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Adverse impacts of high-density microplastics on juvenile growth and behaviour of the endangered tri-spine horseshoe crab *Tachypleus tridentatus*

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ABSTRACT

The impacts of high-density microplastics, namely polyamine 6,6 (nylon), polymethyl methacrylate (PMMA) and polyethylene terephthalate (PET), on growth and behaviour of the endangered tri-spine horseshoe crab *Tachypleus tridentatus* were investigated for 100 days. Negative changes in wet weight and prosomal width of the juveniles were observed in all treatments of microplastics, but significant difference was only detected in prosomal width between control and PMMA. *T. tridentatus* became significantly less active upon exposure to nylon and PET. The extent of burrowing by *T. tridentatus* did not significantly differ among the treatments but was overall significantly reduced towards day 100. *T. tridentatus* exposed to PET significantly showed the lowest survival probability (30 %), compared to the other treatments (70–90 %). In conclusion, high-density microplastics compromised growth and behaviour of juvenile horseshoe crabs. Among the polymers that were tested, PET was considered more harmful and associated with higher mortality.

1. Introduction

Plastics are inexpensive, lightweight, durable and versatile in human societies. More than eight billion t of plastics have been manufactured worldwide since the 1950s, and the mass production of plastics is expected to further increase to meet the ever-growing demand in the future (Thompson et al., 2009; Wright and Kelly, 2017). The rising use comes with the increasing waste of plastics. Apart from a small fraction that can be recycled or incinerated, most of the plastic waste ended up in landfills or natural environments (Geyer et al., 2017; Napper and Thompson, 2020). For instance, over 250,000 t of plastic debris have been reported in the ocean (Eriksen et al., 2014). The situation of plastic pollution could be worsened by the pandemic of COVID-19, during which 26,000 t of additional plastic waste such as disposable masks could be released into marine environments (Bondaroff and Cooke, 2020; Sharma et al., 2020; Peng et al., 2021). Plastic debris can be fragmented into pieces smaller than 5 mm in diameter, which are commonly referred to as microplastics (Arthur and Baker, 2011; Cole et al., 2011). Besides the concerns of plastic ingestion and entanglement classically reported for animals like seabirds and turtles, microplastics are ingestible by smaller organisms and are posing another major threat to marine life.

To date, most of the ecotoxicological studies on microplastics have used low-density (floating) plastic particles, such as polyethylene and polystyrene (Cole and Galloway, 2015; Mao et al., 2018; González-Soto et al., 2019; Piccardo et al., 2020; Guerrera et al., 2021; Raju et al.,

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2022). However, findings from these studies might be less relevant to benthic organisms such as horseshoe crabs, which are apparently more susceptible to sinking microplastics (Wright et al., 2013). Horseshoe crabs are known as "living fossils" and play key roles in marine ecosystems as bioturbators, predators of bivalves, prey of seabirds, and hosts for a large number of epibionts (Botton, 2009; Rudkin and Young, 2009). Horseshoe crabs are also harvested by humans for biomedical applications and traditional rituals, and to a lesser extent as a food source (Hsieh and Chen, 2009; Kreamer and Michels, 2009; Shin et al., 2009). Unfortunately, the populations of horseshoe crabs are generally in decline worldwide, such as that of the tri-spine horseshoe crab *Tachypleus tridentatus* in South China (Kwan et al., 2016; Lee and Morton, 2016), and this species has been recently listed as endangered on the IUCN Red List (Laurie et al., 2019). Microplastics could be one of the factors leading to the population decline, given the growing evidence of plastic pollution in the nursery grounds of *T. tridentatus* in the region (Fok and Cheung, 2015; Cheung et al., 2016; Tsang et al., 2017; Lo et al., 2018; Xu et al., 2020). Moreover, microplastics are often sorbed with other environmental pollutants, e.g., heavy metals, which can be harmful to horseshoe crabs particularly in their early life history (Itow et al., 1998a, 1998b; Kwan et al., 2015).

Juvenile horseshoe crabs burrow in sediment, where they hide from predators and feed on a mixed diet of small invertebrates, meiofauna, plankton and organic matter (Chiu and Morton, 2004; Zhou and Morton, 2004; Kwan et al., 2021; Li et al., 2022a). Their foraging behaviour follows the tidal cycle, i.e., burrowing in sediment during high tide, and emerging and feeding in the surface sediment after the tide has receded (Chiu and Morton, 2004). They crawl with their body half-buried in sediment during low tide, and create the characteristic crawling trail (Rudloe, 1981; Chiu and Morton, 2004). This burrowing nature makes juvenile horseshoe crabs a high-risk group to ingest microplastics that are deposited on the sediment. The assumption of risk is evidenced by the recent findings by Wang et al. (2022), who have observed a large amount of microplastics in the body of juvenile *T. tridentatus* in South China, but the health impacts of these ingested microplastics on horseshoe crabs remain unknown.

The present study aimed to investigate the ecophysiological effects of high-density microplastics on *T. tridentatus*. Three types of microplastics, namely polyamine 6,6 (nylon), polymethyl methacrylate (PMMA) and polyethylene terephthalate (PET), were used as the test models. These particles have densities higher than seawater and tend to sink in the water column. The impacts on growth and behaviour of *T. tridentatus* were determined at its juvenile stage, a fragile and critical life-history stage that determines the population success. Findings from this study would allow us to evaluate the toxicity and ecological risk of microplastics to horseshoe crabs in South China and beyond.

2. Materials and methods

2.1. Experimental design

Juvenile *T. tridentatus* was artificially bred and supplied by the Guangxi Marine Research Institute, South China (Fig. 1a, b). The second-instar juveniles (after their second moult) were used in this study and exposed to three types of high-density microplastics, namely nylon (1.14 g cm^{-3}), PMMA (1.18 g cm^{-3}) and PET (1.38 g cm^{-3}). The particles of nylon, PMMA and PET were made from cleaned plastic waste of cable ties, acrylic sheets and egg cartons, respectively (Fig. 2a), and were pulverised at −196 °C using a Retsch CryoMill cryogenic grinder (Haan, Germany; Fig. 2b). The ultra-low temperature was maintained by liquid nitrogen circulating outside the grinding chamber made of zirconium oxide. The produced microplastics of nylon, PMMA and PET were sieved to reach a particle size range of 30–100 μm before use (Fig. 2c).

Each of the three treatments of microplastics and the control treatment had its own circulation system and a reservoir (150 L) containing filtration materials of cotton and ceramic rings. There were ten replicates per treatment, i.e., ten beakers (0.25 L) with one individual of *T. tridentatus* per beaker. Artificial seawater was made to 15 ‰ (Instant Ocean, Blacksburg, VA) and circulated through the ten beakers and reservoir in each treatment. The salinity was regularly monitored with a portable refractometer. Seawater temperature was maintained at 25 °C throughout the experiment. Suitable sand substrate was provided for *T. tridentatus* in the beakers following Kwan et al. (2015), and microplastics were introduced at an environmentally realistic concentration of 0.1 % w/w in the sand (Carson et al., 2011). The juveniles were daily fed to satiation with freshly hatched brine shrimp larvae (*Artemia* sp.). The exposure experiment lasted 100 days and the sand substrate and microplastics were renewed every 20 days. Growth parameters and behavioural responses of all juveniles were determined during the experiment. The amounts of microplastics ingested by *T. tridentatus* in all treatments were quantified at the end of the experiment.

2.2. Ecophysiological measurements

Wet weight and prosomal width as two growth parameters of *T. tridentatus* were measured every 20 days (Fig. 1a, b). Horseshoe crabs were blotted dry and weighed using a Kern ABT220-5DNM analytical balance (Balingen, Germany; ±0.1 mg). Prosomal width was measured with a caliper (±0.01 mm). Top-view photographs were taken of all juveniles one hour after the renewal of the sand substrate on days 40, 60, 80 and 100. These photographs were processed with the ImageJ software (Schneider et al., 2012) to assess two behavioural parameters of *T. tridentatus*, namely locomotion, which was expressed as the crawling

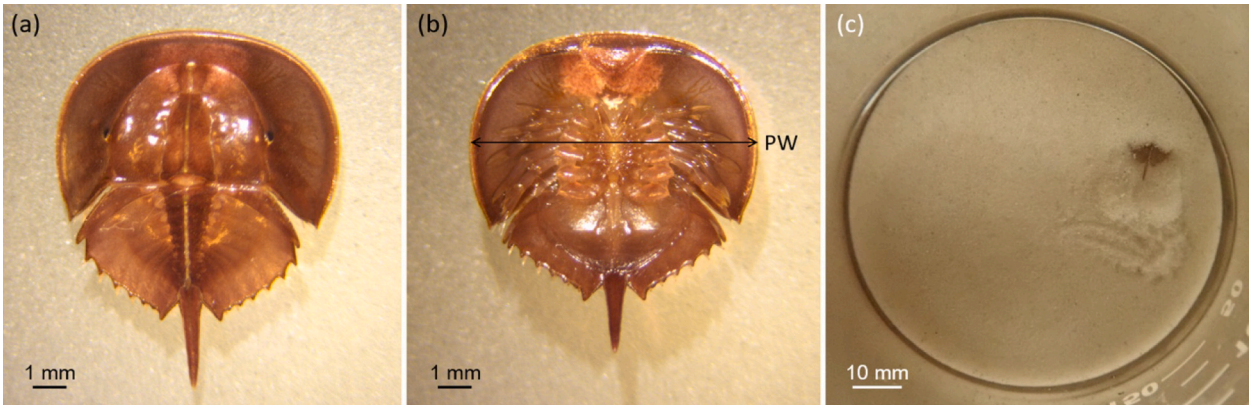

Fig. 1. (a) Dorsal view and (b) ventral view of a second-instar juvenile *Tachypleus tridentatus*. Wet weight and prosomal width (PW) were measured every 20 days. (c) The crawling trail of *T. tridentatus* left on the sand, and its extent of burrowing was observed one hour after the renewal of the sand substrate on days 40, 60, 80 and 100.

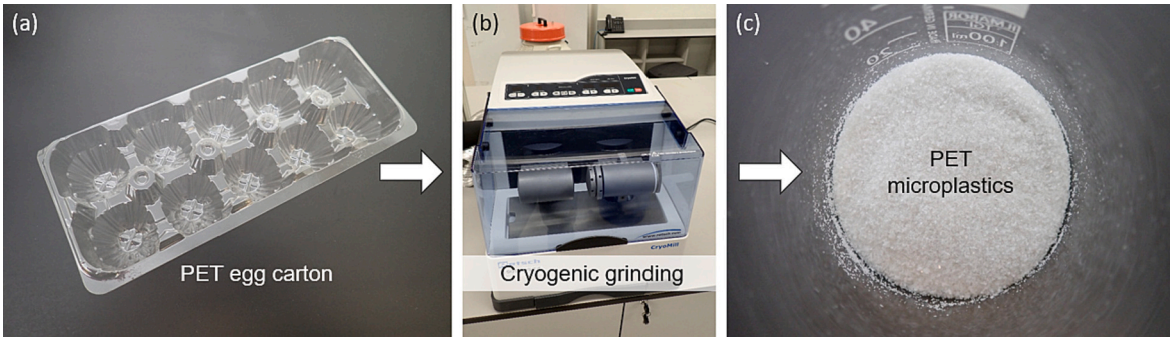

Fig. 2. Preparation of microplastics in this study. (a) Plastic egg cartons represent a common domestic waste of polyethylene terephthalate (PET) and (b) were cryogenically ground at −196 °C using a Retsch CryoMill (Haan, Germany) to produce (c) microplastics of PET. Microplastics of polyamine 6,6 and polymethyl methacrylate were similarly prepared from cable ties and acrylic sheets, respectively.

trail area defined as the total area of ploughed and piled sand made by the movement of each individual during the one-hour period, and the extent of burrowing, which was indicated by the proportion of the animal's top area burrowed in sand (Fig. 1c). The timing of *T. tridentatus* moulting and mortality, if any, was recorded.

The quantities of microplastics ingested by *T. tridentatus* in all treatments were determined after the experiment. Each sample of *T. tridentatus* was digested in a solution containing 10 % potassium hydroxide, 30 % hydrogen peroxide and 14 % ethylenediaminetetraacetic acid disodium salt dihydrate at 40 °C for 48 hours, a process that did not cause any detectable damage on particles of nylon, PMMA and PET in the size range of 412–648 μm and achieved spike recovery rates of 96–100 % in biological matrices, as confirmed by Leung et al. (2021a). Similar digestion treatments had been adopted by other studies to extract smaller-sized microplastics (>30 μm) from biological samples (Leung et al., 2021b; Ho et al., 2022). In the present study, undigested particles in each solution were retained on a stainless-steel sieve with 30 μm pores. Microplastics of nylon, PMMA and PET on the sieves were identified and counted at 785 nm excitation using a Renishaw inVia confocal Raman microscope (Wotton-under Edge, England). The analytical settings of Raman spectrometry were provided in Leung et al. (2021b). A particle recovery test was not performed in the present study, and therefore the determined numbers of ingested microplastics by *T. tridentatus* represented conservative quantities that might be underestimated.

2.3. Statistical analysis

The impacts of microplastics on growth and behaviour of *T. tridentatus* determined at different monitoring time points were compared among the four treatments using two-way repeated measures analysis of variance (RM-ANOVA). Growth rates were expressed as percentage changes over time in wet weight and prosomal width. Behavioural responses were determined in terms of locomotion and the extent of burrowing by *T. tridentatus*. The datasets of wet weight, prosomal width, locomotion and burrowing behaviour did not meet the assumptions for RM-ANOVA, even after data transformation, and were aligned-rank transformed using the *ARTool* package prior to the analysis (see Wobbrock et al., 2011). Tukey's multiple comparison tests were performed when significant differences were detected in RM-ANOVA. The overall relationship between wet weight and prosomal width of *T. tridentatus* was examined by linear regression analysis.

The days of survival of *T. tridentatus* in the four treatments were aligned-rank transformed and analysed with one-way ANOVA. Kaplan-Meir survival analysis with right censoring was used to assess the survival curves of *T. tridentatus*, and pairwise comparisons of the survival probability among the four treatments were performed using log-rank test (Kaplan and Meier, 1958; Lee and Wang, 2003). All statistical tests were performed at a significance level of 5 % using the software Statistica 13 (TIBCO Software, Palo Alto, CA).

3. Results

3.1. Growth parameters

The net changes in mean wet weight of juvenile *T. tridentatus* were 0.9 % in the control, but −6.3 %, −6.2 % and −3.4 % upon exposure to nylon, PMMA and PET, respectively, over 100 days of the experiment (Fig. 3a). However, these changes were not significant among treatments (RM-ANOVA, $F_{3,23} = 0.878$, $p = 0.467$) and exposure time ($F_{4,92} = 0.709$, $p = 0.588$), and in the interaction between treatment and time ($F_{12,92} = 1.198$, $p = 0.297$; Table 1).

The net changes in mean prosomal width of *T. tridentatus* were 2.3 %, −0.6 %, −2.4 % and −0.4 % in the treatments of control, nylon, PMMA and PET, respectively (Fig. 3b). The treatment effect on prosomal width was detected (RM-ANOVA, $F_{3,23} = 3.066$, $p = 0.048$), where the values were significantly lower in the PMMA treatment compared to the control (Tukey's test, $p = 0.049$; Table 1). Exposure time ($F_{4,92} = 0.781$, $p = 0.541$) and the interaction between treatment and time ($F_{12,92} = 1.658$, $p = 0.089$) did not significantly affect prosomal width of *T. tridentatus*. Linear regression analysis revealed that prosomal width could be a fair predictor of the wet weight of *T. tridentatus* across all treatments over time ($r^2 = 0.50$, $p < 0.05$, Fig. 4). Moulting of *T. tridentatus* was not observed during the experimental period.

3.2. Behavioural responses

T. tridentatus moved at a rate of 682 mm^2 per hour on average in the control, which was used as a proxy for locomotion, but the rates decreased to 382, 410 and 342 $mm^2\ h^{-1}$ in the treatments of nylon, PMMA and PET, respectively, after 100 days of exposure (Fig. 5a). Significant difference in locomotion was detected among treatments in RM-ANOVA ($F_{3,23} = 6.310$, $p = 0.003$), where the rates in the nylon and PET treatments were significantly lower than that in the control (Tukey's test, $p = 0.010$ and 0.046, respectively; Table 1). The effects of exposure time ($F_{3,69} = 1.980$, $p = 0.125$) and interaction between treatment and time did not significantly affect locomotion of *T. tridentatus* ($F_{9,69} = 1.126$, $p = 0.276$; Table 1).

On average 54.3 % of the top area of *T. tridentatus* was burrowed in sand under the control conditions, but the extents of burrowing decreased to 37.0 %, 44.4 % and 10.0 % upon exposure to nylon, PMMA and PET, respectively, on day 100 (Fig. 5b). Significant difference in the burrowing behaviour was not detected among treatments (RM-ANOVA, $F_{3,23} = 0.464$, $p = 0.710$), but was detected among monitoring time points ($F_{3,69} = 3.593$, $p = 0.018$; Table 1), where the extent of burrowing determined on day 100 was significantly the lowest (Tukey's test, $p = 0.039$). This time effect might be driven by the decreasing trend in the PET treatment towards day 100 (Fig. 5b). The interaction effect between

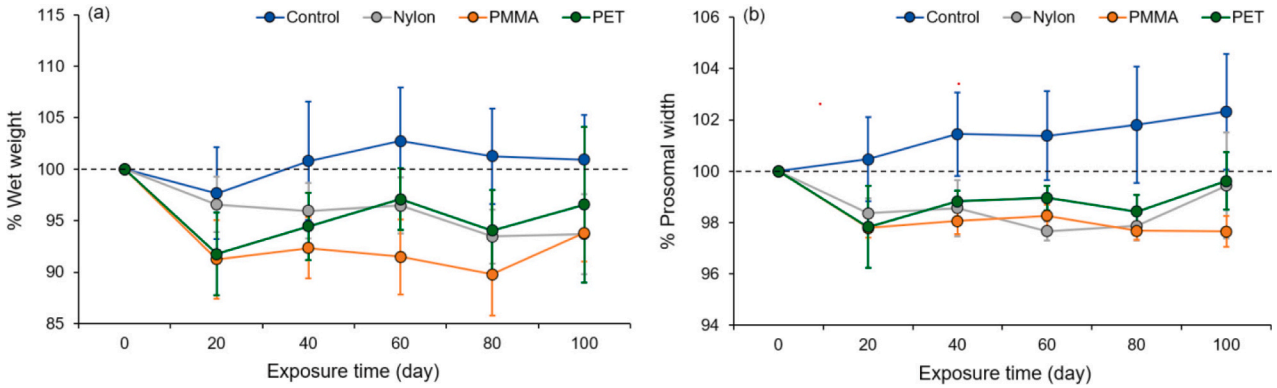

Fig. 3. Relative changes in (a) wet weight and (b) prosomal width of *Tachypleus tridentatus* over 100 days of exposure to polyamine 6,6 (nylon), polymethyl methacrylate (PMMA) and polyethylene terephthalate (PET) compared to the control (mean ± SE). The replicate number of *T. tridentatus* started at 10 per treatment but decreased towards day 100 due to mortality (see Fig. 6). The dashed lines indicate the initial wet weight and prosomal width as 100 % on day 0. Statistical results are provided in Table 1.

Table 1
Results of two-way repeated measures analysis of variance (RM-ANOVA) and, if significant, Tukey's post hoc pairwise comparisons for the aligned-rank transformed data of growth and behaviour of *Tachypleus tridentatus* in the treatments of control, polyamine 6,6 (nylon), polymethyl methacrylate (PMMA) and polyethylene terephthalate (PET) over 100 days of exposure (see Figs. 3, 5). Asterisks indicate significance in the RM-ANOVA ($p < 0.05$). Different italic letters in the brackets indicate significant pairwise differences in Tukey's test ($p < 0.05$).

Variable	Fixed effect	df	*F* value	*p* value	Post hoc comparisons (in descending order)
Wet weight	Treatment	3	0.878	0.467	Not required
	Exposure time	4	0.709	0.588	Not required
	Treatment × time	12	1.198	0.297	
Prosomal width	Treatment	3	3.066	0.048*	Control (*a*), PET (*ab*), nylon (*ab*), PMMA (*b*),
	Exposure time	4	0.781	0.541	Not required
	Treatment × time	12	1.658	0.089	
Locomotion	Treatment	3	6.310	0.003*	Control (*a*), PMMA (*ab*), PET (*bc*), nylon (*c*)
	Exposure time	3	1.980	0.125	Not required
	Treatment × time	9	1.126	0.276	
Burrowed area	Treatment	3	0.464	0.710	Not required
	Exposure time	3	3.593	0.018*	Days 60 (*a*), 40 (*ab*), 80 (*ab*), 100 (*b*)
	Treatment × time	9	1.867	0.072	

treatment and time was not significant on the burrowing behaviour ($F_{9,69} = 1.867$, $p = 0.072$; Table 1).

3.3. Survivorship and ingestion of microplastics

The survival rates of *T. tridentatus* were 80 %, 90 %, 70 % and 30 % in the 100-day treatments of control, nylon, PMMA and PET, respectively (Fig. 6a), which were equivalent to 94.9, 94.8, 95.4 and 85.2 days of survival on average (Fig. 6b). The survival probability of *T. tridentatus* was found to be significantly different among treatments in Kaplan-Meir survival analysis ($\chi^2 = 17.906$, $p = 0.005$; Fig. 6a). Subsequent pairwise comparisons using log-rank test revealed the lowest survivorship of *T. tridentatus* exposed to PET, compared to the treatments of control ($p = 0.001$), nylon ($p = 0.006$) and PMMA ($p < 0.001$). However, significant difference was not detected in the number of survival days using one-way ANOVA ($F_{3,36} = 2.583$, $p = 0.068$; Fig. 6b).

The body burden levels of microplastics were assessed in all live and dead *T. tridentatus* after the 100-day experiment using Raman spectroscopy. The mean numbers were determined to be 7.80 particles of nylon, 2.50 particles of PMMA and 1.10 particles of PET per individual of *T. tridentatus* in the respective treatments (Table 2). These results confirmed ingestion of microplastics by *T. tridentatus* during the experiment.

4. Discussion

Microplastics have formed a major threat to marine life. The impacts of microplastics on model organisms such as fish and bivalves have been extensively studied, but much less attention has been given to horseshoe crabs and other benthic organisms. In this study, juvenile *T. tridentatus*, an endangered species of horseshoe crabs, was exposed for 100 days to three types of high-density microplastics under environmentally realistic conditions. The juveniles showed compromised growth and behaviour in the presence of microplastics, among which PET particles appeared to be more harmful that resulted in higher mortality of *T. tridentatus*.

4.1. Microplastics impairing growth and behaviour

Compared to the positive growth of *T. tridentatus* in the control, negative growth rates were found in the three treatments of microplastics, although the decrease was only statistically significant in prosomal width of the juveniles exposed to PMMA (Fig. 3; Table 1). *T. tridentatus* did not moult during the 100-day experimental period, which was not unexpected, since the moulting interval of *T. tridentatus* could be as long as 13 months under laboratory conditions (Chen et al., 2010). As for the behavioural changes, reduced locomotion of *T. tridentatus* was observed in the treatments of microplastics, and the changes were significant upon exposure to nylon and PET (Fig. 5a). A lower extent of burrowing by *T. tridentatus* across all treatments was also detected on day 100, and this time effect could be largely driven by PET (Fig. 5b). *T. tridentatus* was confirmed to have ingested nylon, PMMA and PET particles in the respective treatments (Table 2).

Ingested microplastics were unlikely to be digestible but could physically damage or obstruct the digestive tract of marine organisms (Wright et al., 2013). Accumulation of microplastics in the digestive tract could disrupt the enzymatic digestion process and result in false food satiation, and thus hampered appetite and energy acquisition (Oliveira et al., 2018; Romano et al., 2018). In this connection, ingestion

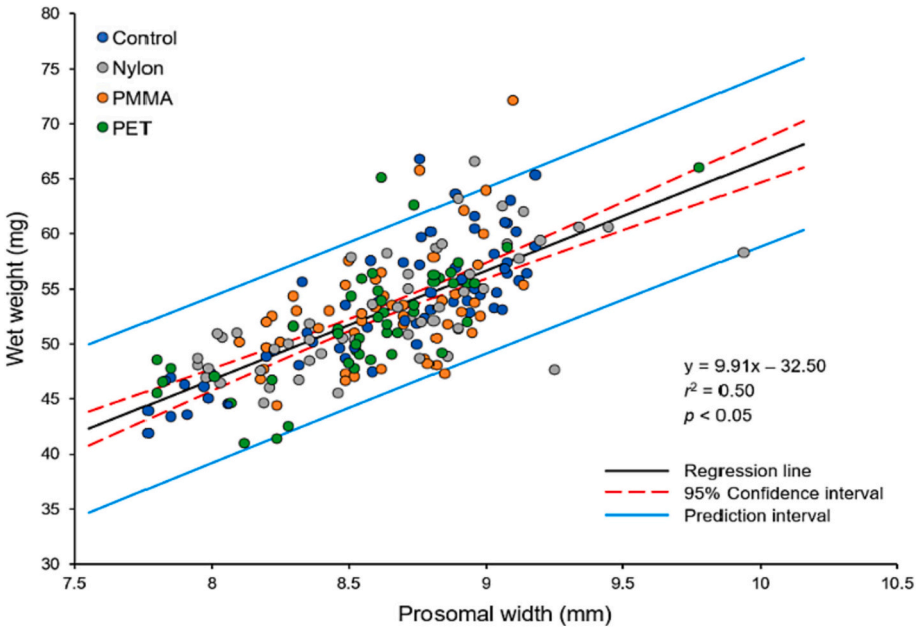

Fig. 4. Linear relationship between wet weight and prosomal width of *Tachypleus tridentatus* across the four treatments reported in Fig. 3. Measurements were taken on live *T. tridentatus* at all sampling time points, leading to 216 pairs of data ($r^2 = 0.500$, $p < 0.001$).

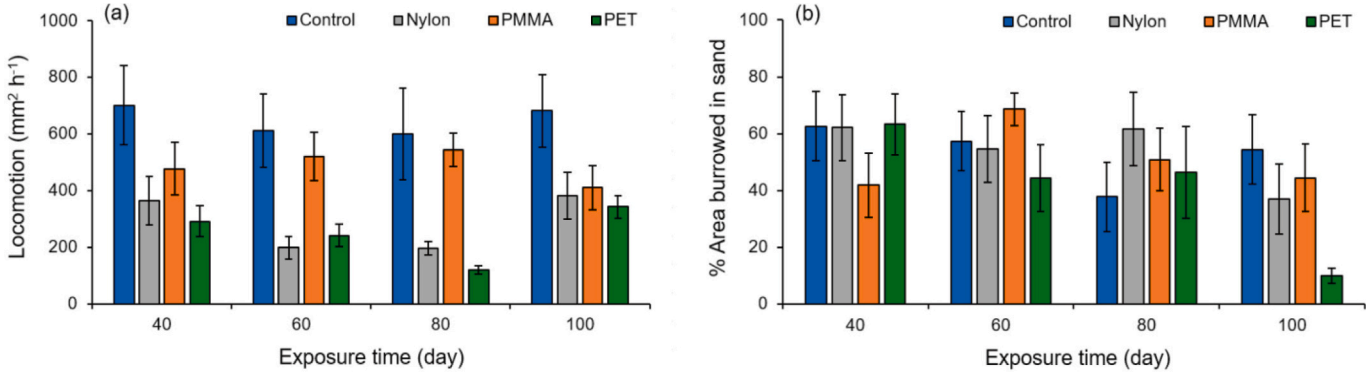

Fig. 5. Behavioural responses of *Tachypleus tridentatus* in terms of (a) locomotion, i.e., the size of crawling trail made on sand per hour, and (b) the extent of burrowing, i.e., the size proportion of its top area burrowed in sand, in the four treatments as in Fig. 3 (mean ± SE). The replicate number of *T. tridentatus* started at 10 per treatment but decreased towards day 100 due to mortality (see Fig. 6). Statistical results are provided in Table 1.

of microplastics was associated with reduced diet diversity and growth of the rocky shore crab *Pachygrapsus transversus* (de Barros et al., 2020). Decreased feeding and absorption efficiencies, accompanied by increased rates of respiration and excretion, were also reported in the Manila clam *Ruditapes philippinarum* that ingested microplastics and showed a diminished scope for growth (Jiang et al., 2022). Likewise, shell growth of the Pacific oyster *Crassostrea gigas* was substantially reduced upon exposure to microplastics (Bringer et al., 2021). In line with these findings, *T. tridentatus* with ingested microplastics might have suffered insufficient energy for growth and became less active, an outcome which might compromise their ability to forage for prey and escape from predators. In this regard, the nursery grounds with a high level of plastic pollution could possibly have additional mortality of juvenile horseshoe crabs due to predation, apart from the toxicity of microplastics.

4.2. Microplastics of PET leading to higher mortality

Survival of *T. tridentatus* was observed the lowest in the PET treatment (Fig. 6). From these results, the toxicity of PET appeared to be higher than the other tested polymers on horseshoe crabs. Likewise, microplastics of PET were found to be harmful to other aquatic life, e.g., inducing oxidative stress and lipid peroxidation in *R. philippinarum* (Parolini et al., 2020). PET exhibited greater effects compared to polyethylene on *C. gigas* in terms of inhibition of lipid metabolism and lesions in the gills and digestive gland (Teng et al., 2021), and compared to nylon, polystyrene and polylactic acid on the freshwater mussel *Dreissena bugensis* regarding its activities of antioxidant enzymes and proteomic alterations (Brehm et al., 2022). Notably, the PET particles used by Brehm et al. (2022) were made from recycled drinking bottles and confirmed to contain a more diverse range of additives compared to the other tested polymers. Some of these additives such as anthranilamide, anthranilonitrile and butylated hydroxytoluene could be toxic to aquatic organisms (Brehm et al., 2022). PET drinking bottles and packaging materials were also found to be a source of estrogen-like endocrine disrupting compounds (Wagner and Oehlmann, 2011). In the present study, the PET particles employed in the experiment were made from used PET containers (Fig. 2a), which possibly contained some of these chemicals that might partly explain the toxicity of PET on *T. tridentatus*, but further studies are required to prove this speculation (Hermabessiere et al., 2017; Gunaalan et al., 2020; Delaeter et al., 2022). It should also be noted that the microplastics used in our experiment had not been

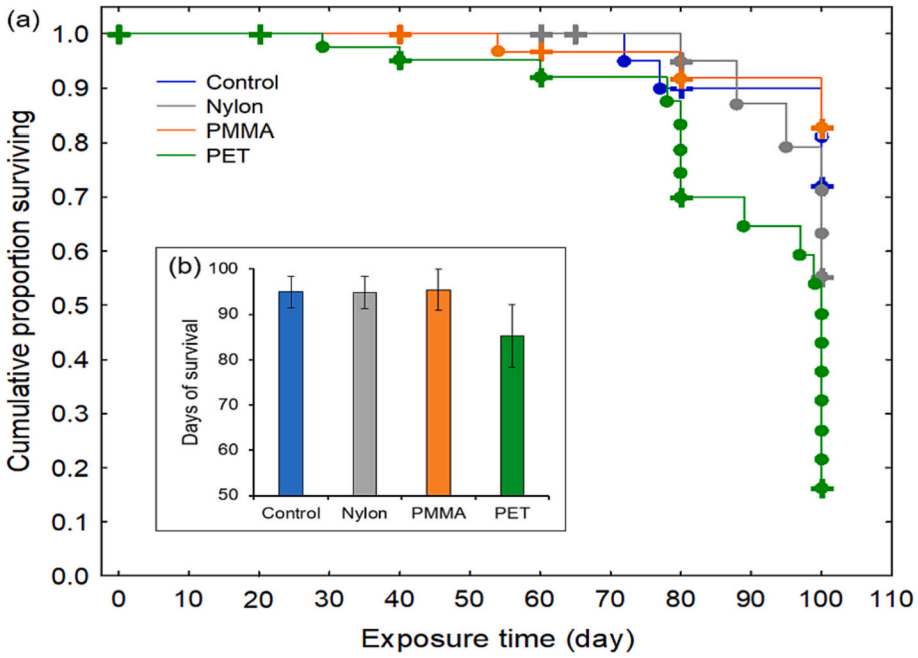

Fig. 6. (a) Cumulative survival curves and (b) days of survival (mean ± SE) of *Tachypleus tridentatus* upon exposure to polyamine 6,6 (nylon), polymethyl methacrylate (PMMA) and polyethylene terephthalate (PET) over 100 days compared to the control. Significance was detected in (a) using Kaplan-Meir survival analysis ($\chi^2 = 17.906$, $p < 0.001$), where *T. tridentatus* exposed to PET showed the significantly lowest survival probability (log-rank test, $p < 0.01$). Crosses are the censored values (alive) and closed circles are the complete response status (dead) recorded during the 100 days of exposure. Significance was not detected in (b) using one-way ANOVA on aligned-rank transformed data ($F_{3,36} = 2.583$, $p = 0.068$).

Table 2
Quantities of microplastics determined in *Tachypleus tridentatus* after 100 days of exposure to polyamine 6,6 (nylon), polymethyl methacrylate (PMMA) and polyethylene terephthalate (PET) compared to the control. Microplastics were extracted and identified from all live and dead juveniles (mean ± SE, n = 10).

Treatment	Ingested microplastics (number per individual)		
	Nylon	PMMA	PET
Control (8 live, 2 dead)	0	0	0
Nylon (7 live, 3 dead)	7.80 ± 2.82	0	0
PMMA (9 live, 1 dead)	0	2.50 ± 0.95	0
PET (3 live, 7 dead)	0	0	1.10 ± 0.38

aged, and therefore the particle surface chemistry and release of chemicals might be different from those occurring in the environment (Ray and Cooney, 2018; Royer et al., 2018; Lomonaco et al., 2020; La Nasa et al., 2021). Moreover, our microplastics made by cryogenic grinding might not have the same physical and chemical properties compared to the plastic particles collected from the field, e.g., in terms of shapes and crystallinity (Li et al., 2022b). Nevertheless, with these limitations in mind, the present findings provided evidence for the negative effects of microplastics on juvenile *T. tridentatus*.

Overall, this study was the first to systematically investigate the ecophysiological impacts of high-density microplastics on horseshoe crabs. In general, the growth and behavioural responses of *T. tridentatus* were compromised by microplastics, among which PET was found to be more harmful that resulted in higher mortality compared to nylon and PMMA. For future research, additional experiments are recommended to examine the presence of additives and potential leachates in these microplastics, in order to identify the causes of toxicity. The use of cellular biomarkers, e.g., on oxidative stress and immune responses (Arif et al., 2022), should also be considered to address the mechanisms associated with the observed ecophysiological changes. Notably, our results about the lethality of PET to *T. tridentatus* would raise serious ecological concerns, since PET was found to be the most abundant type of high-density microplastics in some nursery grounds of horseshoe crabs, e.g., in South China (Lo et al., 2018). Bioaccumulation of PET, along with other microplastics, was also confirmed in juvenile *T. tridentatus* in the wild (Wang et al., 2022). Apart from the high-density microplastics investigated here, we acknowledge that low-density ones, such as polyethylene, polypropylene and polystyrene, also tend to sink over a longer term as a result of photooxidation and biofouling, and therefore their health risks to benthic organisms should not be ignored. All these findings highlight the need for effective waste management in the habitats of horseshoe crabs so as to safeguard these ecologically important living fossils.

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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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