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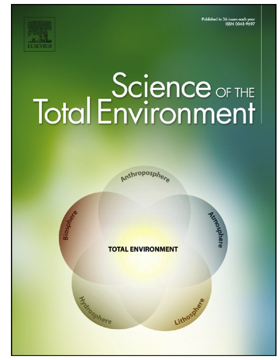

Trace metals exposure in three different coastal compartments show specific morphological and reproductive traits across generations in a sentinel copepod

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Trace metals exposure in three different coastal compartments show specific morphological and reproductive traits across generations in a sentinel copepod.

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Highlights

1. Contrasting sensitivity appeared between exposure compartments and generations.

2. Prosome length and volume were more sensitive to sediment resuspension.

3. Clutch size and egg diameter were more sensitive to dissolved exposure.

4. Seawater spiked trace metal mixtures provided lowest survival.

5. Trace metal and lipid droplet accumulation correlated significantly.

Abstract

The effect of exposure from several compartments of the environment at the level of individuals were rarely investigated. This study reports the effect of contaminants from varied compartments like sediment resuspension, elutriation from resuspended sediment (extract) and seawater spiked trace metal mixtures (TM) on morphological and reproductive traits of the pelagic bioindicator copepod *Eurytemora affinis*. At the population level of *E. affinis*, lowest survival was observed in dissolved exposures (TM and extract) in the first generation (G1), showing some adaptation in the second generation (G2). An opposite trend for resuspended sediment showed higher sensitivity in survival at G2. At the individual level, prosome length and volume proved to be sensitive parameters for resuspended sediments, whereas clutch size and egg diameter were more sensitive to TM and extract. Although the generation of decontamination (G3, no exposure), showed a significant recovery at the population level (survival % along with clutch size) of *E. affinis* exposed to resuspended sediment, morphological characteristics like prosome length and volume showed no such recovery (lower than control, $p < 0.05$). To the contrary, dissolved exposure showed no significant recovery from G1 to G3 on neither survival %, clutch size, egg diameter, prosome volume, but an increase of prosome length ($p < 0.05$). Such tradeoffs in combatting the stress from varied sources of toxicity was observed in all exposures, from G1 to G3. The number of lipid droplets inside the body cavity of *E. affinis* showed a significant positive correlation with trace metal bioaccumulation ($p < 0.01$) along with a negative correlation ($p < 0.05$) with survival and clutch size in each treatment. This confirms the inability of copepods to utilize lipids under stressful conditions. Our study tenders certain morphological and reproductive markers that show specificity to different compartments of exposure, promising an advantage in risk assessment and fish feed studies.

Keywords - *Eurytemora affinis*, dissolved trace metals, sediment resuspension, prosome length, clutch size, lipid droplets.

1. Introduction

Trace metal contamination in several coastal areas worldwide provide significant risks to benthic or pelagic marine organisms (Lesueur et al., 2015; Hamzeh et al., 2016; Buttino et al., 2018; Jeong et al., 2019; Amara et al., 2019). Such toxicities often arise from several subsystems or compartments like the sediment and water column and their interaction. Exposure could be enhanced by the resuspension of sediment into the pelagic phase, accounting for different compartments of contamination in the marine environment, that affect nearby organisms at the individual or population level (Borcier et al., 2019, 2020; Dayras et al., 2020; Kadiene et al., 2022). In Europe, most of the large estuaries are affected by pollution originating from heavy industries (Charry et al., 2018). The Seine estuary in northern France, for example, receives various contaminant loadings, mostly due to dense shipping routes, industries and commercial impacts in the last century (Dauvin 2008; Das et al. 2020, 2022a). In addition, the Seine estuary is characterized by forceful hydrodynamics from natural and anthropogenic processes that exert substantial resuspension of sediments to the pelagic phase, generating high turbidity and release of toxicants (Lesueur et al., 2015; Grasso et al., 2018; Das et al., 2022a).

To monitor the effects of contaminants in such compartments, copepods have been widely used, given their remarkable representation both in the pelagic and/or benthic partitions of the near-bottom transition zone and their suitability as bioindicators (Buttino et al., 2018; Zidour et al. 2019; Das et al. 2020, 2022a, Kadiene et al., 2022). The calanoid copepod *Eurytemora affinis* has been established as a suitable model species and bioindicator for ecosystem health, mainly in the oligohaline part of macrotidal estuaries such as the Seine, playing a crucial role as a keystone species and providing the bulk of secondary production for higher trophic levels in the food web (Kadiene et al., 2017; Zidour et al., 2019; Das et al., 2020, 2022a). Furthermore, *E. affinis* occurs in such highly turbid domains benefitting from an abundance of nutrients but risking the exposure to sediment bound contaminants as well as dissolved toxic agents (Tackx et al., 2003; Cailleaud et al., 2009; 2010). In our previous studies we reported that *E. affinis* can be used as a sentinel to sediment resuspension due to its specific responses in life history traits to contaminants originating from sediment resuspension (both polluted sediment and unpolluted) and trace metal spiked seawater, along with its capacity to bioaccumulate trace metals from the above compartments (Das et al., 2020, 2022a). In addition, studies mentioned that responses of *E. affinis* can be perceived when exposed to acute, chronic and/or multigenerational exposure exemplifying its flexibility to several experimental approaches further highlighting its suitability as a bioindicator (Anderson and Phillips, 2016; Kadiene et al., 2017, 2022; Das et al. 2020, 2022a). Thus, considering the short- and long-term population level responses and the bioaccumulation of trace metals by *E. affinis* from sediment and seawater, reports on individual responses of *E. affinis* to the above matrices are warranted. In addition, individual responses of such keystone species are less reported in the literature despite their importance in providing tools to qualitatively and quantitatively predict the recruitment rate of future copepod populations. This is important for risk assessment but could also benefit the aquaculture industry given their substantial applications as fish feed (Souissi et al., 2008, 2016b; Neffati et al., 2013; Hussain et al., 2020).

Reproductive strategies in the life cycle of a copepod provide a combination of complex and diverse variations when exposed to different environments, making it difficult to comprehend the strategies of stressed individuals and their offspring (Neffati et al., 2013; Alajmi and Zeng, 2015; Hussain et al., 2020). Hence some reproductive and/or morphological biomarkers of trace metal stress in major benthic, pelagic and benthopelagic compartments of a macrotidal estuary can be highly useful to comprehend the mechanism of toxicity and the adaptations of the predominant biota present in such habitats. Few studies on individual responses of copepods exposed to trace metal spiked seawater and/or food, reported significant changes in copepod reproduction and morphological characteristics like loss of egg production, body length, fecundity etc., affecting the quality and the quantity of recruitment to the next generations (Cherif et al., 2015; Souissi et al., 2016b; Kadiene et al., 2019b; Kadiene et al., 2022). Hence, in this study we focused on some rarely addressed biomarkers of morphological and reproductive characteristics of *E. affinis*, further comprehending its strategies when exposed to trace metals in a multigenerational exposure. The inclusion of the multigenerational exposure in our study was to observe the adaptations of *E. affinis* from the early larval stages to the adult across generations that would allow to detect carryover effects and/or inheritance of traits developed in the preceding generation(s) exposed to trace metals. Whether trace metal exposure effects can cease or lead to irreversible vulnerability in reproductive and morphological traits, we studied an additional generation of recovery without any contaminant exposure.

In order to analyze the main compartments, present in a macrotidal estuary, we added an extract of the resuspended sediment collected from the Seine estuary in addition to whole sediment resuspension and trace metal spiked seawater. Hence, our study compared combined trace metals in seawater [cadmium (Cd), lead (Pb), nickel (Ni) and copper (Cu)], an extract (elutriate) of sediment-bound trace metals and a direct exposure of whole sediment samples in resuspension for four generations (G0, G1, G2, and G3). The purpose of focusing on these four trace metals (Cd, Pb, Ni and Cu) is because of the capacity of *E. affinis* to bioaccumulate and bioconcentrate sublethal to lethal concentrations of these metals (when exposed to *E. affinis* (Zidour et al., 2019) from the whole sediment resuspension of the Seine estuary, as reported in our recent study (Das et al., 2022a). In our multigenerational approach, we used the first generation (G0) as acclimated to trace metals and all the parameters being obtained thereafter from G1 until G3 (generation of recovery). Hence, the key objective of this study was to observe the reproductive and morphological strategies of *E. affinis* when exposed to three different compartments: the trace metal extract of sediment resuspension (extract), whole sediment resuspension, and a combination of spiked trace metals through multiple generations. Apart from the key objectives mentioned above, survival (%) correlating to the trace metal accumulation was analyzed in parallel to observe the overall effect of trace metal stress on *E. affinis.*

2. Material and methods

2.1 Model organism – the copepod *Eurytemora affinis* (Poppe, 1880)

A copepod *Eurytemora affinis* population was cultured in a 300 L tank for more than six months under controlled laboratory conditions (18 ^{0}C temperature; 12 h light: 12 h dark; 15 salinity) following Das et al., (2020). Our research group was mass culturing *E. affinis* for the last two decades, which was originally collected in the Seine estuary (low salinity zone). In 2014, our cultures were renewed, to avoid any genetic inbreeding problems that may arise during long term continuous culture. A microalga *Rhodomonas salina* (10 mL with a density of 4×10^6 cells/mL) obtained from the Roscoff culture collection, France (RCC 1537) was mass-cultured and used to feed *E. affinis* every two days, following previous studies (Souissi et al., 2016b; Das et al., 2020).

2.2 Experimental approach

In this study, we used three different exposures (seawater spiked trace metals, whole resuspended sediment and resuspended sediment extract) along with the control comprising of three replicates for each treatment and generation. A multigenerational protocol was designed by following Souissi et al. (2010, 2016b) with specific modifications by Das et al. (2020). After months of acclimation of *E. affinis* to laboratory conditions, 30 ovigerous females were carefully placed in each beaker (4 treatments with 3 replicates, in total 12 beakers of 2 L volume) equipped with a detachable mesh of 200 µm to facilitate the removal of ovigerous females once the clutch was released. The eggs then passed through this mesh due to a smaller diameter (on average <100 µm) and eventually provided nauplii that hatched after 3-4 days of incubation. After the first release of eggs the mesh was removed to elude the possibility of another clutch release, which would alter the population structure (Souissi et al., 2016b). The sequence of development from eggs to nauplii and then to copepodites took around 7 days. We performed the experiment at semi-static conditions, changing the test solutions twice every generation, once at the beginning and the other after the appearance of copepodites to prevent the loss of nauplii as they are sensitive to handle and indistinguishable by the naked eye (Souissi et al., 2016 a, b; Das et al., 2020). In the subsequent week the development of copepodites to adults was observed following the appearance of ovigerous females and signaling the end of one generation with a duration of 2.5 weeks on average (Das et al., 2020). The same procedure was followed for all four generations (G0, G1, G2 and G3) with G0 identified as the acclimation to the respective treatments and G3 as the generation without any contaminants. In our study, the 1st generation (G0) was marked as the generation of acclimation to allow the copepods to get used to the treatments (with trace metals from the dissolved phase and sediment) in a beaker of 2 L volume followed for a complete generation instead of a few hours/days (Souissi et al., 2016 a, b; Das et al., 2020). Thereafter, the 2nd generation (G1) and 3rd generation (G2) was followed with the exposures and control, while the 4th generation (G3) was continued without any exposure, for the recovery. Therefore, it is a study that followed multiple generations of *E. affinis* in exposed and decontaminated conditions. The entire experiment (lasting for approximately 3 months) was carried out in an acclimated chamber at a temperature of 18^0 C, a timer set to 12 h light: 12 h dark. The seawater before any exposure was filtered and autoclaved with the adjustment of salinity to 15.

2.2.1 Preparation of exposure conditions

Three treatments along with the control were prepared with filtered and autoclaved seawater in 2 L beakers at controlled temperature (18^0 C). The concentration of each trace metals used in the combined trace metal treatment (Cd, Pb, Cu and Ni) was with respect to their individual lethal concentrations (10% LC 50%; using metal salts of high grade) when exposed to *E. affinis* as mentioned in Zidour et al., 2019 and Das et al., 2020. Previous studies for such toxicological assays, generally used LC 50% or 1/3 of the LC50% for 96 hours exposure (Tlili et al., 2016; Zidour et al., 2019). Hence, for a long term (multigeneration) observation and to avoid high mortalities, we considered 10% of the LC 50% of each trace metal following Zidour et al. (2019) and Das et al. (2020) [Cd (10.88 μg/L), Pb (41.31 μg/L), Ni (12.55 μg/L) and Cu (3.35 μg/L)]. The concentration for the whole sediment resuspension treatment was also chosen with respect to the lethal concentration (LC 50%) of the Seine estuarine sediment as reported in Das et al. (2020) (5% LC 50 = 0.271g/L) to follow the morphological and reproductive changes for multiple generations, avoiding abrupt mortality rates, given the high bioaccumulation capacity of varied trace metals from the Seine sediments of *E. affinis* (Das et al., 2022a). The collection of sediment samples was done from the low saline, high turbid zone of the Seine estuary (49°26'33.762"N; 0°16'8.475"E), with all basic sediment characterizations reported by Das et al., 2022a. The whole sediment resuspension was kept under constant air bubbling with two glass rods inserted into the sediment at the bottom of the beaker in opposite directions following Das et al. (2022a). The sediment from the bottom got resuspended into the water and a continual circulation of the sediment particles was accomplished. The trace metal extract of the sediment resuspension was prepared according to Buttino et al., 2018 by using the same concentrations of the resuspended sediment (5% LC50) mixed at a ratio of 1:4 (v/v), comprising one part of resuspended sediment and 3 parts of seawater. This mixture was shaken with a magnetic vibrator for 1 hour at 100 rpm speed and later centrifuged for 20 mins at 3000 rpm. The elutriated sample (aqueous supernatant after centrifugation) was freshly prepared each time during the whole experimental period (USEPA-USACOE 1998; ASTM International 2000).

2.2.2 Sample collection

At the end of each generation the rest of the copepod population was homogenized by mixing with a glass rod and rapidly dividing the contents (1800 mL) while in circular motion into two equal parts (900 mL) to avoid any settling of the copepods (Zidour et al., 2019; Das et al., 2020). One half of the samples (900 mL) were stored in alcohol for analyzing the survival% and the morphological characteristics, while the other half (900 mL) was filtered on sterile membrane filters of 0.45 μm and dried to proceed with the trace metal analyses (Devreker et al., 2009; Souissi et al., 2010; 2016 a, b; Souissi et al., 2021). Thereafter each replicate from each treatment was counted and control samples showed on an average 400 to 450 individuals in 900 mL, sediment extract treatment had 200 to 380 individuals/900 mL, whole sediment treatments had 230 to 400 individuals/900mL and trace metal spiked seawater showed 170-350 individuals/900mL. Calculation of survival% followed Souissi et al., 2016a (given in Eq. 1), where S_g is the survival% of any generation, clutch $size_{g-1}$ is the average eggs per female (10 replicates) in the preceding generation (g-1) indicating the theoretical recruitment for the next generation (g). The average clutch size in the preceding generation multiplied with the no. of ovigerous females added in the beginning of each generation (which is 30) provided the total population in theoretical terms. The theoretical estimation of the total population divided by the total population density left in each treatment (in reality after exposure) provided the survival% of the copepods after each generation in the respective treatments.

$$S_g = 100 \times \frac{Clutch\ size_{g-1} \times 30\ ovigerous\ females}{Total\ population_{g-1}} \quad \textbf{Eq. (1)}$$

2.3 Analysis of trace metals

The trace metal concentration was examined by an inductive coupled plasma atomic emission mass spectrometer (ICP-MS) (Agilent 5110 SVDV ICP-OES, United States). To analyze the trace metal concentration in copepods and sediments from each treatment after each generation, copepod samples and sediments were filtered on sterile membrane filters of 0.45 μm and dried to proceed for pre-treatment. The filters with

copepods were digested with 3 mL of nitric acid (HNO_3), whereas the filters containing sediment were digested in Teflon tube with a mixture of nitric acid (HNO_3) and hydrochloric acid (HCl) in a ratio of 1:3 (2 mL of HNO_3: 6 mL HCl) (Ouddane et al., 1990). The final volume in each Teflon tube was adjusted to 5 mL with MilliQ ultrapure water. The suspended particulate matter was removed from the sediment samples by centrifugation (2000 rpm for 20 mins) and the filtrate was collected for trace metal analysis. Trace metal concentration in water was analyzed directly in the ICP-MS without the need of pre-treating the samples. The concentration of the set of trace metals (Cd, Cu, Ni, Pb) studied here was measured by using multiple elemental standards as a reference, along with the maintenance of quality control to obtain precise results following the subsequent works (Ouddane et al., 1990; Zidour et al., 2019; Das et al., 2020; Das et al., 2022a).

2.4 Measurement of morphological characteristics

2.4.1 Prosome length and volume

The prosome length of 10 ovigerous females from each replicate of each treatment was observed under an inverted microscope (OLYMPUS IX71, Tokyo, Japan), and thereafter measurements of the images were taken with the help of the software, Image J 1.41 (see Souissi et al., 2010; 2016a,b; Souissi et al., 2021; Das et al., 2022). The prosome quantifications (length and width) were performed by following Souissi et al. (2010, 2016b). The measurement from the frontal part of the cephalosome until the distal lateral end of the fifth segment of the body of *E. affinis* was attributed as the length of the prosome and the measurement in between the dorsal line until the line between the appendages was considered as the prosome width (Souissi et al., 2010; Choquet et al., 2018). The prosome volume was then calculated from the prosome length and width measurements assuming the shape of the body of *E. affinis* to be ellipsoidal, following Dayras et al. (2020).

2.4.2 Clutch size, clutch volume, and egg volume

After the prosome measurements, the egg numbers were counted by separating the egg sacs from the same (n = 10) ovigerous females separately. This provided the clutch size after each generation in each treatment (Devreker et al., 2009; Souissi et al., 2010; Souissi et al., 2016a, b). The diameter of each egg was measured and the mean egg diameter was then used to calculate the egg volume considering the spherical shape of each egg. The egg volume and the egg numbers yielded the total clutch volume of particular individuals at each generation and treatment.

2.4.3 Lipid residues

The ovigerous females used for the morphological characterizations described above were minutely investigated for the presence of lipids in the form of oil droplets or oil sacs. As explained before in previous studies, the presence of a lipid residue is denoted by droplets of oil or by oil sacs that are clearly visible and occupy the transparent body cavity of different copepod species (Miller et al. 1998; Lee et al., 2006; Souissi et al., 2016, Schmid et al., 2018). The presence of these lipid stores was noted in the same ovigerous females used for morphological measurements from each replicate (no. of females = 10) and their quantity was categorized as high (>3 lipid droplets), low (<3 lipid droplets) and absent (no lipid droplets) inside the body cavity of ovigerous females for each treatment after each generation (Souissi et al. 2016a).

2.5 Statistical analyses

Statistical differences in each treatment in survival%, prosome length, clutch size, prosome volume, and egg diameter were determined by performing one-way analysis of variance (ANOVA). For comparing the significance, Tukey multiple comparisons of means (95% family–wise confidence level) was executed. For testing the condition of ANOVA, Shapiro-Wilk normality test and Levene's Test for homogeneity of variance was used. Linear regression models were used to indicate the relationship between a set of morphological characteristics like prosome length, prosome volume, clutch size, lipid residues, trace metal accumulation and survival. All the above statistical analyses were carried out using R-Studio (version 4.0.4).

3 Results

3.1 Effect of exposures on copepod survival

The survival of the copepod population in each treatment (control, extract, sediment and trace metals) after each generation (G1, G2 and G3) is represented in Fig. 1. Survival of *E. affinis* in the control was higher in comparison to the contaminant exposed treatments in all generations [G1 (36.7%), G2 (40.14%), G3 (42.19%)]. The whole sediment exposed copepods of all treatments showed the lowest survival in G2 (28.24%) although being significantly ($p < 0.05$) lower only when compared to the control. In G3, resuspended sediment had a significant increase in the survival (37.35%, $p < 0.05$), when compared to G2. However, the dissolved exposures showed significant lowest survival in G1 [extract (27.55%) and trace metal mixture (26.42%), $p <0.05$] when compared to the control. No significant increase in survival was observed in extract and trace metal mixture for the generation of recovery (G3). Furthermore, trace metal mixture showed significant lowest survival in all the generations in comparison with the control [(G1 (26.42%), G2 (27.52%), G3 (30.94%), $p < 0.05$].

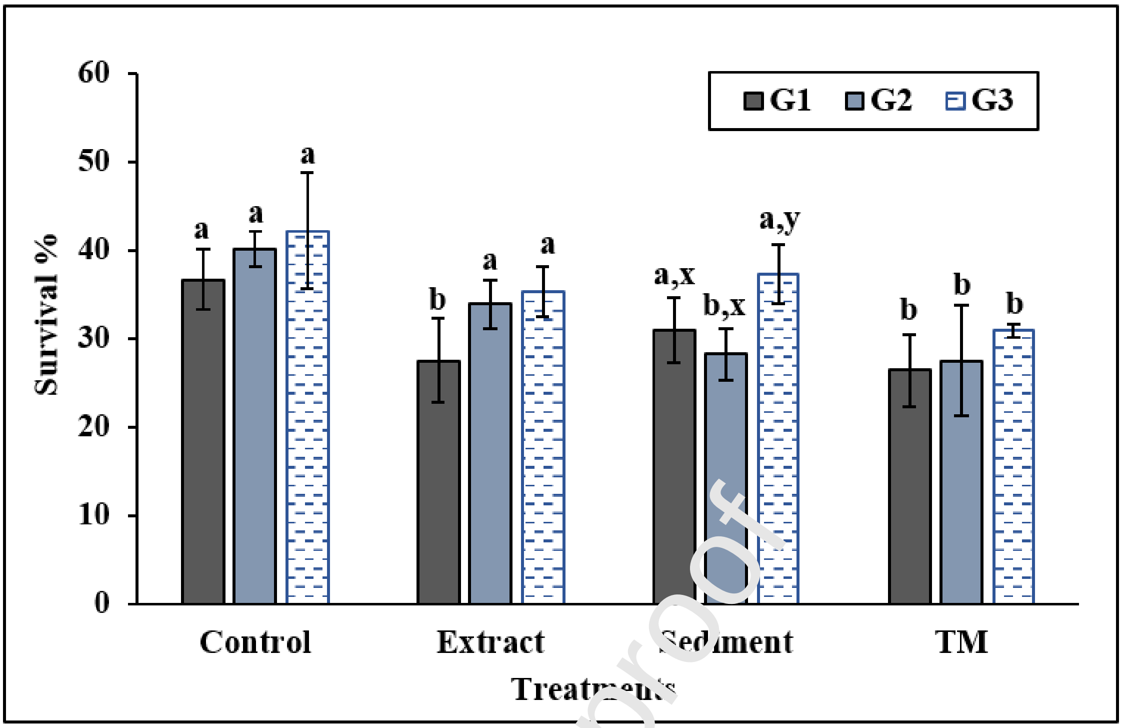

Fig. 1. Survival% of *E. affinis* in each treatment after each generation. Letters a, b denotes significant differences between treatments in one generation and letters x, y denotes significant differences between the three generations in one treatment (p <0.05). Error bars show the standard deviation between the three replicates in each treatment.

3.2 Effect of exposures on the body length and volume of *E. affinis*

The prosome lengths of copepods in each treatment (control, extract, sediment and trace metals) after each generation (G1, G2 and G3) is represented in Fig. 2A. The prosome length of *E. affinis* exposed to the resuspended sediment treatment [G1 (760.66 µm), G2 (741.71 µm) and G3 (767.021 µm)] was significantly lower than the control [G1 (849.73 µm), G2 (864.332 µm), G3 (875.12 µm)] across all generations. However, the two dissolved phase exposures (trace metals and extract) showed significantly lower values of prosome length only in G1 (TM- 781.12 µm; extract – 771.14 µm) when compared to the control. A significant increase of prosome length in G3 was noted in the trace metal mixture (821.28 µm) and extract treatment (855.43 µm), with the only exception visible for the resuspended sediment treatment (767.021 µm), where the exposure to clean water did not significantly increase the prosome length. Fig. 2B shows the prosome volume of the copepods in each treatment and generation, with significantly higher volumes in the control for G2 (0.06 mm^3) and G3 (0.063 mm^3) when compared to contaminant exposed treatments. The least prosome volume was observed in the resuspended sediment treatment for G1 (0.036 mm^3) and G2 (0.032 mm^3), with G2 being significantly lower than the other two exposures, which are the combined trace metal mixture (0.043 mm^3) and the extract treatment (0.046 mm^3). However, in the generation of decontamination (G3), the resuspended sediment exposed copepods showed similar values (0.033 mm^3) of prosome volume like the combined trace metals (0.0432 mm^3) and extract treatment (0.0434 mm^3) showing no significant differences. Notably, the resuspended sediment treatment showed highest sensitivity to both body size related parameters, prosome length and/or volume.

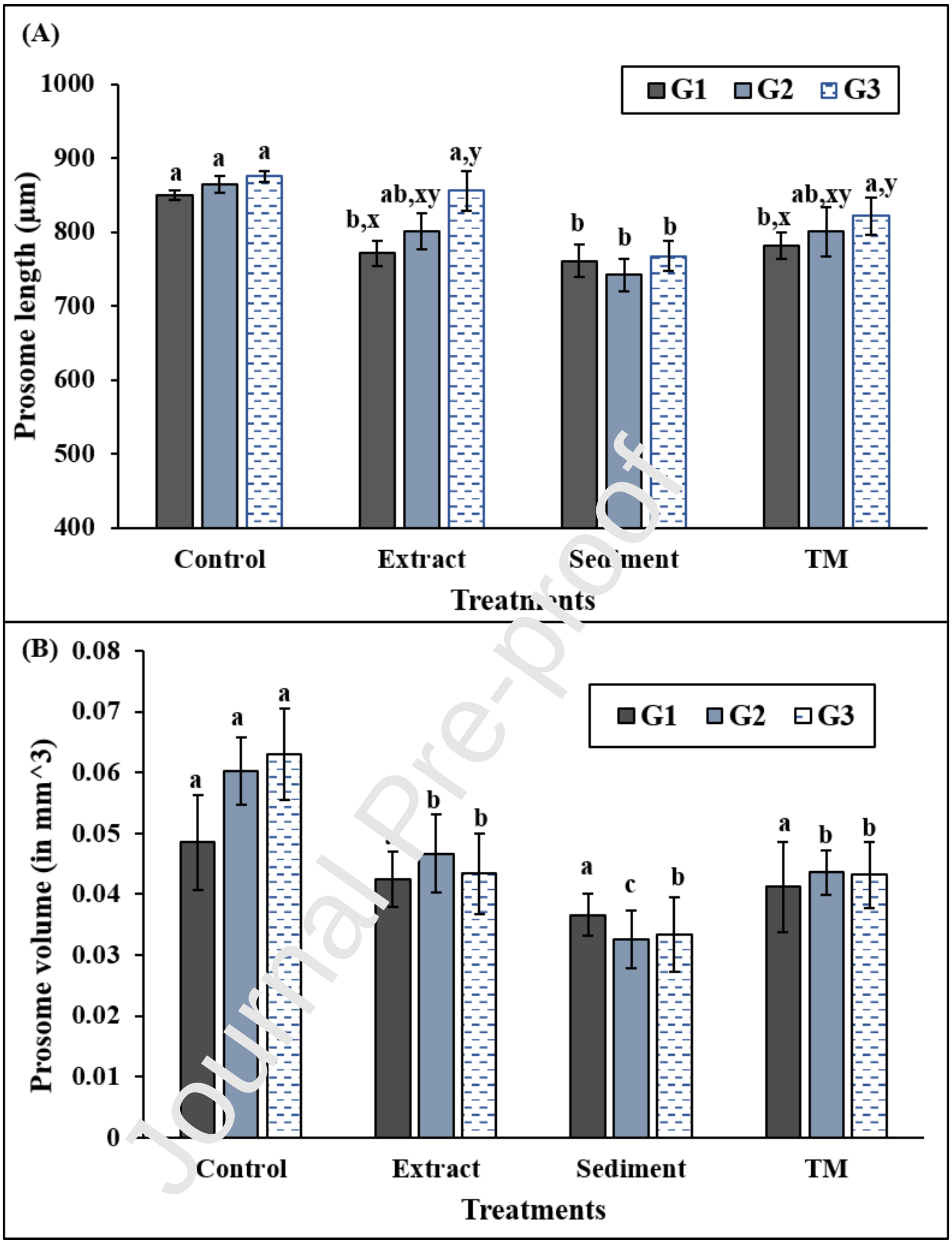

Fig. 2a. Prosome length of *E. affinis* and **Fig. 2b.** The volume of the prosome of *E. affinis* from each treatment after each generation. Letters a, b, c differentiates the significant differences between treatments in one generation and letters x, y differentiates significant differences between the three generations in one treatment ($p < 0.05$). Error bar shows the standard deviation between the three replicates in each treatment.

3.3 Exposure effects on the clutch size of *E. affinis*

The clutch sizes of the copepods when exposed to individual treatments after each generation (G1, G2 and G3) are represented in Fig 3A. The trace metal treatment showed significantly lower clutch sizes (eggs/female) [G1 (20.43), G2 (22.23), G3 (24.61)] in all generations when compared to the control and in G1 when compared to sediment resuspension (28.28) and extract (26.4). The clutch size of the copepods was significantly higher for the resuspended sediment exposure in G3 (32.96) when compared to other contaminant exposed treatments. However, unlike the prosome length, clutch size was more affected by the dissolved trace metal treatments. Fig. 3B shows the egg diameter of the copepods in each treatment and generation showing significantly highest diameter of eggs in the control for G2 (97.21 µm) and G3 (97.47 µm) compared to the other treatments. The statistically least value of egg diameter in G1 was reported in the extract treatment (82.12 µm). However, in G2 both the dissolved exposure [extract (77.53 µm) and trace metal mixture (75.12 µm)] showed significantly least diameter of the eggs when compared to resuspended sediment (84.28 µm) and control. However, the clutch size and egg diameter showed more adverse effects when exposed to a combined trace metal and extract treatment.

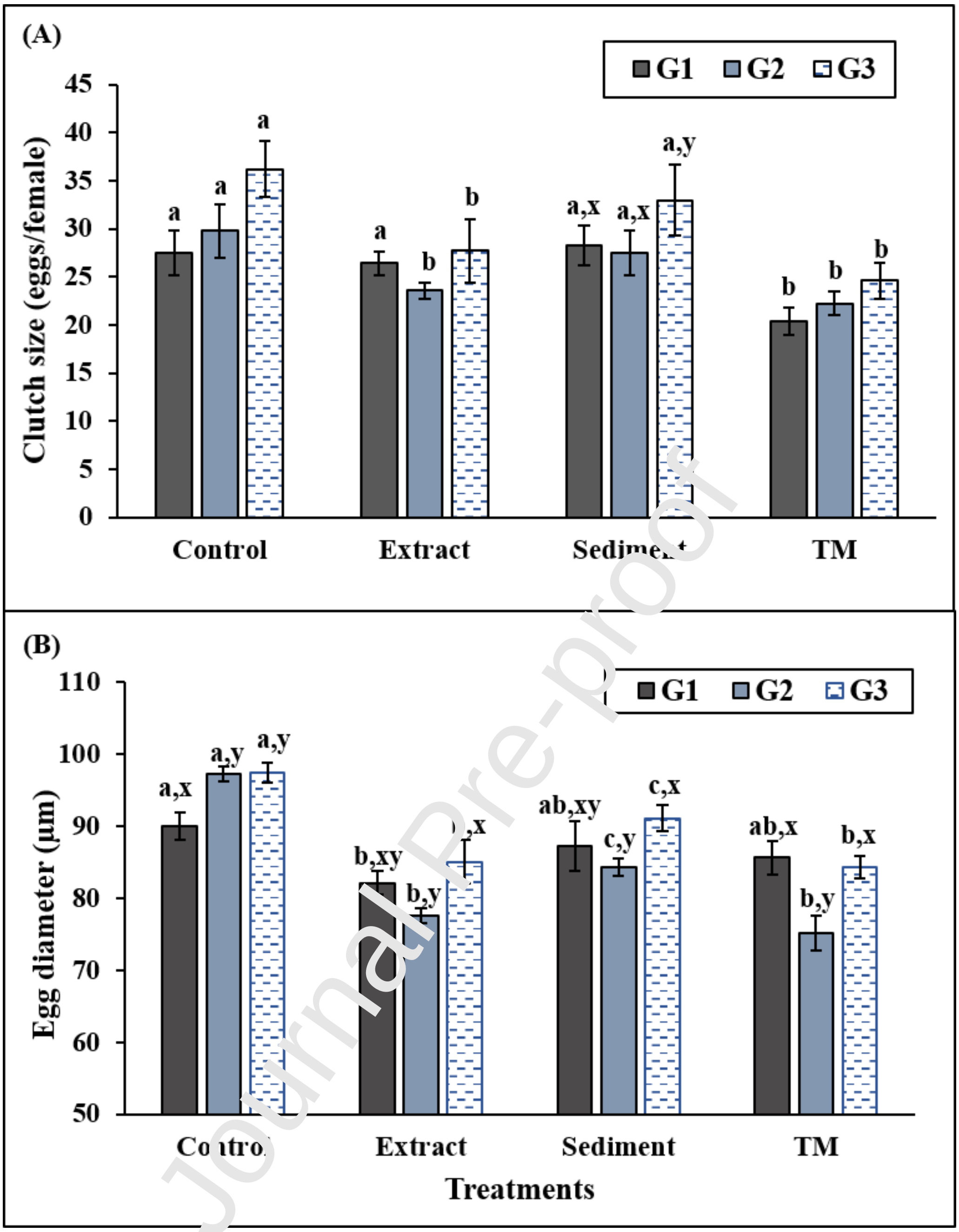

Fig. 3A. Clutch size of copepod *E. affinis*. **Fig. 3B.** Egg diameter of the copepods from each treatment after each generation. Letters a, b, c denotes significant differences between treatments in one generation and letters x, y denotes significant differences between the three generations in one treatment (p <0.05). Error bars indicate the standard deviation between three replicates in each treatment.

3.4 Relationship between morphological and reproductive traits of *E. affinis*

The relationship between different morphological and reproductive variables are shown in Fig. 4. Fig. 4A shows the relationship between the prosome length and the clutch size, where control ovigerous females had the highest no. of eggs and the largest prosome length. A comparison between resuspended sediment and trace metal mixture showed contrasting results, with the resuspended sediment exposed ovigerous females showing higher clutch sizes, but lower prosome length and the trace metal mixture exposed individuals showed lower clutch sizes, but higher prosome lengths. The sediment extract treatment, however showed variable results but had resemblance with the trace metal mixture treatment showing higher prosome length and lower no. of eggs, marking the specificity of each exposure; dissolved trace metals differing from the pattern noted for resuspended sediment. Fig. 4B shows the relationship between the prosome volume and the clutch volume that corresponded to the prosome length and clutch size showing the highest values for control and treatment-specific variability in morphology and reproductive characteristics for the respective exposures.

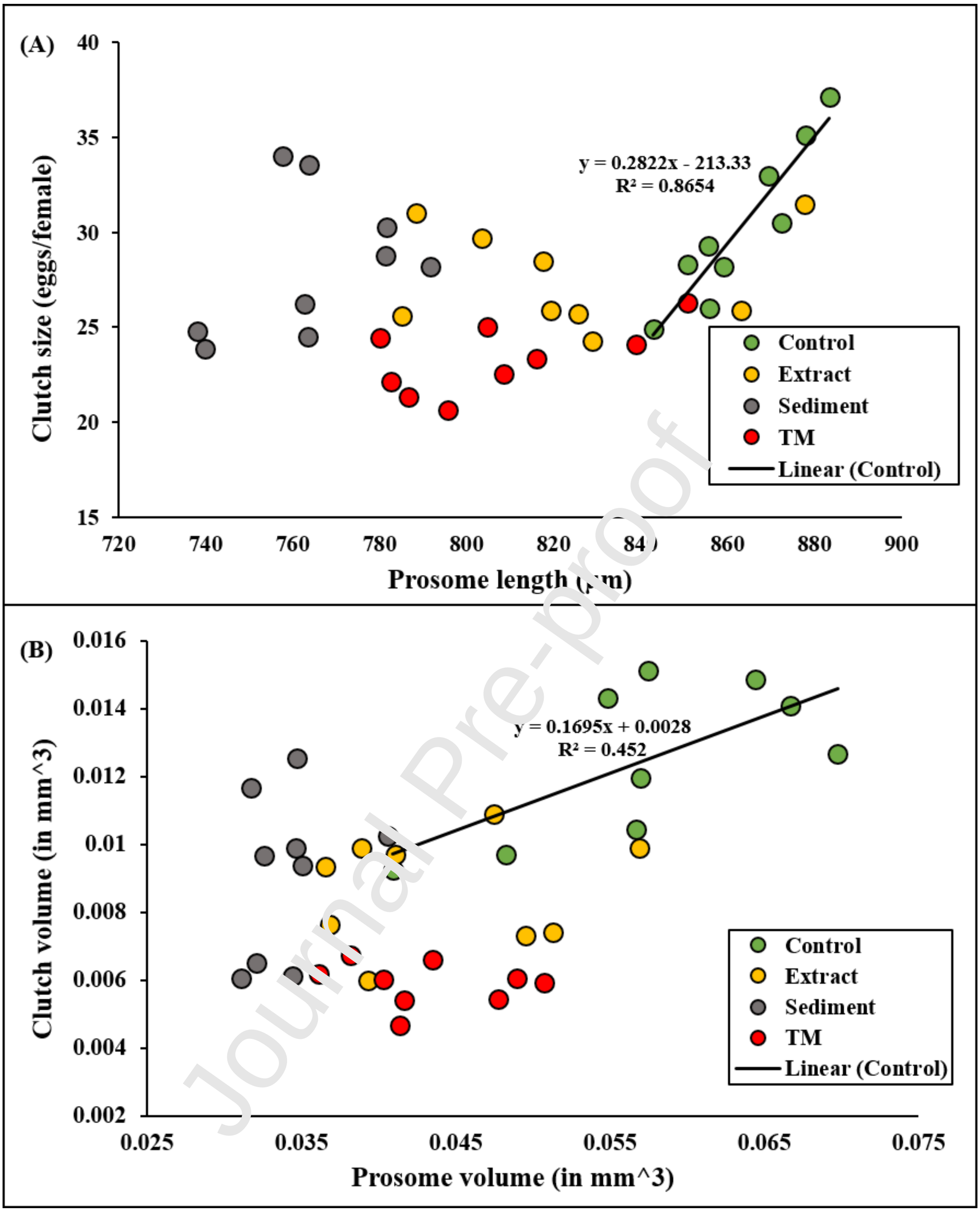

Fig. 4A. Relationship between prosome length and clutch size of *E. affinis* in all treatments with the only significant trend ($p < 0.01$) shown for the control after combining all generations (G1, G2 and G3). **Fig. 4B.** The relationship between prosome volume and clutch volume of *E. affinis* in all the treatments with the only significant trend shown ($p < 0.05$) for control after combining all generations (G1, G2 and G3).

3.5 Relationship between lipid droplets and trace metal accumulation

The relationship between lipid droplets and Log 10 of the summation of trace metal bioaccumulation (Cd, Pb, Ni and Cu) is shown in Fig. 5. The highest % of lipid droplets were noted in the trace metal mixture followed by resuspended sediment > extract treatment. The least amount of high % of lipid droplets was noted for the control ovigerous females. The higher amount of both the lipids and trace metal accumulation in each of the exposed treatments was observed for the first two generations of exposure G1 and G2, with a reduction in G3 (generation of decontamination). The lipid droplets and the trace metal accumulation, individually, were positively correlated for only the contaminant exposed treatments ($p < 0.01$) showing no significant relationship in the control. In the generation of decontamination (G3) the lipid droplets and the trace metal concentrations decreased for each treatment indicating its usage in metabolism, exemplifying higher survival and clutch size in G3 (Supplementary information Fig. 1S and 2S). The concentration of trace metals studied in here (Cd, Pb, Ni and Cu) were analyzed in each compartment as semi-static exposure and are listed in Table 1S (Supplementary information).

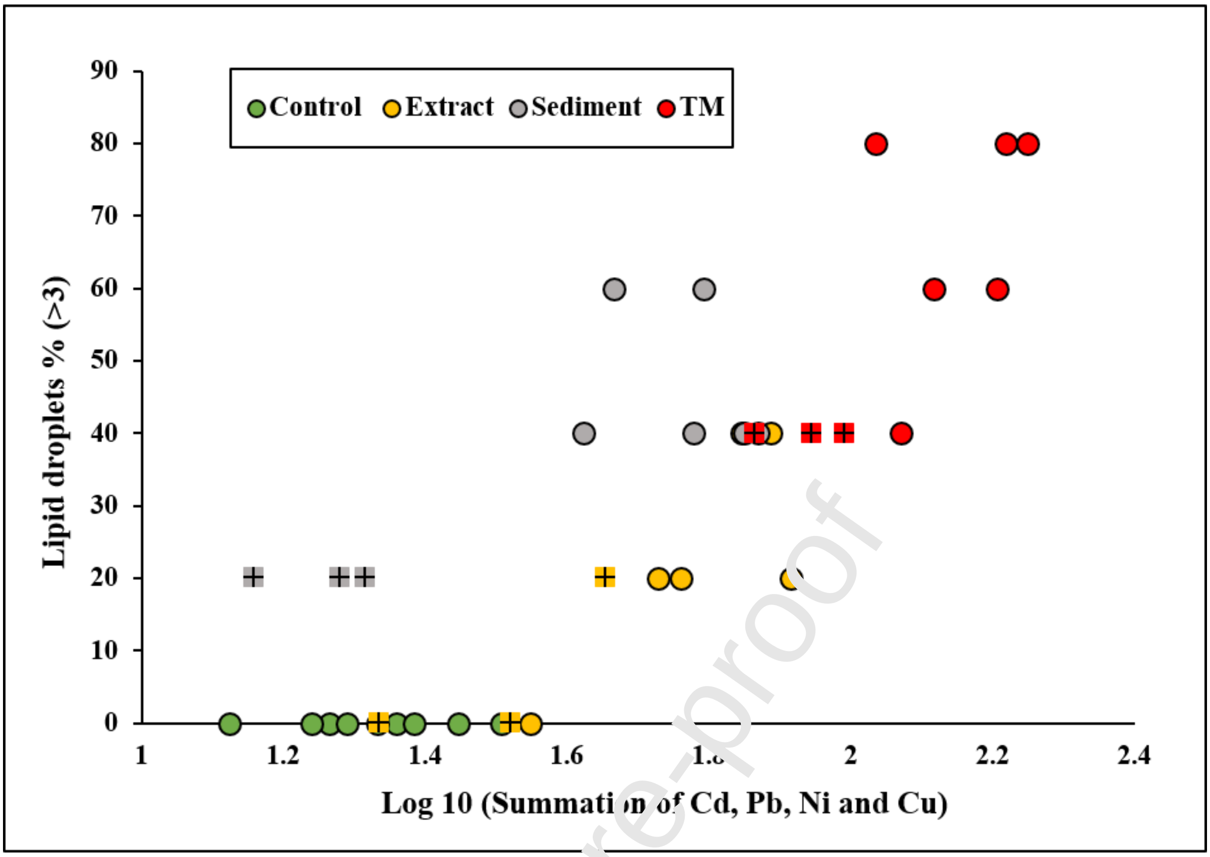

Fig. 5. The % of ovigerous females carrying >3 lipid droplets (out of 10 replicates) along with the Log 10 value of the summation of the spiked trace metals (Cd + Pb + Cu + Ni) bioaccumulated by the copepod *E. affinis* in each treatment. The box icon with plus (+) denotes the generation of recovery G3 (no added contaminants).

4. Discussion

4.1 Survival of *E. affinis* when exposed to trace metals

Studies on the multigenerational exposure of various contaminants to copepods or other aquatic invertebrates usually reported adverse effects on the population structure mostly affecting the parental generation (Kwok et al., 2009; Dietrich et al., 2010). Such studies further reported that pre-exposed offspring in subsequent or successive generations are often capable of developing resilience to environmental pollutants or contaminants by genetic or physiological adaptations

(Kozlowsky-Suzuki et al., 2009; Kwok et al., 2009; Dietrich et al., 2010, Wang et al., 2018). Our results of copepod survivorship document a similar observation made in previous studies, where the survival% of the population of *E. affinis* in G1 was lowest and was mostly affected by trace metal concentrations from the dissolved phase (trace metal mixture and sediment extract) showing significantly lower survival than in the control, but later survival% recovers in G2 (Kim et al., 2012; Das et al., 2020; Kadiene et al., 2022).

After the initial exposure to environmentally relevant concentrations of contaminants many aquatic invertebrates show certain physiological responses and/or activate detoxifying enzymatic activities for resisting the metabolic challenges of, e.g. trace metals, hydrophobic compounds and micro-plastics (Bao et al., 2018; Das et al., 2018; Araujo et al., 2019a, b; Zhang et al., 2019; Das et al., 2020; Kadiene et al., 2022). In this study a similar pattern was noted where, G1 showed the lowest survival in the dissolved treatments (trace metal mixture and sediment elutriate) but G2 produced more resilient offspring signalling a progressive adaptation incurred while being exposed to contaminants during G0 and G1. On contrary, *E. affinis* exposed to resuspended sediment treatments had lowest survival in G2. This phenomenon is presumably due to the filter feeding behaviour of *E. affinis*, promoting higher chance of trace metal exposure from dissolved phases during G0 and G1, and limited encounter rates between pollutants resuspended from the sediment and the pelagic copepod *E. affinis*. According to Cailleaud et al., 2009, tidal scale and vertical positioning of copepods in the aquatic column (surface or near bottom) significantly affected the bioaccumulation of organic contaminants, markers of enzymatic alterations and the population structure of *E. affinis*. In addition, the resuspension of the natural sediment from the Seine estuary is often a supply of nutrients along with a combination of contaminants (trace metal, organic pollutants, hydrophobic compounds, etc) (Cailleaud et al., 2009, 2010). Hence, the resuspension of the sediment into the water column released an array of diverse substances causing an eventual effect on the survivorship of *E. affinis* in the subsequent generation (G2) marking the specific effect of an indirect exposure of contaminants through resuspension. Some previous studies discussed the effect of contaminants on the subsequent generations and also notified the importance of faster depuration routes being activated when exposed to certain specific toxicants, especially originating from sediments and/ or lethal diets (Landrum and Scavia 1983; Paul-Pont et al., 2016, Das et al., 2020). Some studies showed that hatching rate and hatching success was impaired in copepods exposed to resuspended sediment with increasing no. of generations under continuous exposure leading to lower population density and survival% in subsequent generations (Stringer et al., 2014; Buttino et al., 2018; Hussain et al., 2020). In the generation of decontamination (G3), the survival% increased in all the treatments but a significant increase was only observed for resuspended sediment treatments. The finding of higher recovery in a resuspended sediment exposure has been observed in a few studies where particle bound exposures (e.g., microplastics and/or sediments) showed an ability to activate higher depuration routes when compared to dissolved exposures (Lotufo, 1998; Paul-Pont et al., 2016; Das et al., 2020). In our study, the generation G3 (no contaminants) showed considerably lower concentration of trace metals in *E. affinis* exposed to resuspended sediment than dissolved exposures. The presence of combined organic fractions along with trace metals in the sediment column can be considered as responsible for such a phenomenon, for e.g., Iron (Fe) oxides (being high in the Seine estuary, Das et al., 2022a) often forming complexes with a range of trace metals like Cd, Pb, Ni, Zn, Cu. Some studies reported that such binding behavior in combined exposures alters the bioconcentration factors and bioavailability of contaminants in model organisms (Yu et al., 2001; Gao et al., 2019; Guan et al., 2020). In addition, our study reports that sediment resuspension exposure did not reflect any maternal carryover effects in G3 on the survival of *E. affinis* at the population level. However, such indications are species specific and largely depend on the prevailing contaminants present in the macrotidal estuaries (Lotufo, 1998; Paul-Pont et al., 2016; Rügner et al., 2019).

4.2 Morphological and reproductive traits

Morphological and reproductive traits of copepods in several stressed conditions have proven to provide sensitive parameters for predicting the survival% and the overall health of an organism (Svetlichny et al., 2012; Nefati et al., 2013; Wang et al., 2018; Yi et al., 2019; Hussain et al., 2020; Lenz et al., 2021). Studies have reported a reduction in the time for development including a decrease of hatching rate and hatching success over generations when exposed to a range of contaminants (Gutierrez et al., 2010; Hussain et al., 2020). Our study refers to a wide range of morphological and reproductive traits like the prosome length, prosome volume, clutch size, egg diameter and lipid droplets present inside the body cavity of *E. affinis*. The prosome length at G1 was low in all the contaminant exposed treatments, but eventually at G2, the dissolved toxic exposures (trace metal mixture and sediment extract) showed adaptability by possibly inducing plasticity in their phenotype to withstand the effects of trace metals (Gutierrez et al., 2010; Svetlichny et al., 2012; Souissi et al., 2016a; Hussain et al., 2020). To the contrary, copepods exposed to resuspended sediments still showed significantly lower prosome length in G2 and G3 when compared to the control. Notably this study reports that although the transfer of *E. affinis* to G3 could significantly increase the survival% in resuspended sediment exposure at the population level, the morphological parameters studied at an individual level did not show any such trend. Previous studies discussed the relationship between absorption of a wide variety of substances with copepod size, and reported that higher nutrient content in the copepod guts corresponded to larger size, whereas small sized copepods had a lower nutrient content (Chen et al., 2018; Sew et al., 2018; Dinh et al., 2021; Svendheim et al., 2021). Our results reflect similar observations where ingestion of the low nutrient contaminated sediment by *E. affinis* (assuming the resuspension of sediment particles as food particles) caused potential effects on the size of the copepods. A classical model proposed by Lam and Frost (1976), which was later used by Koski et al. (2017) assumed that the exposure to dispersed or suspended food particles increased filtration and feeding rate which further affects copepod body length (Lam and Frost 1976; Koski et al., 2017; Dinh et al., 2021). In our study, the resuspended sediment particles may have caused such an effect on the prosome length of *E. affinis*. This was similarly described in a recent study where resuspension of fine particles had the most adverse effect on the body length of *Calanus finmarchicus* when compared to other regimes (Svendheim et al., 2021). In addition to calanoids, previous studies reported the effect of sediment spiked fungicides on the body length of harpacticoids to be one of the most prominent factors (Turesson et al., 2007; Sancho et al., 2016). In a recent study, the exposure of tetrabromodiphenyl ether

(BDE 47) to the marine rotifer *Brachionus plicatilis* contracted the gastric cells in the stomach wall and led to their shrinkage, suggesting for decreased digestion and nutritional state (Yang et al., 2021). Such organic pollutants are highly prevalent in the sediment of the Seine Estuary and the lower body length from the resuspended sediment exposure can be attributed to the huge line-up of contaminants present in our study area (Cailleaud et al., 2009, 2010).

However, in the present study the clutch size and egg diameter of *E. affinis* was mostly affected by the dissolved exposure (trace metal mixtures and extract from the sediment) together showing a lower no. of egg production and also reflecting a significantly smaller egg diameter in G1 and G2 compared to the control and resuspended sediment exposure. Previous works focused on the dissolved exposure of toxicants and inferred a lower fecundity caused by trace metals like Cu in *Pseudodiaptomus* sp. (Dinh et al., 2021), Cd in *Pseudodiaptomus annandalei* (Kadiene et al., 2022) and three species of *Brachionus* sp. (Kang et al., 2019). However, when comparing trace metal mixtures and extract treatments, our study showed that trace metal mixture had the most adverse effects on clutch size (eggs/female) but with a larger egg diameter in G1. To the contrary, extract treatment showed that females with larger clutch sizes had smaller egg diameters, compared to those experiencing just trace metal spiked exposure. Such tendencies of compensation or tradeoffs to combat the stressed conditions have been discussed as a reproductive trait to increase adaptability and fitness of an organism. This happened either by quantitative assurance of increasing the no. of possible progeny or by qualitative enhancement (larger egg diameter) ensuring fitness and adaptability (Aránguiz-Acuña and Pérez- Portilla, 2017; Aránguiz-Acuña et al., 2018; Djebbi et al., 2021; Souissi et al., 2021; Kadiene et al., 2022).

The above phenomena can be interpreted with the classical theory of r/k selection, in a quality *vs*. quantity approach using r-strategists showing investment to the no. of progenies and k strategists showing investment to their quality (Cassill, 2019, 2021; Souissi et al., 2021). To our knowledge, this is the first study showing specific adaptations to exposure of toxicants from different compartments (i.e. resuspended, dissolved, sediment extract). Furthermore, the differential gene expressions of *E. affinis* when exposed to the main estuarine compartments can explain the specific markers reported in this study and aid to comprehend the mechanisms of toxicity prevalent in such ecosystems, through ecotoxicogenomics. Previous studies reporting standardization of morphological measurements and/or quantitative analysis of shape and size in model zooplankters are rare in evaluating population size in several habitats and also to monitor water quality parameters (Souissi and Souissi, 2021; Kadiene et al., 2022). Our study uses certain biometrics that can be applied to individuals exposed to anthropogenic stress from different compartments. The combination of morphological and reproductive characteristics that can easily be procured from model copepods can be used as a fitness index or quality index to analyze the health of an ecosystem. A recent study used a robust allometric correlation between clutch size and prosome length to introduce a quality index using *E. affinis* that can give valuable details on overall ecosystem health (Souissi and Souissi, 2021). Our study supports the findings and used such morphological and reproductive characteristics to show the incongruity between several stressed conditions and control individuals when exposed for multiple generations. In addition, our study reports a higher number of lipid droplets in contaminant exposed treatments than in the control, exhibiting a significant positive correlation with trace metal accumulation from the different compartments of exposure. Such observations are similar to previous reports where, trace metals among others, Cr, Cd, Ag, Li, Zn, Pb mostly accumulate in lipid droplets, showing a higher affinity in between the two parameters that indicate stressed conditions (Lobus et al., 2018; Wang et al., 2019; Canavate et al., 2020; Bezzera et al., 2022). Previous studies showed the tendency in copepods to accumulate lipid droplets inside their body cavity, and their inability to utilize them for metabolic responses under stressful physiological and/or environmental conditions further depending on the time of exposure due to the complex pathways of mobilization and degradation of lipid droplets (Kim et al., 2016; Lobus et al., 2018; Oh et al., 2019, Wang et al., 2019; Bezzera et al., 2022). Similarly, our study reports a higher accumulation of lipid droplets in treatments, presumably due to the stress encountered at constant exposure across 3 generations (G0, G1 and G2). We further report that the generation of decontamination (G3) decreased the amount of lipid droplets when transferred to unexposed filtered seawater. This phenomenon presumably indicates the utilization of the lipid storages by ovigerous females of *E. affinis* to increase the fitness of the individuals. The recruited progenies that are well implied in our results showed an increase in survival% and larger clutch sizes for G3, in all exposures. Collectively, this study reports certain morphological and reproductive markers of a sentinel copepod *E. affinis* that are possibly transmitted to successive generations signaling the ecosystem health and impacting the plasticity induced in the progenies, when exposed to the main compartments of an estuary. Such markers can help to assess potential threats caused from varied pathways of toxic exposures in zooplanktons. This study further introduces biometric relationships for stressed conditions showing plasticity in the phenotype of bioindicator organisms inquiring an added constraint on the overall population structure including physiological and reproductive efforts.

5. Conclusion

Our study reports morphological and reproductive characteristics of *E. affinis* when exposed to resuspended sediment from the Seine estuary, an extract of the same sediment and a dissolved trace metal mixture (TM). The effects caused by dissolved exposure (TM and extract) and resuspended sediment were contrasting and showed some specificity in each treatment. Survival % was mostly affected in the first generation G1 for TM and extract, whereas resuspended sediment exposure showed significantly lower survival% in G2 (second generation of exposure). The effect of each exposure on an individual level also showed specificity between the exposures from different compartments. The prosome length and volume showed high sensitivity to resuspended sediment, whereas clutch size, clutch volume and egg diameter showed higher sensitivity to dissolved trace metal exposure (TM and extract). The lipid droplets inside the body cavity of *E. affinis* showed higher accumulation at all exposure conditions and a significant positive correlation with the amount of trace metals bioaccumulated by *E. affinis*. In all generations (from the first generation, G0 to generation of decontamination, G3), a proneness to combat the toxicity from varied sources through tradeoffs was observed. This is the first documentation of morphological and reproductive traits of *E. affinis* when exposed to different compartments of the environment. Our study establishes new morphological and reproductive markers of toxicity from varied sources on an individual level in the pelagic bioindicator copepod *E. affinis*. However, gene expressions and/or molecular responses of *E. affinis* in each generation, exposed/adapted to different compartments of a polluted area should be targeted in future toxicological investigations to get a deeper insight in the mechanisms of toxic action, pathways and responses.

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Credit author statement

Shagnika Das: Performed the experiment, analysis and drafted the manuscript.

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Sami Souissi: Conceived the project and supervised the MS drafting.

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Declaration of competing interest

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

Graphical abstract