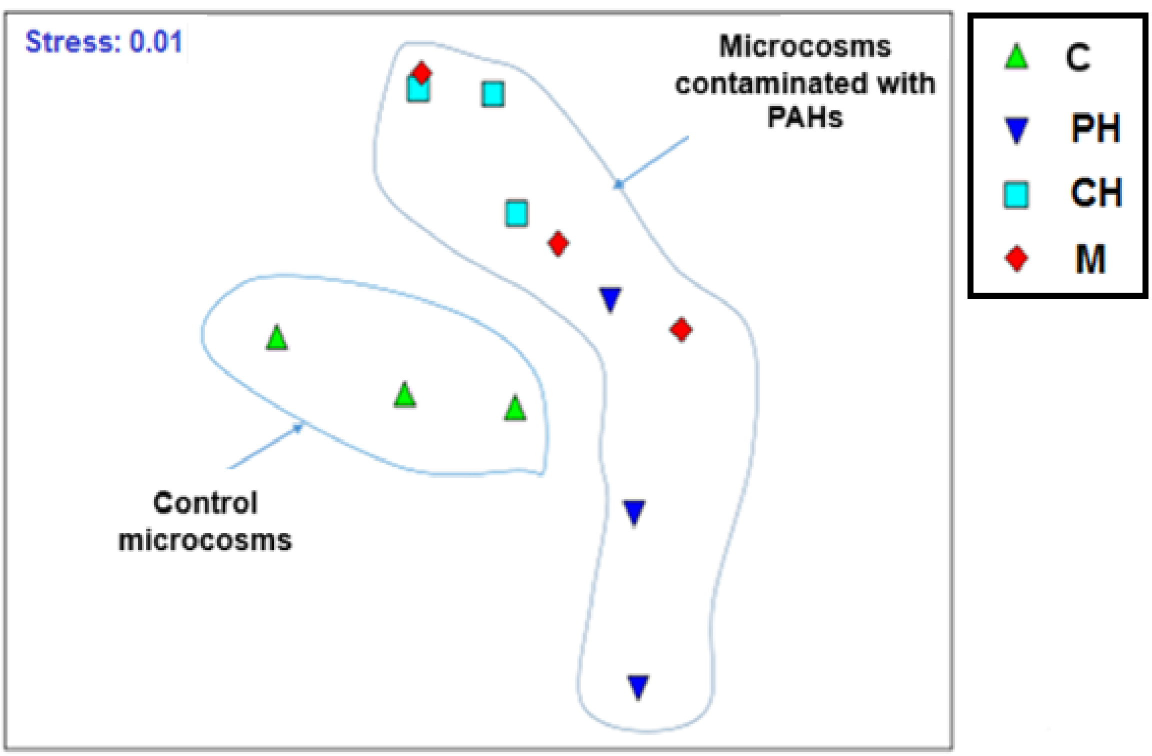

Figure 3. Ordination of the various microcosms (controls and contaminated by the HAP) according to the nMDS method based on species abundances. C: Control; PH: phenanthrene; CH: Chrysene; M: mixture of phenanthrene and chrysene.

DISCUSSION

This research aimed to investigate the impact of polycyclic aromatic hydrocarbons on the migratory behavior of harpacticoid and cyclopoid copepods. The bioassay was designed to connect two distinct types of sediments in open microcosms: one natural (inhabited by meiofauna and untreated) positioned at the top, and the other contaminated with PAHs—chrysene, phenanthrene, or a combination of both—located at the bottom. The city of Menzel Jemil, Tunisia, was chosen due to its abundance of copepods. At the end of the experiment, *C. furcigera* and *Enhydrosoma* sp. were predominant in the natural sediment, with these two species exhibiting distinct responses to the considered hydrocarbons. *C. furcigera* experienced a notable decline in average numbers, decreasing from 17 individuals in natural sediment to only 5 individuals in sediment contaminated by phenanthrene, and 4 individuals in sediment affected by chrysene and the combination of both hydrocarbons. This suggests that *C. furcigera* can somewhat endure pollution from PAHs. Conversely, *Enhydrosoma* sp. completely vanished from the phenanthrene-contaminated sediment but persisted in the sediment contaminated with chrysene and the phenanthrene/chrysene mixture. This suggests that *Enhydrosoma* sp. is highly responsive to phenanthrene, and an antagonistic interaction appears to regulate the relationship between the two mixed hydrocarbons. Morelis et al. (2007) examined the chemical interactions of chrysene and phenanthrene in marine sediments. They found that the combination of these PAHs was less toxic than when either chemical acted independently. These interactions may be linked to the variations in hydro-solubility of the two compounds, which are caused by their differing molecular weights: chrysene contains four aromatic rings, whereas phenanthrene has three. Additionally, two other species, present in natural sediment, displayed different behaviors toward PAHs: *Heterolalophonte stromeii stromei* and *Amphiascus parvulus*. Both copepod species, *C. furcigera* and *H. stromeii stromei*, were found in all compartments, regardless of whether they were contaminated with PAHs or not. However, there was a notable reduction in the number of individuals in the contaminated compartments compared to the control one. In contrast, *A. parvulus* exhibited behavior similar to that of *Enhydrosoma* sp.; this species was absent from compartments contaminated with phenanthrene but was present in microcosms containing sediments spiked with chrysene and in those with a mixture of phenanthrene and chrysene.

The presence of *C. furcigera* and *H. stromeii stromei* in various contaminated compartments may be attributed to several factors, including their potential tolerance to the PAHs tested or their entrapment in sediment compartments, which theoretically could be stickier, as they attempt to navigate through these altered conditions. The significance of the body surface area of copepods is heightened due to their native appendages. The absence of *Enhydrosoma* sp. and *A. parvulus* in compartments contaminated with phenanthrene—contrasting with their presence in compartments affected by chrysene and the combined PAHs—may indicate greater sensitivity of these copepod species to phenanthrene. The effects of phenanthrene and chrysene appear to be opposed. Species such as *Asellopsis hispida* and *Bulbamphiascus imus* were only found in untreated compartments, suggesting their potential sensitivity to phenanthrene and/or chrysene. These findings imply that these species might be capable of sensing stress induced by nearby PAHs and could be actively avoiding such conditions.

The results of this study are consistent with those found by Lotufo (1997), who observed high mortality rates in response to phenanthrene exposure and utilized fluoranthene alone. Conversely, Lotufo's study demonstrated that the phenanthrene/fluoranthene mixture did not result in significant mortality when compared to the respective control. Our findings, however, contradict those of Pavillon et al. (2003), who reported that phenanthrene had acute toxicity at a sediment concentration of 60 mg/kg DW for copepods, with no survivors noted after 96 hours of exposure.

CONCLUSION

Polluted sediments in the natural environment represent a significant stressor for sensitive species of copepods, rendering their survival in such conditions unviable; these species tend to avoid habitats that are unsuitable for them. In contrast, these same polluted sediments can create ideal conditions for more tolerant species or opportunists, leading to an increase in their populations. This research aimed to assess how two hydrocarbons (phenanthrene and chrysene), used individually or in combination, influenced the distribution of mechanistic copepod species in interconnected compartment microcosms. By observing the migratory patterns of these copepods in response to PAH exposure, we aimed to understand their specific behaviors under such stressors.

The findings underscore that each compartment had a unique species composition. Sediments not exposed to stress exhibited the highest species richness, while those polluted by phenanthrene displayed the lowest diversity. Our research identified the most sensitive species to PAHs, such as *A. hispida* and *B. imus*, which were only present in control sediments. These species showed evident avoidance of PAHs, likely through distant chemo-detection. Conversely, species like *C. furcigera* and *H. stromeii stromei* demonstrated a degree of tolerance to PAHs by migrating into all contaminated areas.

Notably, exposure to phenanthrene alone was found to be more detrimental to specific species in terms of their abundance than exposure to the combination of phenanthrene and chrysene, suggesting that these PAHs interact and imply potential antagonism between chrysene and phenanthrene. Ultimately, the presence of PAHs in sediments involved in ecotoxicological research significantly affects the abundance and diversity of copepods. Of the 19 species identified in the initial survey, only four displayed a relative capability to tolerate the presence of PAHs in the sediment.

From the perspective of this study, it is important to note that (1) the experiment should be replicated to track also the horizontal movement of copepods, which will allow for a more thorough confirmation of our findings, and (2) the impacts of additional vector pollutants like nanoparticles or microplastics should be analyzed using the same methodologies employed in this research.

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CONFLICT OF INTERESET STATEMENT

We declare that we have no conflict of interest.

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