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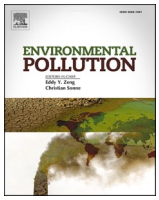

Evaluation of precopulatory pairing behaviour and male fertility in a marine amphipod exposed to plastic additives☆

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ABSTRACT

Plastics contain a mixture of chemical additives that can leach into the environment and potentially cause harmful effects on reproduction and the endocrine system. Two of these chemicals, N-butyl benzenesulfonamide (NBBS) and triphenyl phosphate (TPHP), are among the top 30 organic chemicals detected in surface and groundwater and are currently placed on international watchlist for evaluation. Although bans have been placed on legacy pollutants such as diethylhexyl phthalate (DEHP) and dibutyl phthalate (DBP), their persistence remains a concern. This study aimed to examine the impact of plastic additives, including NBBS, TPHP, DBP, and DEHP, on the reproductive behaviour and male fertility of the marine amphipod *Echinogammarus marinus*. Twenty precopulatory pairs of *E. marinus* were exposed to varying concentrations of the four test chemicals to assess their pairing behaviour. A high-throughput methodology was developed and optimised to record the contact time and re-pair time within 15 min and additional point observations for 96 h. The study found that low levels of NBBS, TPHP, and DEHP prolonged the contact and re-pairing time of amphipods and the proportion of pairs reduced drastically with re-pairing success ranging from 75% to 100% in the control group and 0%–85% in the exposed groups at 96 h. Sperm count declined by 40% and 60% in the 50 μg/l and 500 μg/l DBP groups, respectively, whereas TPHP resulted in significantly lower sperms in 50 μg/l exposed group. Animals exposed to NBBS and DEHP showed high interindividual variability in all exposed groups. Overall, this study provides evidence that plastic additives can disrupt the reproductive mechanisms and sperm counts of amphipods at environmentally relevant concentrations. Our research also demonstrated the usefulness of the precopulatory pairing mechanism as a sensitive endpoint in ecotoxicity assessments to proactively mitigate population-level effects in the aquatic environment.

1. Introduction

The use of plastic additives in manufacturing plastics and associated products, including packaging materials, medical devices, and personal care products, is likely to continue to show an upward trend because of their numerous benefits (Pfaendner, 2006; Hermabessiere et al., 2017). This is because these additives are commonly used to improve product the plasticity, durability, and overall performance of the final product while also reducing production costs and even to reduce production costs (Hansen et al., 2013; Mafuta et al., 2021; Costa et al., 2023). Consequently, the ubiquitous use of plastics in daily life has resulted in the leaching and accumulation of plastic waste in the environment, where it persists and causes severe ecological harm (Thompson et al., 2009; Rochman et al., 2013; Jambeck et al., 2015; Rochman et al., 2013; Jang et al., 2023).

The European Union has identified more than 20% of plastic compounds used globally as substances of concern because of their persistence, bioaccumulation, or toxicity (Wiesinger et al., 2021). Some of

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these plastic addtives of concern include phthalates, stabilisers, flame retardants, and fillers (Hansen et al., 2013; Hahladakis et al., 2018). Numerous studies have investigated their effects using various endpoints to determine the response of organisms of interest. For instance, reproductive endpoints have been used to evaluate the potential adverse effects of plastic additives on the male-female behaviour of amphipods, oocyte development, and sperm parameters (Wisniewska and Szaniawska, 2015; Yu et al., 2018; Matuszczak et al., 2019). In addition, behavioural endpoints, such as locomotor activity, aggression, and anxiety, have been used to assess the impact of plastic additives on the nervous system of model organisms (Gore et al., 2015; Tao et al., 2022). Meanwhile, molecular endpoints, including gene expression and epigenetic modifications, have been used to examine the mechanisms underlying the toxicity of plastic additives (Alam et al., 2010; Sree et al., 2023). These studies provide valuable insights into the toxicity of plastic additives and the mechanisms involved in their mode of action to understand and predict their impact on human exposure and the environment.

Echinogammarus marinus is a common marine amphipod found along the coastlines of northwestern Europe, from Norway to southern Portugal. It is an intertidal estuarine species and a crucial food source for wading birds (Martins et al., 2002). This species has been used in several ecotoxicological studies (Bossus et al., 2014; Vannuci-Silva et al., 2019). In Crustacea, precopulatory pairing is a common mate-guarding strategy, with copulation typically occurring shortly after the female moults. Previous studies have used precopulatory pairing as a quantitative measure by recording the time taken for the disruption and reformation of pairs (Malbouisson et al., 1995; Blockwell et al., 1998). Since the early 2000s, this mechanism has been used to evaluate the effects of various compounds including sex hormones, pesticides, heavy metals, hydrocarbons, and waste effluents (Heckmann et al., 2005; Pandey et al., 2011; Pedersen et al., 2013; Love et al., 2020). Environmental pollutants can affect the precopulatory behaviour of amphipods at environmentally relevant concentrations, resulting in an immediate disruption of pairs and a prolonged re-pairing time (Cold and Forbes, 2004). However, time constraints in experimental setups and the availability of animals with a sufficient sample size have limited the use of precopulatory pairing behaviour as an endpoint.

Sperm count, viability, and motility are the most common measures used to evaluate sperm quality in amphipods (Harlıoğlu et al., 2018). It is essential to monitor sperm counts in amphipods because low counts have been correlated with decreased egg fertilisation and smaller broods (Lewis and Ford, 2012). Both animal and in vitro studies have confirmed adverse effects on male fertility. For example, exposure to dibutyl phthalate (DBP) and diethylhexyl phthalate (DEHP) adversely affects spermatogenesis and male reproduction in fish species such as *Pseudetroplus maculatus* and *Oryzias melastigma* (Ye et al., 2014; Sruthi et al., 2021). A decrease in the number of spermatozoa in fish has also been reported in response to DBP and DEHP exposure (Uren-Webster et al., 2010; Sruthi et al., 2021). Invertebrate studies on molluscs and echinoderms have shown that toxicants can impair sperm motility. For example, Pacific oysters (*Crassostrea gigas*) exposed to 1 μg/l nonylphenol showed a 70% decrease in sperm motility after 72 h (Nice, 2005). Other studies on fresh and marine water amphipods have documented negative impacts on sperm quality and quantity in response to environmental stressors, such as insecticides (Trapp et al., 2015), radiation (Fuller et al., 2019), and wastewater effluents (Yang et al., 2008; Botelho et al., 2021). Modelling studies have indicated that sperm counts below certain levels can adversely affect amphipod populations (Ford et al., 2012).

In this study, we selected four plastic additives of concern: (a) n-butyl benzenesulfonamide (NBBS), (b) triphenyl phosphate (TPHP), which are sulfonamide- and phosphate-based plasticisers, respectively, and among the ten selected for in-depth evaluation by the National Toxicology Program (NTP) (Oltmanns et al., 2019), (c) di-ethylhexyl phthalate (DEHP), and (d) dibutyl phthalate (DBP), which belongs to the phthalate group, although banned, they are still of concern and are listed as priority chemicals in the Oslo and Paris Convention (OSPAR) to protect the marine environment (Peijnenburg, 2008; USEPA, 2019). Furthermore, three of these compounds (DEHP, TPHP, and NBBS) have been identified as among England's top 30 organic chemicals detected in surface water and groundwater (Spurgeon et al., 2022). This study aimed to investigate the effects of these plastic additives on the precopulatory pairing behaviour and sperm count of *E. marinus*. We hypothesised that exposure to these plastic additives would impair the pairing behaviour of male and female *E. marinus* initially in precopula and that exposed males would have lower sperm count.

2. Materials and method

2.1. Test chemicals and reagents

The tested chemicals NBBS (CAS No. 3622-84-2 purity ≥98.5%), TPHP (CAS No. 115-86-6 purity ≥99.0%), DEHP (CAS No. 117-81-7, purity ≥99.5%), DBP (CAS No. 84-74-2, purity ≥99%), and dimethyl sulfoxide (DMSO) (CAS No. 67-68-5, purity ≥99.9%) were purchased from Sigma-Aldrich (Merck Life Science U.K.) Limited. Pharmaceutical grade sea salt was purchased from Tropic Marin® Classic sea salt (Wartenberg, Germany). Stock solutions were prepared using DMSO, and working solutions were prepared in artificial seawater (ASW). DMSO was maintained at 0.01% for each concentration. We reported the nominal values of the test compounds owing to the limitations of the analytical methods for these volumes.

2.2. Collection of animals

Echingammarus marinus specimens were collected from Langstone Harbor (50.789624, −1.042419), Portsmouth, U.K., during low tides under seaweed (*Fucus vesiculosus* and *Ascophyllum nodosum*) and stones. The animals were then transported in a bucket containing seawater to the Institute of Marine Sciences, Portsmouth, U.K., where they were sorted into adult males and females using a stereomicroscope. Males and females with incomplete body parts, juveniles, and trematode-parasitised animals were excluded from the study (Guler et al., 2015). Male and female animals were acclimatised in separate buckets containing artificial seawater (ASW) with a salinity of 33 ± 2 ppt and incubated at 10 ± 1 °C with a 24 h dark regime to eliminate potential effects of circadian rhythm (Kohler et al., 2018) for 14 days prior to the start of the experiment. The ASW was changed every three days, and the animals were fed *A. nodosum* (Bossus et al., 2014). The animals used were healthy, acclimated to the laboratory environment, and had no known preexisting conditions that could affect the study outcomes.

2.3. Selection of test concentrations

The compound concentrations selected for this study were based on reported environmental concentrations of up to 4000 μg/l for NBBS, 95 μg/l for TPHP, 3203 μg/l for DEHP, and 182 μg/l for DBP in surface water, groundwater, and wastewater effluents across Europe and Asia (Spurgeon et al., 2022; Saha et al., 2022; Yin et al., 2021).

2.4. Experimental design for pairing experiment

A preliminary study was conducted to optimise the test chambers and video-recording techniques. To maximise the number of available males and females for pairing, a pre-pairing period following acclimatisation was adopted to ensure that most of the females were at the initial stages of precopula and available for re-pairing for the experiment (Malbouisson et al., 1995). Thus, re-pairing was not constrained by the female reproductive stage. Precopulatory pairs were randomly selected using a spoon and placed on tissue paper for separation. The animals were separated naturally within a few minutes or gently pulled apart

manually without causing harm.

A total of 480 pairs (960 individuals) were exposed to one of four test compounds, NBBS, TPHP, DBP, and DEHP, each at six different concentrations, including two controls: a blank control (artificial seawater), a solvent control (DMSO), and four compound concentrations of 0.5, 5, 50, and 500 μg/l (Fig. 1). Each of these 24 compound*concentration treatment combinations had 20 replicates (one pair per replicate). Exposure was carried out in rectangular dishes (80 × 20 mm) with eight chambers and a separator; all eight chambers contained the same treatment combination (Fig. 1). The males and corresponding females were isolated on opposite sides of the separator and incubated at 10 ± 1 °C in the dark. After 1 h of exposure, the separator was removed, and four dishes, each containing a randomly chosen concentration of a given treatment, were video-recorded using the Zantiks LT unit (Zantiks Ltd., Cambridge, UK) for 15 min. Hereafter, the dishes were kept at 10 ± 1 °C under a 24-h dark photoperiod for another 96 h.

Two variables were recorded in the videos: contact time and re-pairing time. Contact time is the time when the animal unsuccessfully attempts to establish a precopulatory pair. The re-pairing time is the time point for the successful formation of precopulatory pairs. Not all pairs had an unsuccessful pairing attempt, and these were excluded from the data analyses investigating the contact time. Not all pairs re-paired successfully during the 15 min, and these were excluded from data analyses investigating re-pairing time. Both measurements were recorded in seconds. Pairs were visually inspected again after 24 h, 48 h, 72 h and 96 h and new successfully formed pairs were recorded.

2.5. Sperm count

To investigate the effect of the selected plastic additives on the sperm count of amphipods, 345 adult male *E. marinus* were exposed to one of the 23 different treatment combinations. These treatment combinations were the same as described above, excluding TPHP at 500 μg/l with over 80% mortality at day 14. Each treatment combination had 15 replicates (one male per replicate). Males were individually placed in a polypropylene dish (10.5 cm diameter) containing 80 ml of the test solution and exposed for 14 days at 10 ± 1 °C in the dark.

The method for quantifying the sperm count was adapted from Yang et al. (2008). The animals were anaesthetised in a bath of a working solution of clove oil and weighed individually using an analytical balance. Testes were dissected using micro-dissecting scissors and tweezers under a stereomicroscope. The spermatozoa in the seminal vesicles were smeared, dilacerated onto the cavity slide, and mixed with 30 μL of PBS. The mixture was transferred to a pre-weighed Eppendorf tube containing 30 μL of PBS. The cell suspension was mixed by pipetting, and 10 μL of sperm solution was added to each side of the Neubauer haemocytometer, with a coverslip on top. The number of sperm cells was manually counted under the microscope with inverted light at ×40 magnification. Each organism had three technical replicates and the average was used for the analysis. The total number of spermatozoa was calculated by multiplying the sperm counts per μl by the volume of sperm solution. A typical sperm cell (spermatozoon) consists of a main body and a straight non-motile pseudoflagellum (Fig. 2). The counted sperm cells were grouped according to the exposure conditions and normalised using fourth root transformation (Yang et al., 2008). The male weight was included in the data analysis to eliminate the effect of weight on sperm count since sperm count is proportional to body weight. Animals with no visible sperm (1.4%) and dead animals (10.7%) were excluded from data analysis.

2.6. Statistics

Data analyses were performed using IBM SPSS Statistics 28 software. For the pairing experiment, a Kruskal-Wallis (K.W.) test was performed separately for each test compound to examine the effect of concentration on the two dependent variables, contact time and re-pairing time (N ranging from 87 to 104 for each K.W. test). Dunn's procedure for pairwise comparison with Bonferroni correction was performed for post-hoc analysis. Fisher's exact test was used to determine whether there was an

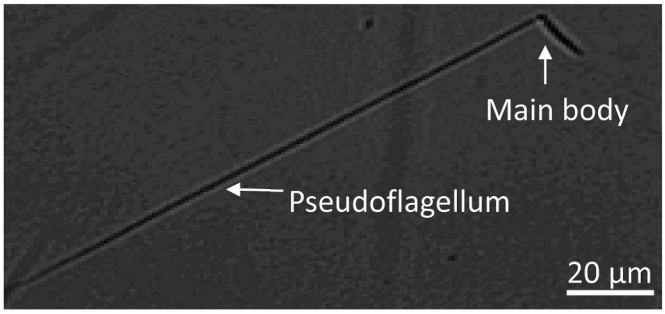

Fig. 2. A spermatozoon of *Echinogammarus marinus* as observed under a fluorescence microscope at ×40 magnification.

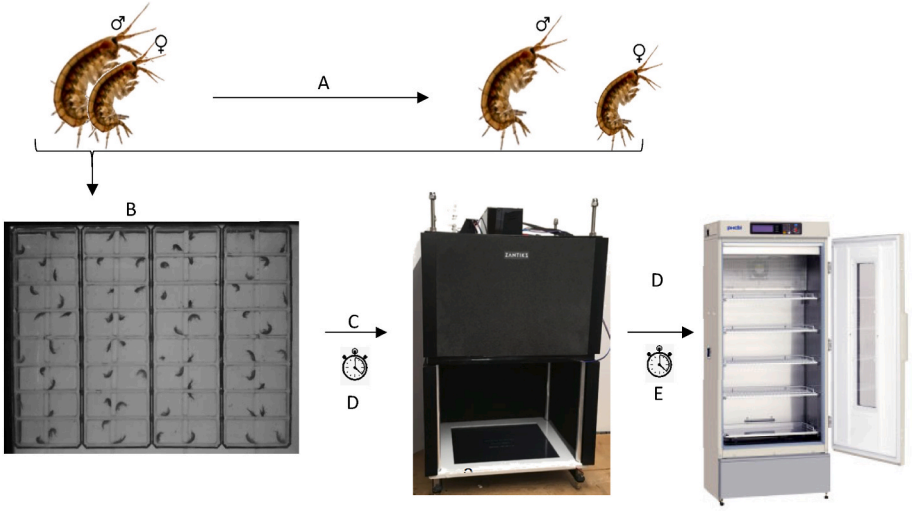

Fig. 1. Experimental design for the precopulatory pairing experiment of *Echinogammarus marinus* showing separation of paired males and females (A) that were introduced into rectangular dishes containing the exposure medium (B) and transferred into the Zantiks LT system (C) for video recording of the contact time and re-pair time for 15 min (D), and transferred into the incubator maintained at 9 ± 1.2 °C (D) to allow for observation every 24 h till 96 h.

association between concentration and whether pairs reformed within 15 min of observation (binary response). The effect of concentration and number of days of exposure as predictors of whether pairs had reformed over the 96 h were assessed using binomial logistic regressions conducted separately for each compound (N = 120 per model). Model significance and comparison were determined using a $\chi 2$ omnibus test and Nagelkerke's R^2, while B coefficients and odds ratios were reported for both predictors. The effects of concentration on sperm count normalised by the fourth root were evaluated using one-way ANCOVAs for each compound, with male body weight as the covariate (N ranging from 58 to 89 per model). A pairwise comparison was used as a post hoc when there was a significant difference. Statistical significance was set at $P < 0.05$.

3. Results

3.1. Effects of plastic additives on precopulatory behaviour

3.1.1. Solvent effect

We found no significant difference between the blank control and DMSO control for all the tested compounds. The data for the DMSO control group were compared with the exposed groups to determine significant differences.

3.1.2. NBBS

To investigate the effect of NBBS on pairing behaviour, we assessed the proportion of animals that re-paired after 15 min of the initial observation. Compared to the DMSO control (100%), there were no statistically significant differences in the proportion of pairs reformed at NBBS concentrations of 0.5 μg/l (100%), 5 μg/l (100%), 50 μg/l (90%); however, there was a significant decrease in 500 μg/l (60%) group (Fisher, $p = 0.003$).

The median time to make contact and re-pair in all treatment groups ranged from 17 to 147 s and 25–581 s respectively. Kruskal-Wallis tests showed a significant effect of concentration on contact time ($H(5) = 88.86$, $p < 0.001$) and re-pairing time ($H(5) = 95.01$, $p < 0.001$). Post hoc analyses, adjusted by Bonferroni correction, confirmed that apart from contact time in 0.5 μg/l, all exposed groups took a significantly longer time to make contact (5–500 μg/l $p < 0.001$) and re-pair (0.5 μg/l, $p = 0.014$, 5–500 μg/l, $p < 0.001$) than the DMSO control group (Fig. 3A) (Supplementary Table 1).

Continuous observation for 96 h showed that 95% of pairs in the control groups were still in pairs after four days, while the exposed groups declined with time (Fig. 3E). Binary logistic regression was used to model the effect of concentration and duration of exposure on the odds of observing no pairs over 96 h. The binomial regression model was significant ($\chi^2(8) = 235.12$, $p < 0.001$). All concentrations were statistically significant predictor variables affecting the re-pairing rate, with the greatest odds of no pair formation observed at 500 μg/l. The hours of exposure were not significant predictors in the model (Supplementary Table 2A).

3.1.3. TPHP

Following exposure of *E. marinus* to increasing concentrations of TPHP, compared to the DMSO control (100%), there was no statistically significant difference in the proportion of animals that re-paired in TPHP concentrations of 0.5 μg/l (100%), 5 μg/l (95%), 50 μg/l (80%). In comparison, there was a significant decrease in 500 μg/l (75%) group (Fisher, $p = 0.047$).

The effect of concentration on contact time ($H(5) = 83.56$, $p < 0.001$) and re-pairing time ($H(5) = 90.62$, $p < 0.001$) were statistically significant. The median contact time and re-pairing time ranged between 18 – 194 s and 45–581 s, respectively. Animals took significantly longer to make contact in all the exposed groups (5–500 μg/l, $p < 0.001$), apart from those in 0.5 μg/l ($p = 0.137$) compared to the DMSO control group. A concentration-response relationship existed between concentration and re-pairing time, such that re-pairing time was significantly prolonged in the exposed groups (5–500 μg/l, $p < 0.001$) except for those in 0.5 μg/l ($p = 0.205$) (Fig. 3B).

After 96 h, the control group achieved 95% pair formation, whereas the exposed groups showed a lower percentage of pairs (Fig. 3F). The overall binomial logistic regression model was significant ($\chi^2(8) = 146.08$, $p < 0.001$). The different concentrations were significant predictors, with the highest odds of pairs not reforming observed at 500 μg/l. However, hours of exposure were not significant predictors in the model (Supplementary Table 2B).

3.1.4. DBP

Upon assessing the effect of DBP on pair formation, a significantly lower proportion of pairs was recorded at 500 μg/l (30%) (Fisher, $p < 0.001$), while a higher proportion of pairs reformed at lower concentrations of 0.5 μg/l (100%), 5 μg/l (90%), 50 μg/l (90%) which was not significant compared to the DMSO control group (100%).

Concentration had a statistically significant effect on pairing behaviour (contact time ($H(5) = 48.802$, $p < 0.001$) and re-pairing time ($H(5) = 24.78$, $p < 0.001$)). The median contact time and re-pairing time ranged between 18 – 229 s and 43–396 s, respectively. The establishment of contact was not different in 0.5 μg/l ($p = 0.351$), while the rest of the exposed groups took a significantly longer time to make contact than the DMSO control group (5 μg/l, $p = 0.043$); 50 μg/l, $p = 0.001$; and 500 μg/l, $p < 0.001$) (Fig. 3C).

Continuous observation within 96 h showed that the percentage of pairs reformed increased with time, with the control group attaining higher than 90% re-pairing success; those in 5 μg/l and 50 μg/l remained the same, while no pairs were found in 500 μg/l after 96 h of exposure (Fig. 3G). The binomial regression model was significant ($\chi^2(8) = 171.43$, $p < 0.001$) with concentration and only 48 h exposure duration were found to be significant predictors in the model (Supplementary Table 2C).

3.1.5. DEHP

The effect of DEHP on pairing behaviour showed no significant difference in the proportion of animals that re-paired in 0.5 μg/l (95%), 5 μg/l (100%), 50 μ g/l (100%), while there was a significant decrease in 500 μg/l (45%) group (Fisher, $p < 0.001$).

The effect of concentration on contact time ($H(5) = 51.50$, $p < 0.001$) and re-pairing time ($H(5) = 55.50$, $p < 0.001$) was statistically significant. The median contact time and re-pairing time ranged between 15 – 130 s and 47–320 s respectively. Animals took significantly longer to make contact in all the exposed groups (5–500 μg/l, $p < 0.001$) apart from those in the 0.5 μg/l ($p = 0.137$) group compared to the DMSO control group (Fig. 3D). Post hoc tests further revealed significant differences between the DMSO control group and exposed groups for contact time (0.5 μg/l, $p = 0.002$; and 5–500 μg/l, $p < 0.001$) and re-pairing time (0.5–500 μg/l, $p < 0.001$).

The control group achieved more than 95% pair formation, while the exposed group achieved less than 90% after 96 h (Fig. 3H). The binomial logistic regression model was significant ($\chi^2(8) = 104.24$, $p < 0.001$). The different concentrations were significant predictors while the hours of exposure were not significant predictors in the model (Supplementary Table 2D).

3.2. Sperm count

3.2.1. General

The control and DSMO groups did not differ significantly for any of the tested compounds. Thus, the DSMO control group was compared with the exposed groups to determine significant differences. Mortality was recorded in all exposed groups, including the controls, but this was not more than 20% of the sample size apart from TPHP (50 μg/l) and DBP (500 μg/l) with 60% mortality.

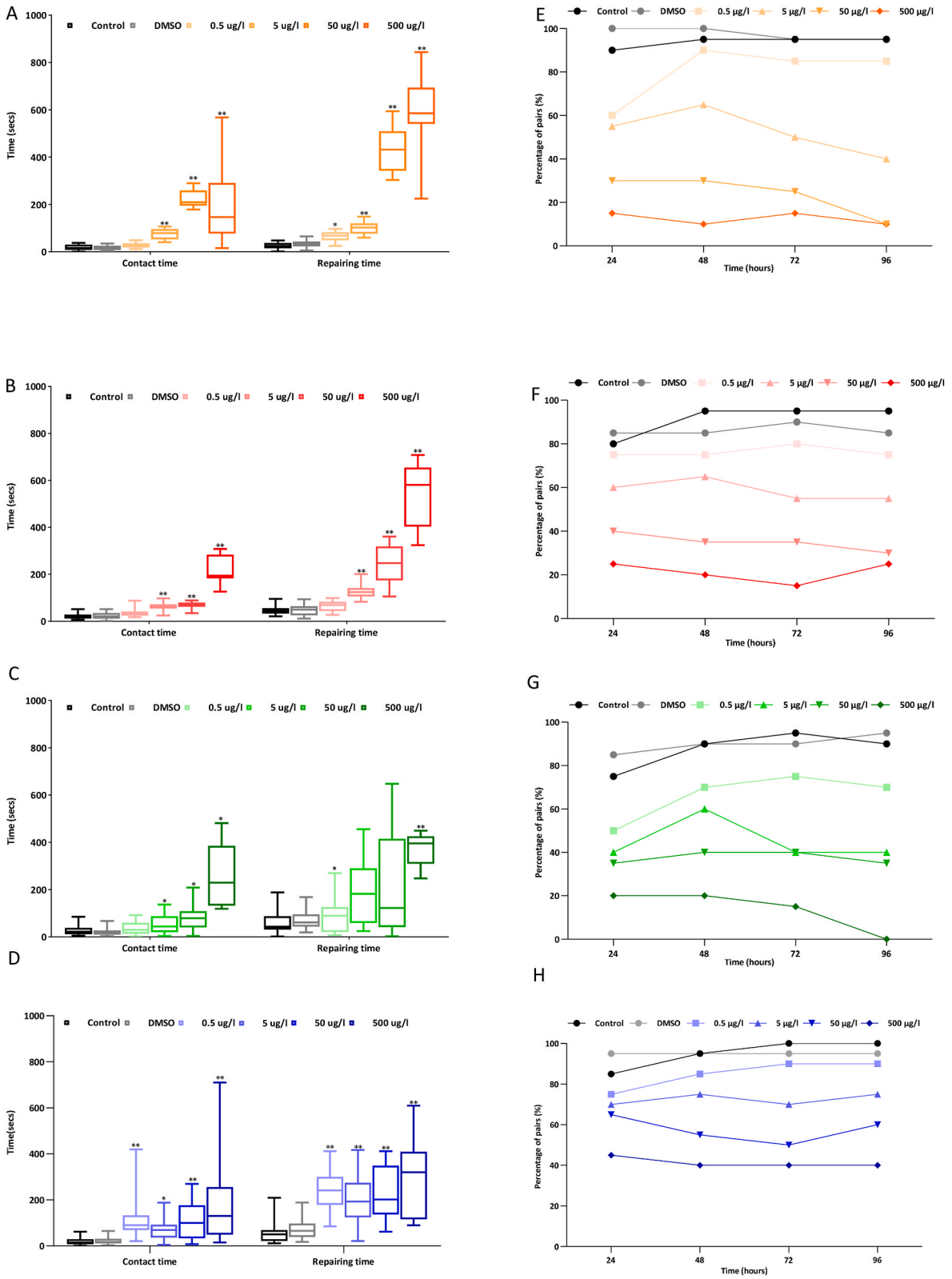

Fig. 3. (A–H): Contact time and re-pairing time of *Echinogammarus marinus* within 15 min after 1 h exposure to NBBS (A), TPHP (B), DBP (C) and DEHP (D); and percentage of pairs reformed after 96 h exposure to NBBS (E), TPHP (F), DBP (G) and DEHP (H). (Bars = boxplot of contact time and re-pairing time; Line in box = median; box = 25th to 75th percentiles; bars = min and max values; * denote p value < 0.05 and ** for $p < 0.001$).

3.2.2. NBBS

The mean sperm count recorded was 16,514 ± 1152 (S.E.) or (10.86 ± 0.22) (S.D.) following fourth root transformation (N = 79) (Fig. 4A). NBBS concentration did not affect the sperm count of *E. marinus* (ANCOVA F (5) = 0.834, p = 0.528). Male weight (M.W) did not significantly correlate with sperm count (Supplementary Graph 1 A). Likewise, concentration (C) and interaction between concentration and male weight were not statistically significant within the model (ANCOVA, M.W: F (1) = 0.505, p = 0.480; M.W vs C: F (5) = 0.903, p = 0.485).

3.2.3. TPHP

Males exposed to TPHP had a mean sperm count of 16,350 ± 1416 or 11.09 ± 0.39 after the fourth root transformation (N = 55). The number of sperm produced and counted was significantly affected by TPHP concentration (ANCOVA: F (4) = 5.965, p < 0.01). The exposed group had a concentration effect with a significantly low count in 50 μg/l after 14 days of exposure (Fig. 4B). Although there was no significant correlation between male weight and sperm count (Supplementary Graph 1 B), the interaction between male weight and concentration was significant (ANCOVA, M.W: F (1) = 0.236, p = 0.630; M.W vs C: F (4) = 4.838, p = 0.002).

3.2.4. DBP

The mean sperm count of males exposed to DBP was 15,390 ± 838 or 10.99 ± 0.16, following the fourth root transformation (N = 79). There was a concentration-response relationship in sperm count, but the effect of concentration was not significant (ANCOVA, F (5) = 0.915, p = 0.477) (Fig. 4C). There was no significant correlation between male weight and sperm numbers (Supplementary Graph 1C), and the interaction between male weight and concentration was not significant in the model (ANCOVA, M.W: F (1) = 0.013, p = 0.909; M.W vs C: F (5) = 1.417, p = 0.230).

3.2.5. DEHP

The mean sperm count recorded was 24,135 ± 1474 or 11.80 ± 0.28 after the fourth root transformation. (N = 89). Sperm counts were not significantly affected by DEHP concentration (ANCOVA, F (5) = 1.304, p = 0.271, Fig. 4D). Male weight significantly correlated with the sperm count of the exposed group (ANCOVA, F (1) = 5.285, p = 0.024) (Supplementary Graph 1D). The interaction between male weight and concentration was not significant (ANCOVA, F (5) = 1.326, p = 0.262).

4. Results summary

Table 1 summarises the no observed effect concentration (NOEC) and low observed effect concentration (LOEC) of the tested plastic additives on precopulatory pairing behaviour and sperm count. The NOEC was not determined for NBBS, TPHP, and DEHP after evaluating the results of reproductive behaviour and sperm count data. However, DBP had a LOEC of 5 μg/l for contact time, 50 μg/l for re-pairing time and 5 μg/l for sperm count. The LOEC for NBBS and DEHP were 0.5 μg/l for contact time and re-pairing time. TPHP had LOEC of 5 μg/l and 0.5 μg/l for contact time and re-pairing time respectively, while DBP had 50 μg/l and 500 μg/l for contact time and re-pairing time respectively. No LOEC was determined for sperm count after exposure to NBBS, TPHP, and DEHP; however, DBP had an LOEC of 50 μg/l. The NOEC of the proportion of pairs reformed was not determined, whereas the LOEC of the proportion of pairs reformed was observed at 0.5 μg/l for all tested compounds at 96 h.

5. Discussion

Although previous studies that have assessed the impact of plastic additives on reproduction have focused on vertebrates (Kay et al., 2014; Li et al., 2018; Deng et al., 2021; Sendra et al., 2021; Witchey et al., 2023), there is still a knowledge gap regarding their effects on aquatic invertebrates. Thus, this study examined the effects of selected plastic additives on the reproductive behaviour of marine amphipods and male fertility. We hypothesised that environmentally relevant NBBS, TPHP, DBP, and DEHP concentrations would negatively affect the precopulatory pairing behaviour of male and female amphipods and sperm count in males. Supporting our hypothesis, we found that the selected plastic additives prolonged the contact time and re-pairing within 15 min of observation. To the best of our knowledge, this study is the first to measure contact time as an additional endpoint in the mechanism of

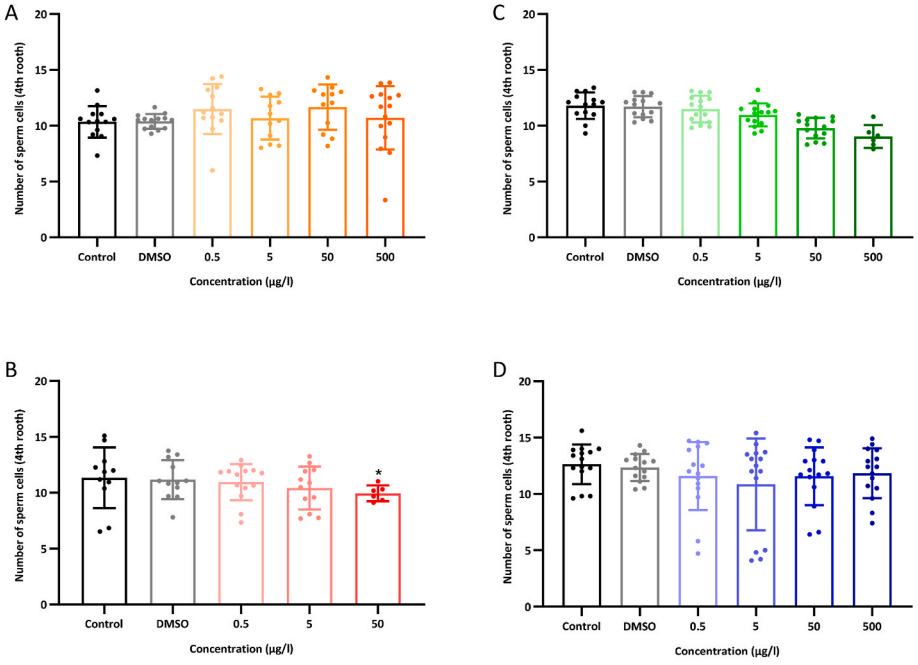

Fig. 4. (A–D): Mean fourth root-transformed sperm counts of *Echinogammarus marinus* exposed to NBBS(A), TPHP (B), DBP (C), and DEHP (D) for 14 days. (Bar = Mean values ± SD, Dots = distribution of the data; * = p < 0.05).

Table 1
Summary table for NOEC and LOEC for selected compounds and endpoints.

	NOEC (μg/l)				LOEC (μg/l)			
	Contact time	Re-pairing time	Sperm count	% of pairs reformed	Contact time	Re-pairing time	Sperm count	% of pairs reformed
NBBS	ND	ND	ND	ND	0.5 ↑	0.5 ↑	ND	0.5
TPHP	ND	ND	ND	ND	5 ↑	0.5 ↑	ND	0.5
DBP	5	50	5	ND	50 ↑	500 ↑	50 ↓	0.5
DEHP	ND	ND	ND	ND	0.5 ↑	0.5 ↑	ND	0.5

(ND- Not determined; ↑ - increase; ↓ - decrease).

precopulatory pairing. We found that contact time (defined as an unsuccessful attempt at re-pairing) provided data points for this failed event, which would have otherwise been excluded if re-pair did not occur within the observation time. Contact time may also provide insights as to whether re-pairing will likely occur since animals with a shorter contact time will most likely re-pair at a corresponding earlier time. In other cases, prolonged contact led to animals not reforming pairs within the observation time.

Furthermore, the concentrations of all the tested compounds reduced re-pairing rates by approximately 30% at 5 μg/l (90–100% in controls down to 60–70% successful pairs in 5 μg/l), whereas concentrations >5 μg/l reduced re-pairing rates even further by >50% (generally down to 0 and 40% successful re-pairing events). The decline in re-pairing rate was observed at concentrations lower than those previously reported in freshwater and marine systems. For example, in a Chinese river, DBP and DEHP were reported to have concentrations of 20.3 and 7 μg/l, respectively (Ren et al., 2023). Similarly, in England's groundwater, NBBS and TPHP were reported to have concentrations of 4000 μg/l and 7.2 μg/l, respectively (Spurgeon et al., 2022). In marine systems, NBBS was reported to have a concentration of 0.5 μg/l in Italy (Di Carro et al., 2018), while DEHP and DBP were reported to have concentrations of 8.0 and 5.0 μg/l, respectively, in the East China Sea (Zhang et al., 2020). These findings provide evidence of the environmental relevance of our study. The prolonged re-pairing time observed in our study is consistent with that reported by Wisniewska and Szaniawska (2015). Their study was on marine amphipod *Gammarus tigrinus* exposed took up to 445 s to reform pairs at 0.5 μg/l 17α-ethinylestradiol. Similarly, earlier studies have reported that the reproductive behaviour of the freshwater amphipods *Gammarus pulex* (Cold and Forbes, 2004) and *Hyalella azteca* (Pedersen et al., 2013) exposed to chemical contaminants is impaired, thus prolonging the re-pair time.

The experimental design used here makes it difficult to distinguish whether the behavioural effects manifested in males, females, or both. The effects of low/environmentally relevant concentrations of NBBS, TPHP, and DEHP may impair the performance of sex pheromones in the presence of these compounds, which are known environmental stressors. Amphipods primarily rely on chemical signals and cues (distance and contact) for decision-making on a series of events starting from initial attraction and pair formation, eventually leading to mating (Zhang et al., 2011). These tested compounds might have suppressed the info-chemicals necessary to stimulate reproduction and other physiological responses (Hay, 2009). Alternatively, it may be an olfactory response by the male or female overriding or enhancing their desire to re-pair, causing a deviation from the natural response. For instance, Love et al. (2020) reported that *E. marinus* males exposed to wastewater effluent for 21 d paired significantly faster with unexposed females. Interestingly, Negro et al. (2021) reported that *H. azteca* exposed to pesticide chlorpyrifos did not disrupt precopulatory pairs at high concentrations; however, upon transfer to a clean medium, the exposed animals reformed pairs faster than the control group.

This study found that the increased concentrations of the tested plastic additives drastically reduced the proportion of pairs that successfully re-paired. Generally, if animals did not pair within a day or two, they did not manage to do so over the next two to three days. Thus, we found that the percentage of pairs declined at 96 h or remained as they were at 24 h, except for DBP, where no pairs were found after 96 h. This may be attributed to the reduction in energy reserves for reproduction as exposure time increases (Cold and Forbes, 2004) or impaired homeostasis due to a direct toxic effect of test compounds (Heckmann et al., 2005), thus impairing precopulatory behaviour at high concentrations over time. For instance, Watts et al. (2001) found that plastic additive bisphenol (BPA) and synthetic oestrogen ethinyl-estradiol disrupted pairs of *G. Pulex* only at an almost acutely toxic concentration. Although terms such as 'decisiveness', 'courtship', and 'tenacity' have been applied to describe the precopulatory behaviour of *G. Pulex* (Dick and Elwood, 1989), it is difficult to assess which of these activities is most affected by environmental stressors (Malbouisson et al., 1995).

Furthermore, the mode of action of the plastic additives used in this study differs because of their unique properties, some of which are capable of inhibiting the oestrogenic and androgenic effects of AChE inhibition in mammals and aquatic animals (Papaioannou et al., 2010; Chen et al., 2014; Waidyanatha et al., 2020; Hu et al., 2023; Zhang et al., 2023). Studies have established disruptive effects on reproduction in vertebrates, such as DEHP and DBP in zebrafish (*Danio rerio*) (Chen et al., 2014; Yuan et al., 2022), TPHP in zebrafish (*D. rerio*) (Li et al., 2018; He et al., 2021) and rats (Witchey et al., 2023), and NBBS in rats (Iuclid, 2007; Rider et al., 2020). Precopulatory pairing behaviour, in the form of pair formation or separation, is imperative for mating success. The inability of animals to re-pair can potentially preclude opportunities for copulation, thus influencing population-level effects and interconnected food webs. In future studies, these results will be used to assess whether reproductive impairment is permanent or reversible in post-exposure assays.

This study also aimed to assess the impact of selected plastic additives on the sperm count of *E. marinus* after 14 days of exposure, with the hypothesis that sperm quantity would be reduced. We found that sperm count declined with increasing concentration in animals exposed to TPHP and DBP, as predicted, but not in animals exposed to NBBS and DEHP. The decline recorded for two of the compounds tested might be linked to endocrine-disrupting effects. For instance, Gismondi et al. (2017) found that *G. Pulex* exposed to the anti-androgen compound cyprotene acetate (CPA) significantly reduced sperm production and sperm counts. Their study suggested that CPA could inhibit or stimulate androgenic and gonadal hormones necessary for producing spermatozoa. A study on the impact of cadmium and pyriproxyfennon *Gammarus fossarum*, carried out by Trapp et al. (2015), found a significant decline in sperm production in a concentration-dependent manner.

Field studies have also assessed whether environmental pollutants can lead to low sperm count in invertebrates. For example, Ford et al. (2012) studied the impact of industrial pollutants on *E. marinus* collected from an industrially impacted site and compared to those from two reference sites. They found that animals from the contaminated site had 20% lower sperm counts than those from the reference site, but it was uncertain if this reduction was due to endocrine disruptive effects. Notably, studies have confirmed that anti-androgenic chemicals can reduce spermatozoa owing to their impact on the androgenic gland, which is widely responsible for spermatogenesis and male differentiation in amphipods (Yang et al., 2008). Another field study conducted by Botelho et al. (2021) reported low sperm counts in *E. marinus* in Langstone Harbor compared to six other locations in the U.K., suspected to be

caused by environmental stressors. Since the animals used in our study were collected from this site, it is unknown whether this pre-existing low sperm condition influenced our results. However, this findings emphasizes the risks for these animals and the need for their inclusion in marine monitoring programs.

This study found that sperm count in NBBS and DEHP did not show a concentration-response relationship. The count at low concentrations was similar to that at high concentrations, with an approximately 10% difference from that of the control group. This could be due to the high individual variability in the number of sperms retrieved from individual animals within the same concentration. This kind of variability in sperm count has also been previously reported by Trapp et al. (2015), who found that low concentrations of methoxyfenozide showed high inter-individual variability in sperm counts of *G. fossarum*. Although this study only assessed sperm quantity, environmental compounds can also affect sperm quality, depending on the specific properties of the pollutant and the methodology used for assessing sperm quality. For example, Fuller et al. (2019) studied the impact of ionising radiation on sperm quantity and quality of *E. marinus*. They found no effects on sperm quantity but a reduction in sperm quality after exposure to 1 and 10 mGy/d.

Furthermore, numerous studies have examined sperm parameters after exposure to plastic additives in humans and animals, including fish, rats, and other vertebrates (Rider et al., 2020; Mondal & Bandyopadhyay, 2023; Basili et al., 2023)). For example, Chen et al. (2020) found no apparent decline in sperm concentration or vitality in adult male minnows *(Gobiocypris rarus*) after exposure to TPHP for 28 d. However, they recorded a difference in sperm velocity and motility compared with the control group. Aly (2016) reported a significantly reduced sperm count, motility and testicular weight in male rats fed DBP for 15 days. However, a previous study by Jarmołowicz et al. (2010) reported no significant effect of DBP at concentrations as high as 10,000 μg/l on pikeperch sperm motility. DEHP has been associated with adverse reproductive outcomes such as a decrease in egg production of copepods (Heindler et al., 2017), delay in the first-day reproduction of crustaceans (Jung et al., 2020), histological alteration in the testes and ovaries of fish (Ye et al., 2014), and decreased number of spermatozoa in zebrafish (Uren-Webster et al., 2010). A study on the reproductive effects of NBBS reported a significant decrease in the weight of the testes and epididymides in male rats orally fed 400 mg/kg of NBBS for two weeks before mating (Iuclid, 2007). These studies serve as a baseline for studies assessing marine amphipod sperm counts, and future work will investigate the degree of damage to sperm parameters.

6. Conclusion

Precopulatory pairing behaviour was sensitive in showing compound effects after just an hour of exposure, with increasing concentration-dependent severity by prolonging re-pairing time within the first 15 min of observations and preventing an increasing proportion of pairs from re-pairing over 96 h of observation. The optimisation of data collection techniques for assessing precopulatory pairing reduced the duration of the experiment and the use of contact time as an additional endpoint provides data that might have been lost in the event of an unsuccessful re-pair attempt. All four tested plastic additives significantly affected reproductive behaviour, even at environmentally relevant concentrations. A decline in sperm count was recorded in TPHP and DBP after 14 days of exposure. NBBS and DEHP exposure increased variability around the mean sperm count in the exposed and control groups. While behavioural and reproductive experiments provide helpful information on population-level effects, it is difficult to ascertain the modes of action of these compounds, which may differ from those of fish and other invertebrates. However, the effects found at environmentally relevant concentrations indicate the possibility of negative implications on higher ecological levels, since environmental protection aims to protect aquatic life over the long term.

CRediT authorship contribution statement

Bidemi Green-Ojo: Conceptualisation, Methodology, Investigation, Formal Analysis, Visualisation, Writing – Original Draft, Writing – Review, and Editing. Marina Tenório Botelho: Conceptualisation, Methodology, Investigation, Data Curation, Formal Analysis, Writing – Original Draft, Writing – Review, and Editing. Gisela De Aragão Umbuzeiro: Supervision, Resources, Formal Analysis, Writing – Review and Editing. Vicente Gomes: Supervision, Resources, Formal Analysis, Writing – Review and Editing. Mathew O Parker: Supervision, Formal Analysis, Resources, Writing – Review, and Editing. Lena Grinsted: Supervision, Formal Analysis, Resources Writing – Review, and Editing. Alex T Ford: Conceptualisation, Methodology, Supervision, Data Curation, Formal Analysis, Resources, Writing – Review, and Editing.

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.envpol.2023.122946.

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