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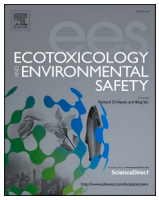



Microplastics increases the heat tolerance of *Daphnia magna* under global warming via hormetic effects

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ABSTRACT

The ecological risk assessment of microplastics under global warming receives increasing attention. Yet, such studies mostly focused on increased mean temperatures (MT), ignoring another key component of global warming, namely daily temperature fluctuations (DTF). Moreover, we know next to nothing about the combined effects of multigenerational exposure to microplastics and warming. In this study, *Daphnia magna* was exposed to an environmentally relevant concentration of polystyrene microplastics (5 μg L^{-1}) under six thermal conditions (MT: 20 ℃, 24 ℃; DTF: 0 ℃, 5 ℃, 10 ℃) over two generations to investigate the interactive effects of microplastics and global warming. Results showed that microplastics had no effects on *Daphnia* at standard thermal conditions (constant 20 °C). Yet, microplastics increased the fecundity, heat tolerance, amount of energy storage, net energy budget and cytochrome P450 activity, and decreased the energy consumption when tested under an increased MT or DTF, indicating a hormesis effect induced by microplastics under warming. The unexpected increase in heat tolerance upon exposure to microplastics could be partly explained by the reduced energy consumption and/or increased energy availability. Overall, the present study highlighted the importance of including DTF and multigenerational exposure to improve the ecological risk assessment of microplastics under global warming.

1. Introduction

The ecological risk assessment of microplastics has attracted great attention as they are ubiquitous and hard to degrade in natural environments, and thought to have negative effects on organisms (Guilhermino et al., 2021). Most studies, however, exposed organisms to unrealistically high concentrations of microplastics, potentially overestimating the effects of microplastics at environmentally relevant concentrations. The few studies exploring the exposure to microplastics at environmentally relevant concentrations mainly showed little or no effects on organisms (Guven et al., 2018; Revel et al., 2020). Yet, recent studies demonstrated that multigenerational long-term exposure to microplastics can cause stronger biological effects even at low concentrations (Liu et al., 2020; Chang et al., 2022). For instance, two generations of exposure to polystyrene (5 μg L^{-1}) decreased the heartbeat rate of *D. magna* (Chang et al., 2022), and three generations of exposure to polystyrene (1 μg L^{-1}) increased the growth rate and reproduction of *D. pulex* (Liu et al., 2020). Given their long persistence in natural systems, multigenerational exposure to environmentally relevant concentrations is therefore essential for assessing the real effects of microplastics.

The effects of toxicants are influenced by other environmental stressors, of which temperature is a critical one to consider given our warming planet (Noyes et al., 2009; Dinh et al., 2023). Indeed, the toxicity of many contaminants is higher under warming, which is known as the "climate-induced toxicant sensitivity" (CITS) concept (Noyes and Lema, 2015). Most studies on the effects of warming on the toxicity of contaminants focused on traditional contaminants (e.g. pesticides: Verheyen et al., 2019a; metals: Zhang et al., 2018), whereas only recently attention went to emerging contaminants such as microplastics. The few studies on this typically investigated the effect of a higher mean temperature (MT) on microplastics' toxicity and resulted in mixed findings (Jaikumar et al., 2018; Sadler et al., 2019; Lyu et al., 2021). Sadler et al. (2019) found that an elevated temperature (18 ℃ vs 24 ℃)

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did not affect the toxicity of microplastics, while Jaikumar et al. (2018) reported that the acute sensitivity of *Daphnia* to microplastics increased sharply with temperature (18 ℃, 22 ℃ and 26 ℃), and similarly Lyu et al. (2021) reported that the toxicity of microplastics in *Daphnia* was more prominent at higher mean temperatures (15 ℃, 20 ℃ and 30 ℃). While these studies provided valuable insights, they did not consider another key factor of global warming: the increase in daily temperature fluctuations (DTF). The increase in DTF may be more harmful than the increase in MT, moreover the effect of DTF may be larger at a high MT because more extreme temperatures, and associated costs, will be experienced during a DTF cycle (Colinet et al., 2015). However, we currently know next to nothing about how DTF together with an increase in MT affects the toxicity of microplastics (but see Chang et al., 2022). Note that nearly all tests on the toxicity of microplastics used constant temperatures, thereby ignoring the widespread DTF in current thermal regimes (Colinet et al., 2015). Therefore, including DTF is of crucial importance to increase realism in ecological risk assessment of microplastics.

The interplay between ecotoxicology and global change biology can go in both directions. Warming cannot only alter the toxicity of toxicants, toxicants can also affect the sensitivity of organisms to warming that is known as the "toxicant-induced climate change sensitivity" (TICS) (Noyes et al., 2009; Verheyen et al., 2019a). Previous studies demonstrated that traditional toxicants in general decreased the heat tolerance of aquatic organisms (e.g. Patra et al., 2007; Noyes et al., 2009; Verheyen et al., 2019a). For instance, the critical thermal maximum (CT_{max}, the high temperature whereby animals lose locomotor abilities) was reduced by exposure to the metals cadmium and chromium in marine prawn *Macrobrachium rosenbergii* (Rosas, 1993), and by exposure to the pesticides endosulfan and chlorpyrifos in different freshwater fish species (Patra et al., 2007). Evaluating the effects of toxicants on the heat tolerance of organisms is crucial to understand the fate of natural populations under global warming in the presence of toxicants (Verheyen et al., 2019a). Yet, whether and how emerging contaminants like microplastics affect the heat tolerance of organisms remains unknown.

There is increasing attention for energy budgets in ecotoxicology as these may explain effects on life history traits (Verheyen and Stoks, 2020) and are thought to be the underlying drivers of interactions between stressors (Sokolova, 2013). Stressors such as toxicants and warming may indeed negatively affect the balance between energy gains and costs, thereby impairing the net energy budget. They may reduce energy availability (e.g. because of reduced feeding), as well as increase energy consumption (e.g. because of costly defense mechanisms) (Sokolova, 2013). For example, it was shown that the negative effect on the net energy budget contributed to increased toxicity of the pesticide chlorpyrifos under warming (Verheyen and Stoks, 2020). Effects of microplastics on energy budgets are still not fully understood. In a recent example study, Shang et al. (2021) showed that microplastics suppressed the net energy budget of the mussel *Mytilus coruscus*. Yet, how microplastics and warming jointly shape the net energy budget remains to be studied.

Taking into account the issues raised above, in this study, we investigated the single and combined effects of warming (an increase in MT) and DTF on the sensitivity to microplastics (CITS concept), and vice versa, how exposure to microplastics changes the sensitivity to warming (TICS concept). To increase realism, we imposed a two-generation exposure to an environmentally relevant concentration of polystyrene, a common source of microplastics pollution. We studied this in the water flea *Daphnia magna* as it plays a key role in aquatic ecosystems. We here extend the study by Chang et al. (2022) that documented thermal evolution of effects of MT, DTF and microplastics on life history. To address the CITS concept, we here report on fecundity, an important fitness component of *Daphnia,* while to address the TICS concept we quantified CT_{max} as measure of heat tolerance. To start exploring insights in the mechanisms underlying how microplastics and warming may affect fecundity and CT_{max}, we determined net energy budgets. Moreover, to get further insights in the toxicity effects of microplastics under warming we also quantified the activity of cytochrome P450, an important detoxification enzyme (Ding et al., 2018). Overall, our study design allowed to investigate the interactive effects of an increase in MT, DTF and microplastics, therefore providing novel insights for the CITS and TICS concepts for this group of emerging contaminants, and benefiting a more realistic ecological risk assessment of microplastics at environmentally relevant concentration under global warming.

2. Materials and methods

2.1. Study organisms

Three *Daphnia* populations were sampled from shallow water bodies in the high-latitude regions of the species in Sweden (Lake Bysjön), Norway (Pond Asklund), and Denmark (Lake Ring). From each population, one *Daphnia* clone was cultured separately in glass jars filled with dechlorinated tap water under standard conditions (20 ± 1 ℃, light: dark period = 16:8). The culture medium was refreshed every two days, and *Daphnia* was daily fed with the green algae *Acutodesmus obliquus* (1×10^5 cells mL^{-1}). To start the experiment, neonates of the third brood neonates were used as the first generation to minimize the maternal effects.

Shallow water bodies inhabited by *Daphnia* in this region currently experience a mean temperature of 20 ℃ and a low DTF level (maximum 5 ℃, i.e. daily temperature fluctuations between 17.5 ℃ and 22.5 ℃) that are predicted to increase to a mean temperature of 24 ℃ and a higher DTF level (maximum 10 ℃; i.e. daily temperature fluctuations between 19 ℃ and 29 ℃) by the end of this century under scenario RCP (Representative Concentration Pathway) scenario 8.5 of the IPCC (Intergovernmental Panel on Climate Change) (IPCC, 2013). The current thermal regime in the shallow water bodies in the study region was based on temperature data obtained from the Flake model (Simmons et al., 2007) and confirmed by in situ measurements with data loggers (Debecker and Stoks, 2019). The future DTF under scenario RCP 8.5 was determined as in Verheyen and Stoks (2019b) based on daily maximum and minimum temperatures predicted for 2080 using BCC_CSM 1.1 (Beijing Climate Center Climate System Model 1.1). While the IPCC scenarios predict future air temperatures, these are tightly linked to water temperatures in shallow bodies. It has been modelled that climate change-induced increases in air temperature from 1.5 to 4.3 ℃ will correspond with slightly lower increases in water temperatures (from 1.3 to 3.7 ℃) in shallow ponds (Ali et al., 2016). So the future scenario of 24 ℃ with 10 ℃ DTF in the studied Swedish region is likely to occur by 2100 based on IPCC scenario RCP8.5.

2.2. Polystyrene microplastics

The polystyrene microplastics (1 % w/v, 10 mL; with as additive 2.5 % sodium azide) used in this experiment were purchased from BaseLine Chromtech Research Centre, Tianjin, China. The dialysis of polystyrene microplastics was performed using a dialysis bag (MWCO 1000 Da) in a 2 L beaker which was changed five times over three days to minimize the interference of the additives. The average size of the polystyrene microplastics particles (mean ± SE = 154.1 ± 2.9 nm) after dialysis was determined by DLS (dynamic light scattering) (Zetasizer Nano ZS 90, Malvern Instrument, UK). This fits the lower size range of microplastics according to the definition by Wallace et al. (2016). The shape of the polystyrene microplastics (microspheres, Fig. S1) was observed by scanning electron microscopy (Quanta 250 FEG, FEI, USA). Dialyzed polystyrene microplastics were diluted to make a stock solution (100 mg L^{-1}) with MilliQ water, and the stock solution was further diluted to 5 μg L^{-1} to obtain the exposure concentration used in the experiment. The stock solution was stored at 4 °C under darkness, and ultrasonicated for 30 min to keep particles scattered before use each time.

2.3. Experimental design

A full factorial experiment was conducted with 12 treatment combinations: two microplastics treatments (absence vs presence) × two mean temperatures (MT: 20 ℃ vs 24 ℃) × three daily temperature fluctuations (DTF: constant 0 ℃, low 5 ℃, high 10 ℃) to investigate the single and interactive effects of microplastics, MT and DTF on *Daphnia*. A low exposure concentration of polystyrene microplastics (5 μg L^{-1}, ~ 2.7×10^6 particles mL^{-1}) was used as this is within the reported range of environmentally relevant concentrations (Wang et al., 2019; Liu et al., 2020; Chang et al., 2022) and the predictions by Lenz et al. (2016). There were three replicates per clone for each treatment combination resulting in a total of 108 experimental units. In the first generation (F1), each replicate started with 12 neonates (< 48 h) that were reared in a 730 mL glass jar containing 500 mL cultured medium. *Daphnia* were fed enough food (1×10^5 cells mL^{-1} *Acutodesmus obliquus*) to avoid food shortage (Brans et al., 2017), and the culture medium was changed every two days. The neonates (< 48 h) of the third brood of the first generation (F1) were used to start the second generation (F2). In the second generation, again 12 neonates were tested per jar in each replicate and these were exposed to the same conditions as their mothers experienced in the first generation. Experimental water temperature data were recorded with an Elitech (GSP-6 thermometer) data logger (Fig. S2). The experiment ended when all *Daphnia* in a jar had released their second clutch (the scheme of the exposure design is shown in Fig. S3).

2.4. Response variables

All response variables were measured in the second generation. The fecundity was quantified as the total number of offspring produced by each mother *Daphnia* in the first and second clutches. At the end of the experiment, on a subset of three *Daphnia* per vial the heat tolerance was measured. In addition, three *Daphnia* (F2) per jar were flash frozen in liquid nitrogen and stored in a − 80 ℃ refrigerator to measure physiological variables.

The critical thermal maximum (CT_{max}), the temperature where *Daphnia* loses its locomotor capacity and faints, was measured following the protocol by Brans et al. (2017). Briefly, one individual was transferred to a 1.5 mL Eppendorf tube containing dechlorinated tap water. The tubes were then randomly placed in a thermal heater (Huxi HX-20G2, Shanghai) at 20 ℃ and heated at a constant heating rate of 1 ℃ per 30 s *Daphnia* activity was continuously observed and CT_{max} was recorded as the temperature where *Daphnia* fainted and sank to the bottom of the tubes. After fainting, the *Daphnia* were immediately transferred to water at 20 ℃ to check their recovery; all tested individuals recovered. The three CT_{max} values obtained per jar were averaged for later statistical analysis.

To quantify physiological variables, three frozen *Daphnia* per clone were first pooled and then were homogenized in 90 μL phosphate-buffered saline (PBS, pH = 7.3) on ice and then centrifuged for 5 min at 13,000 rpm at 4 ℃. The supernatant was used to measure the activity of cytochrome P450 and the variables associated with the energy budget. All assays were done spectrophotometrically using an Infinite 200 pro plate reader (Austria).

The cytochrome P450 was considered an essential detoxification enzyme and used to assess the toxicity effects of contaminants on organisms (Uno et al., 2012; Verheyen and Stoks, 2019a). The cytochrome P450 activity was quantified with an ELISA (enzyme linked immunosorbent assay) kit based on the double antibody sandwich method according to the kit protocol. Briefly, 40 μL of sample dilution was added to the antibody-coated 96-well plate, followed by 10 μL of supernatant. Next, 100 μL of enzyme reagent was added to each well and incubated at 37 ℃ for 60 min. After incubation, the liquid was discarded and drained, then each well was filled with washing solution and left to stand for 30 s and discarded, and that was repeated 5 times and tapped dry. Followed by adding 50 μL of chromogen A and then 50 μL of chromogen B to each well and incubating at 37 ℃ for 15 min under darkness. Lastly, 50 μL of termination solution was added to each well to stop the chromogenic development. The absorbance of the supernatant was measured at 450 nm, and then converted into cytochrome P450 activity based the absorbance of standard samples provided by the cytochrome P450 ELISA kit (Jingmei biotechnology).

To estimate effects on the net energy budget we quantified the available energy reserves (Ea), and the energy consumption (Ec) and integrated these in the cellular energy allocation (CEA) using the equation CEA = Ea – Ec (Samanta et al., 2020). The CEA is an important indicator that not only estimates the net energy budget, but can also be used as a biomarker for toxicant stress (De Coen and Janssen, 2003). The available energy reserves were estimated by the sum of the amounts of energy stored in lipids, proteins and carbohydrates that were obtained by quantifying body contents of these storage molecules and then converting these to energy contents using their energetic equivalents (39,500 mJ per mg lipid, 24,000 mJ per mg protein, and 17,500 mJ per mg carbohydrate) (De Coen and Janssen, 2003). The lipid content was measured following Zhang et al. (2016). Briefly, 8 μL supernatant and 56 μL sulfuric acid were firstly mixed in a fat-free, acetone-rinsed glass vial, where after the vial was heated at 150 ℃ for 20 min. After cooling to room temperature, 350 μL MilliQ water was added and the absorbance measured at 340 nm. Absorbances were converted into fat contents based on the standard curve of glyceryl tripalmitate. The protein was measured using the Protein Assay Kit based on the Bradford method. 10 μL supernatant was dyed with 300 μL G250 Dyeing solution, and the absorbance of the solution was measured at 595 nm. The absorbance was converted into protein content based on the absorbance curve of the protein standard sample provided by Protein Assay Kit. The content of carbohydrate was measured by the glucose kit of Beyotime company. To transform all glycogen into glucose, 5 μL supernatant was combined with 2 μL amyloglucosidase and 13 μL MilliQ water and this mixture was incubated for 30 min at 37 ℃. Then, we mixed 3 μL of this with the glucose assay reagent with A, and the new mixture was incubated for 15 min at 37 ℃. Thereafter, the absorbance at 505 nm was measured and converted into the carbohydrate content based on the absorbance curve of the glucose standard sample provided by the glucose Assay Kit.

The amount of energy consumed (Ec) was estimated using the activity of the electron transport system (ETS) based on the stoichiometric relationship that 1 μmol O_2 is consumed for each 2 μmol formazan formed in the ETS system (De Coen and Janssen, 2003). ETS activity was measured following Verheyen and Stoks (2019b). Briefly, 5 μL supernatant and 15 μL buffered substrate solution (0.13 M Tris-HCl, 0.3 % Triton X-100, 1.7 mM NADH, 250 μm NADPH, pH = 8.5) were mixed in a 384-well plate, and then 10 μL iodonitrotetrazolium (INT, 8 mM p-iodonitrotetrazolium) was added. The increase in absorbance of formazan at 490 nm every 30 s was measured at 20 ℃ during 5 min. The amount of formazan formed was calculated by using a molecular extinction coefficient of 15.9 mM^{-1} cm^{-1}. The quantity of oxygen consumed was transformed into energetic equivalents by using the oxyenthalpic equivalents (484 kJ mol^{-1} O^2).

2.5. Statistical analyses

We used generalized liner models (GLMs) to analyze the single and combined effects of microplastics, mean temperature and daily temperature fluctuations on the response variables. In addition, we specifically investigated whether the environmentally relevant microplastics concentration had effects at the standard thermal ecotox test conditions (20 °C MT, 0 °C DTF) using a priori contrast tests. Finally, to investigate in a powerful way how the effects of microplastics were affected by DTF, separate GLMs were conducted at each mean temperature. The GLMs assumptions of normality and homoscedasticity were confirmed by using Kolmogorov-Smirnov tests and Levene's tests, respectively. Fisher's LSD post-hoc tests were conducted to test for differences among

treatment combinations in case of significant interactions. P values < 0.05 were considered significant, while P values < 0.10 were considered a trend. All analyses were performed in IBSS SPSS statistics 26, and graphs were plotted with Origin pro 2018 C.

3. Results

3.1. Fecundity

No mortality of *Daphnia* was found during the experiment. The effects of microplastics depended on the mean temperature (MP × MT: Table S1, Fig. 1). While microplastics had no effects on fecundity at 24 ℃ MT (LSD: $P = 0.868$), at 20 ℃ MT exposure to microplastics increased the fecundity only at 10 ℃ DTF (LSD: $P < 0.01$). Yet, the focused a priori test showed that MP did not affect fecundity at the standard thermal test conditions (20 ℃ MT, 0 ℃ DTF) ($P = 0.924$, Table S2, Fig. 1). The fecundity decreased when *Daphnia* were reared at 24 ℃ MT than at 20 ℃ MT (main effect of MT, Table S1, Fig. 1). At 20 °C MT, the fecundity was increased at 5 ℃ DTF (LSD: $P < 0.01$) but not at 10 ℃ DTF (LSD: $P = 0.328$) compared to 0 °C DTF (MT × DTF interaction, Table S1, Fig. 1).

3.2. Heat tolerance (CT_{max})

Overall, microplastics exposure increased CT_{max} (main effect MP, Table S1, Fig. 2). Yet, the focused a priori test showed that MP did not affect CT_{max} at the standard thermal test conditions (20 ℃ MT, 0 ℃ DTF, Table S2, Fig. 2). The effects of microplastics on the heat tolerance depended on MT and DTF. At a mean temperature of 20 ℃, exposure to microplastics increased the heat tolerance of *Daphnia* only at 10 ℃ DTF (LSD: $P = 0.044$, Fig. 2). Meanwhile, at 24 ℃ exposure to microplastics increased the heat tolerance of *Daphnia* at 0 ℃ and 5 ℃ DTF (LSD: both $P < 0.01$, Fig. 2). Additionally, *Daphnia* generally had a higher heat tolerance when reared at 24 ℃ than at 20 ℃ (main effect of MT: Table S1, Fig. 2). Moreover, at 24 ℃, the heat tolerance increased at 5 ℃ and 10 ℃ DTF compared to 0 ℃ DTF (MT × DTF, LSD: both $P < 0.01$, Table S1, Fig. 2).

3.3. Energy metabolism

Exposure to microplastics had no effects on the energy available (Ea), energy consumed (Ec) and the net energy budget (cellular energy allocation, CEA) at standard thermal conditions (20 ℃ MT, 0 ℃ DTF, Table S2, Fig. 3). *Daphnia* had a higher Ea when reared at 20 ℃ than at 24 ℃ (main effect of MT, Table S1, Fig. 3a). Notably, separate GLMs at each mean temperature showed the effect of microplastics on Ea was affected by DTF: at a mean temperature of 20 ℃, microplastics increased the Ea of *Daphnia,* yet only at 10 ℃ DTF ($P = 0.038$, Table S3, Fig. 3a), while at a mean temperature of 24 ℃, DTF did not change the microplastics effect on Ea ($P = 0.514$, Table S4, Fig. 3a).

Exposure to microplastics decreased the Ec, yet only at 24 °C ($P < 0.01$) resulting in a significant MP x MT interaction (Table S1, Fig. 3b). The effect of microplastics on Ec also depended on DTF (MP × DTF: Table S1, Fig. 3b). Exposure to microplastics decreased the Ec of *Daphnia* only at 0 ℃ and 5 ℃ DTF (LSD: both $P < 0.01$, Fig. 3b). Additionally, the effects of DTF on *Daphnia* depended on MT resulting in a DTF × MT interaction (Table S1, Fig. 3b): at a mean temperature of 20 ℃, a lower Ec was observed only at 5 ℃ DTF (LSD: $P < 0.01$), while at 24 ℃, a lower Ec was observed only at 10 ℃ DTF (LSD: $P < 0.01$).

Daphnia had in general a higher CEA when reared at 5 ℃ DTF than at 0 ℃ DTF (LSD: $P = 0.011$, Table S1, Fig. 3c). Notably, separate GLMs per MT showed that there was a trend for a MP x DTF interaction for CEA only at 20 ℃ ($P = 0.052$, Table S3, Fig. 3c): microplastics significantly increased the CEA of *Daphnia* only at 10 ℃ DTF under 20 ℃ MT (LSD: $P = 0.025$, Fig. 3c). Besides, Pearson correlation analysis showed a significant relationship between CEA and the fecundity ($r = 0.524$, $P = 0.001$).

3.4. Cytochrome P450

In general, microplastics increased the cytochrome P450 activity (main effect of MP, Table S1, Fig. 4). While microplastics did not significantly change cytochrome P450 activity at standard thermal conditions (20 ℃ MT, 0 ℃ DTF, Table S2). Notably, separate GLMs showed that there was a trend for a MP x DTF interaction at 24 ℃ ($P = 0.051$, Table S4, Fig. 4): microplastics increased the cytochrome P450 activity of *Daphnia* when reared at 5 ℃ (LSD: $P = 0.033$) and 10 ℃ DTF (LSD: $P = 0.015$) under the MT of 24 ℃.

4. Discussion

Our results showed no effects of the here tested environmentally realistic concentration of microplastics at the standard thermal regime (mean of 20 °C and 5 °C DTF). Yet, when combined with the increase in mean temperature (MT) and/or daily temperature fluctuations (DTF) the exposure to microplastics had beneficial effects on *Daphnia* indicating an hormetic response. Moreover, microplastics increased the heat tolerance of *Daphnia*, partly by affecting the energy budget, thereby improving our mechanistic understanding of the interaction of between

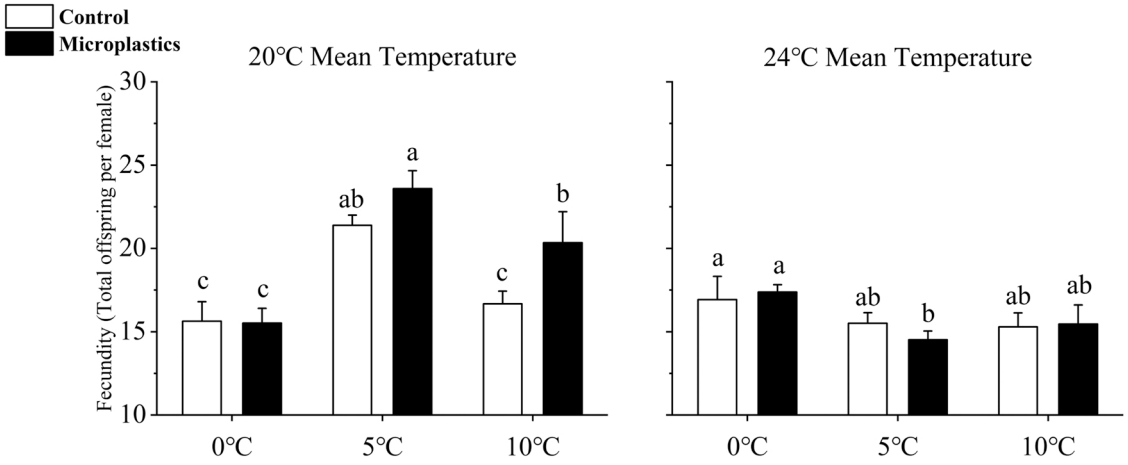

Fig. 1. Effects of microplastics (MP) on the fecundity (Total offspring per female) of *Daphnia magna* as a function of mean temperature (MT) and daily temperature fluctuations (DTF). Given are the means + 1 standard error (SE, n = 3 replicates per treatment). Black and white bar represent microplastics and control treatment respectively. Lowercase letters indicate significant differences between treatments at the same mean temperature.

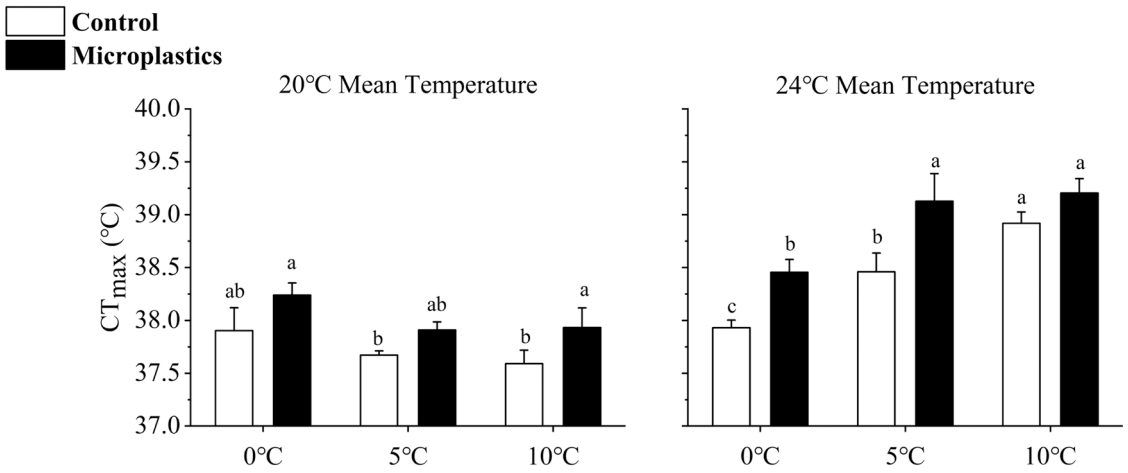

Fig. 2. Effects of microplastics (MP) on the critical thermal maximum (CT_{max}) of *Daphnia magna* as a function of mean temperature (MT) and daily temperature fluctuations (DTF). Given are means + 1 standard error (SE, n = 3 replicates per treatment). Black and white bar represent microplastics and control treatment respectively. Lowercase letters indicate significant differences between treatments in the same mean temperature.

microplastics and warming.

4.1. Effects of microplastics depended on warming and/or DTF

At standard toxicological thermal conditions, none of the tested variables of *Daphnia* were affected when exposed to an environmentally relevant concentration of microplastics. Nevertheless, the microplastics did affect the *Daphnia* but this strongly depended on warming and/or DTF. This deviates from the CITS concept that climate change related stressors can increase the toxicity of contaminants (Noyes and Lema, 2015). Indeed, we found that under warming or higher DTF microplastics could increase the fecundity, heat tolerance, Ea, CEA and cytochrome P450 activity, and decrease the Ec. Previous studies exploring the effects of microplastics and temperature only considered elevated mean temperatures. For instance, Lyu et al. (2021) reported that the toxicity of microplastics for *D. magna* was prominently enhanced when reared at 30 ℃ than at 20 ℃ and 15 ℃, and Jaikumar et al. (2018) found that the acute sensitivity of *D. magna* and *D. pulex* to microplastics increased sharply with elevated mean temperature, yet Sadler et al. (2019) did not observe an increased toxicity of microplastics on *D. magna* under an elevated mean temperature. The underlying mechanisms for warming to change the effects of microplastics are mostly likely related to energy trade-offs between defenses against thermal stress and against microplastics (Lyu et al., 2021). Energetic trade-offs, however, do not seem to explain in our study why MP only increased the fecundity at 20 ℃ but not at 24 ℃. Indeed, we did not detect a higher energy consumption or lower amount of available energy when *Daphnia* were exposed to microplastics at 24 ℃. One important difference with the study of Lyu et al. (2021) is that we did a multi-generational microplastics exposure while they only exposed *Daphnia* for a single generation to microplastics.

How effects of microplastics are shaped by DTF, another important thermal factor that is also predicted to increase under global warming, has been rarely studied (but see Chang et al., 2022). Here we confirmed the findings by Chang et al. (2022) that DTF can change the effects of microplastics in *Daphnia*. For example, in current study we showed that exposure to microplastics increased the fecundity, CT_{max}, Ea and CEA of *Daphnia* when reared at 10 ℃ DTF and a mean temperature of 20 ℃, but not at a constant temperature of 20 ℃ (0 ℃ DTF). Notably, microplastics also increased the cytochrome P450 enzyme activity after two generations of exposure to DTF (5 ℃ and 10 ℃) at the elevated mean temperature (24 ℃). This is consistent with previous studies suggesting cytochrome P450 to be involved in physiological pathways influenced by microplastics at high temperatures (Ding et al., 2018). Cytochrome P450 is a widely used biomarker for environmental contamination and involved in the detoxification of various xenobiotics (Uno et al., 2012). The mechanisms underlying the increased cytochrome P450 enzyme activity by microplastics is still unknown. It was hypothesized the higher cytochrome P450 enzyme activity was related to an adaptation period under microplastics exposure (Ding et al., 2018).

Most exposure experiments with microplastics were conducted at high concentrations and showed negative effects (Lenz et al., 2016; De Felice et al., 2019). For example, Bosker et al. (2019) found exposure to a concentration of 100,000 particles mL^{-1} to result in a 21 % reduction in total biomass of *D. magna*, and Schür et al. (2020) reported concentrations of 2000 and 10,000 particles mL^{-1} to reduce survival of *D. magna*. While informative, these studies are less relevant for the ecological risk assessment of microplastics. High concentrations of microplastics are expected to more often cause negative effects, whereas more environmentally realistic low concentrations of microplastics more likely induce no effects, or may even induce positive effects on organisms (Agathokleous et al., 2021). Studies have indeed shown that microplastics at low concentrations caused positive effects (Gambardella et al., 2018; Liu et al., 2020; Agathokleous et al., 2021; Chang et al., 2022). For instance, exposure to polystyrene microplastics (1 μg L^{-1}) for three generations significantly increased the body length and fecundity of *D. pulex* (Liu et al., 2020), and exposure to polystyrene particles (1 μg L^{-1}) for 48 h increased the swimming speed of neonates of the rotifer *Brachionus plicatilis* (Gambardella et al., 2018). These beneficial effects could be explained by hormesis whereby very low concentrations of microplastics may generate overcompensatory effects (Mattson, 2008). Our results added the novel insight that exposure to microplastics at environmentally relevant concentrations may cause beneficial effects (e.g., increased fecundity, CT_{max}, Ea, CEA and cytochrome P450) via hormesis but only under warming and/or DTF. Further studies with different types of microplastics, concentrations and test organisms are warranted to verify this conclusion.

4.2. Microplastics increased the heat tolerance by changing energy metabolism

When test organisms are reared at elevated temperatures, they typically develop a higher heat tolerance. For instance, in *D. magna* a higher CT_{max} has been shown when exposed to + 4 ℃ warming (Geerts et al., 2015). This was consistent with our results that *Daphnia* had a higher CT_{max} when reared at 24 ℃. Much less studies tested effects of DTF on CT_{max}. Verheyen et al. (2019a) found a tendency for the CT_{max} of larvae of the damselfly *Ischnura elegans* to increase with increasing DTF.

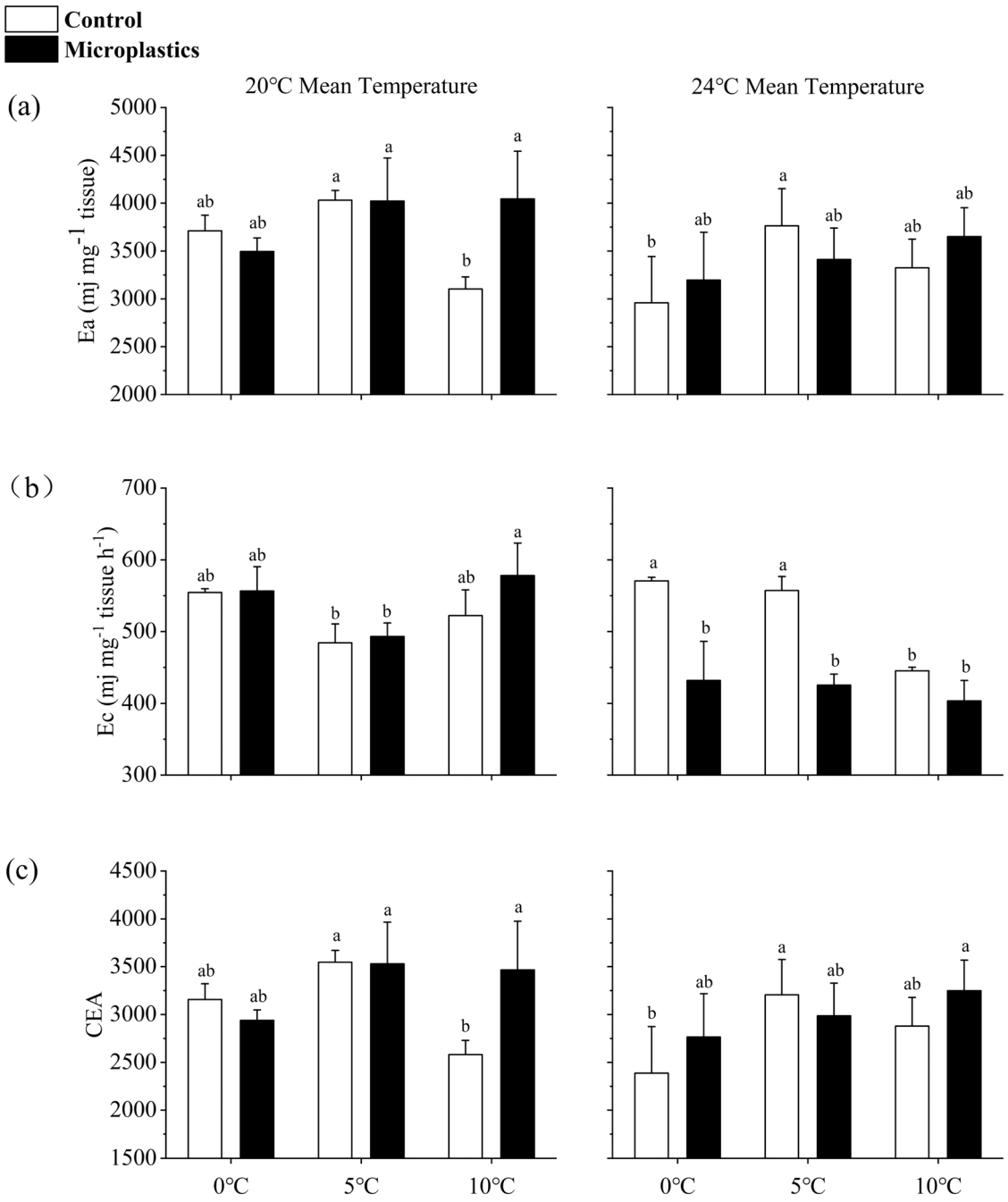

Fig. 3. Effects of microplastics (MP) on energy metabolism of *Daphnia magna* as a function of mean temperature (MT) and daily temperature fluctuations (DTF): (a) available energy reserves (Ea), (b) energy consumption (Ec), and (c) cellular energy allocation (CEA). Given are the means + 1 standard error (SE, n = 3 replicates per treatment). Black and white bar represent microplastics and control treatment respectively. Lowercase letters indicate significant differences between treatments in the same mean temperature.

We also found a similar trend that CT_{max} increased with increasing DTF ($P = 0.062$ for 5 ℃ DTF, $P < 0.01$ for 10 ℃ DTF).

In contrast with the TICS concept that exposure to toxicants makes organisms more sensitive to climate change (Noyes et al., 2009; Noyes and Lema, 2015), exposure to microplastics increased the CT_{max}. A toxicant-induced reduction in CT_{max} is thought to be driven by an increased energy demand combined with a reduced energy supply, resulting in a mismatch between oxygen and supply occurring at lower temperatures, thereby lowering the heat tolerance (Bagwe et al., 2015). Our results, however, do not suggest an increased oxygen demand and, if anything, the energy consumption decreased and the amount of energy available increased under microplastics exposure. As a result, exposure to microplastics increased the net energy budget (CEA), which indicated *Daphnia* had sufficient energy supply to defend against the extreme temperature. Specifically, exposure to microplastics only increased the CT_{max} at the MT of 20 ℃ combined with a DTF of 10 ℃, and at the MT of 24 ℃ combined with a DTF of 0 ℃ or 5 ℃ (all $P < 0.047$). In support of the energy metabolism contributing to this pattern, microplastics only increased the Ea at the MT of 20 ℃ with 10 ℃ DTF, and only decreased Ec at the MT of 24 ℃ with 0 ℃ or 5 ℃ DTF. We speculate that the higher available energy might be caused by increasing the swimming speed as previously observed in *Daphnia* (Chang et al., 2022). Higher swimming speeds may lead to a higher food intake as *Daphnia* is a filter feeder (De Felice et al., 2019). Moreover, the presence of microplastics also causes

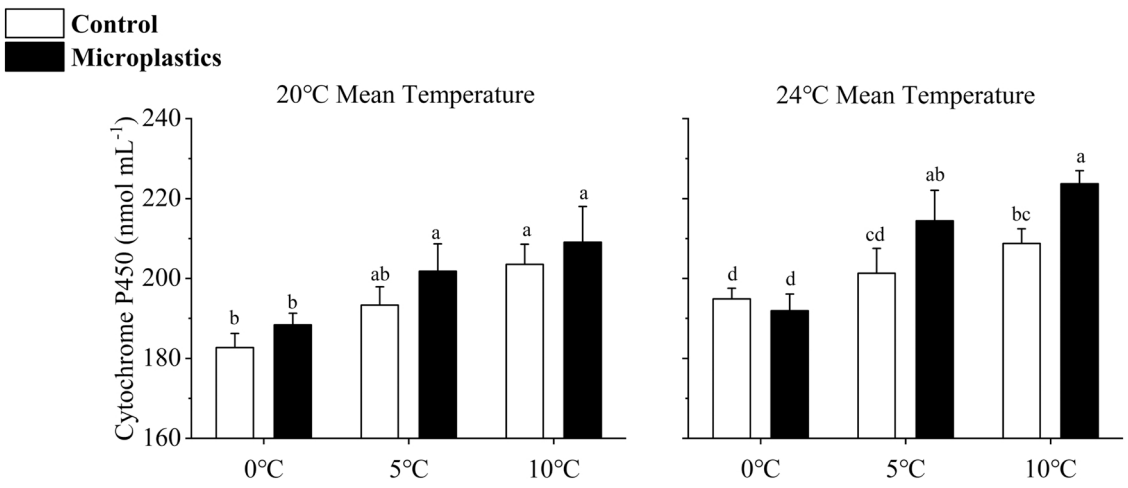

Fig. 4. Effects of microplastics (MP) on cytochrome P450 of *Daphnia magna* as a function of mean temperature (MT) and daily temperature fluctuations (DTF). Given are the means + 1 standard error (SE, n = 3 replicates per treatment). Black and white bar represent microplastics and control treatment respectively. Lowercase letters indicate significant differences between treatments in the same mean temperature.

D. magna to widen filtering gapes to obtain more algae (Chen et al., 2020). Hence, bioenergetics responses may explain why *Daphnia* had an unexpected higher heat tolerance when exposed to microplastics.

It was noteworthy that exposure to microplastics did, however, not increase the heat tolerance of *Daphnia* under the most challenging thermal condition: at 24 ℃ with 10 ℃ DTF. This was most likely because *Daphnia* had reached the highest physiologically achievable heat tolerance, whereby exposure to microplastics was not able to further increase its heat tolerance. In line with this, the CT_{max} in the control group at this thermal condition was the highest among all temperature treatments.

5. Conclusions

In conclusion, multigenerational exposure to an environmentally relevant concentration of microplastics revealed two important insights into the effects of these emerging contaminants in a warming world. First, the observation that effects of microplastics only became apparent under warming and/or DTF is highly relevant for current risk assessment. Indeed, DTF are widespread, and both the higher mean temperatures and DTF levels can currently already be encountered by *Daphnia* at lower latitudes or in urban areas (Verheyen et al., 2019b). Studies ignoring these thermal dependencies may therefore miss important, yet environmentally relevant, effects of microplastics. Ideally, future studies should include a larger gradient of temperatures as stressor interaction patterns may depend on the stressor gradient length (Mack et al., 2022). For the same reason, the interactive effects with warming should be tested along a range of concentrations of microplastics, as the toxicant concentration used may determine the interaction type with warming (Mack et al., 2022). Second, we demonstrated for the first time that, in contrast to the widespread pattern among traditional toxicants such as pesticides and metals, microplastics can increase the heat tolerance. This in intriguing and needs further empirical studies, ideally also on the potential mechanisms underlying this effect. As heat tolerance is thought to be driven by the balance between oxygen supply and demand (Verheyen et al., 2019a), future studies should quantify these processes. More general, to obtain better mechanistic insights in the here observed interaction patterns between microplastics and thermal factors we advocate follow-up studies that integrate metabolomics and transcriptomics. These techniques have indeed been successful applied to identify mechanisms underlying interactions between traditional toxicants and warming. Taken together, our results indicate that the here studied microplastics seem to violate the predictions of both the TICS and CITS concepts, and thereby highlight the importance of using both environmentally relevant concentrations and thermal conditions when assessing the ecological risk of microplastics in future work.

CRediT authorship contribution statement

Mengjie Chang: Experimental work, Methodology, Data curation, Writing – orignal draft. **Mingyang Li:** Help with the experimental work. **Wencheng Xu:** Help with the experimental work. **Xin Li:** Supervision. **Jian Liu:** Supervision. **Robby Stoks:** Writing – review & editing. **Chao Zhang:** Conceptualization, Methodology, Writing – review & editing, Supervision, Funding acquisition.

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. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.ecoenv.2022.114416.

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