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Combined effects of polystyrene microplastics and thermal stress on the freshwater mussel *Dreissena polymorpha*

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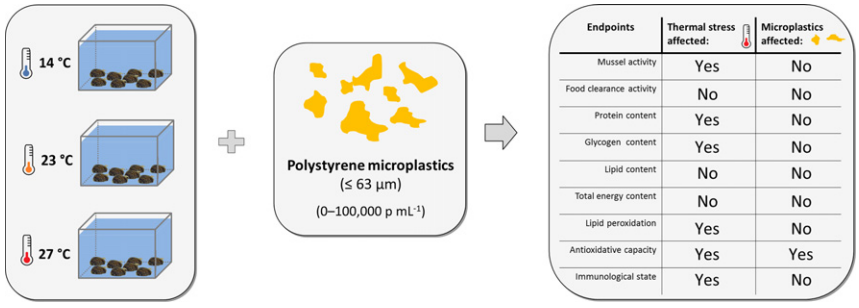
HIGHLIGHTS

- Thermal stress induces stronger effects than microplastics in *Dreissena polymorpha*.
- Thermal stress affects behavior, metabolism and immune function.
- Microplastics only affect the antioxidative capacity.
- Microplastic and diatomite effects only differ for the antioxidative capacity.

GRAPHICAL ABSTRACT



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ABSTRACT

Human-induced changes in the environment have increased the number of stressors impacting aquatic organism. In the light of climate change and plastic pollution, thermal stress and microplastics (MP) have become two of the most intensively studied stressors in aquatic ecosystems. Previous studies, however, mostly evaluated the impacts of thermal and MP stress in isolation, thereby neglecting joint effects.

To examine the combined effects of both, we exposed the freshwater mussel *Dreissena polymorpha* to irregular polystyrene MP (6.4, 160, 4000, 100,000 p mL^{-1}) at either 14, 23 or 27 °C for 14 days and analyzed mortality, mussel activity and clearance rate, energy reserves, oxidative stress and the immunological state. Further, we exposed the mussels to diatomite (natural particle equivalent, 100,000 p mL^{-1}) at each of the three water temperatures to compare MP and natural particle toxicity.

An increase in water temperature has a pronounced effect on *D. polymorpha* and significantly affects the activity, energy reserves, oxidative stress and immune function. In contrast, the effects by MP are limited to a change in the antioxidative capacity without any interactive effects between MP and thermal exposure. The comparison of the MP with a diatomite exposure revealed only limited influence of the particle type on the response of *D. polymorpha* to high concentrations of suspended particles.

The results indicate that MPs have minor effects on a freshwater mussel compared to thermal stress, neither alone nor as interactive effect. Limited MP toxicity could be based on adaptation mechanism of dreissenids to suspended solids. Nonetheless, MP may contribute to environmental impacts of multiple anthropogenic stressors, especially if their levels increase in the future. Therefore, we suggest integrating MP into the broader context of multiple stressor studies to understand and assess their joint impacts on freshwater ecosystems.

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1. Introduction

Since the beginning of the Anthropocene, industrialization has enhanced the demand for resources causing vast global changes, including human-induced environmental impacts (Steffen et al., 2007). Anthropogenic impacts on the environment are diverse, range from regional to global scale and they include, amongst others, the emission of pollutants (e.g., plastics), the change in land use, the exploitation of natural resources as well as climate change. In combination with naturally occurring stressors, anthropogenic changes, thus, lead to increasingly complex and challenging living conditions for biota.

Previous meta-analyses for marine, coastal and freshwater ecosystems have shown that relevant aquatic stressors (e.g., temperature or water chemistry change, habitat disturbance, chemical pollution, invasive species) do not only affect aquatic ecosystems individually but also in combination (Crain et al., 2008; Jackson et al., 2016). These joint effects may be additive synergistic or antagonistic. Thus, experiments with multiple stressors are important to understand their joint impact and interaction. This also adds more "realism" in ecotoxicological research (Beyer et al., 2014) and improves our understanding of the impacts of global change on aquatic ecosystems.

Global warming is a highly relevant stressor in aquatic ecosystems (Sommer et al., 2012) with freshwater habitats being particularly susceptible (Williamson et al., 2009). Global mean surface temperatures are projected to increase by 0.9–5.4 °C by 2100 relative to 1850–1900 likely causing an increase in water temperature (IPCC, 2019). In addition to gradually increasing water temperatures, rapid changes will be caused by more frequent and intense acute heatwaves (IPCC, 2019). Since the impacts due to global warming may further be altered due to additional stressor in aquatic ecosystems. Investigating combinatorial effects of climate change-associated factors and anthropogenic stressors is highly relevant (Przeslawki et al., 2015).

In this respect, microplastics (MP) might be one of these relevant anthropogenic stressors. As MP are ubiquitous in aquatic ecosystems (Chae and An, 2017), concerns over potential impacts on biota have been raised. First risk assessments concluded that current environmental concentrations of MP are much lower than the ones required to induce adverse effects in aquatic species (Burns and Boxall, 2018; Adam et al., 2018; Besseling et al., 2018). However, Adam et al. (2018) and Besseling et al. (2018) also stated that MP may pose an environmental risk in hotspots of pollution, especially in Asia. Furthermore, these assessments looked at MP as a single stressor and did not consider the interaction with other stressors. As effects of multiple stressors can add up, the risk MP pose to aquatic environments may be underestimated.

Previous studies focusing on combinatorial effects of MP and a second stressor mostly investigated the influence of chemical pollutants such as polycyclic aromatic hydrocarbons and polychlorinated biphenyls, pharmaceuticals, pesticides or heavy metals (e.g., Guven et al., 2018; Horton et al., 2018; Lin et al., 2019; Oliveira et al., 2013; Zhang et al., 2019). In comparison, the interaction of MP and thermal stress has been much less studied. Current knowledge is restricted to several studies which investigated thermal stress in combination with polyethylene (PE) and polyvinyl chloride (PVC) particles in fish, daphnids and mollusks (Ferreira et al., 2016; Fonte et al., 2016; Jaikumar et al., 2018; Lenz, 2016; Wen et al., 2018). As with MP research in general (Eerkes-Medrano and Thompson, 2018), little work has been done on freshwater biota.

To fill this knowledge gap, we analyzed the combined effects of thermal stress and MP by exposing the freshwater bivalve *D. polymorpha* at 14, 23 and 27 °C water temperature to irregular polystyrene (PS) MP (≤ 63 μm, 6.4, 160, 4000 and 100,000 particles mL^{-1} (p mL^{-1})). After 14 d of exposure, we examined the effects on the energy reserves (proteins, glycogen, total lipids), the antioxidant system (lipid peroxidation, remaining antioxidant capacity), the immunological function and the mortality of *D. polymorpha*. During the experiment, we additionally determined the activity of the mussels (individuals with open valves) as well as the clearance rate of *D. polymorpha*. Finally, we analyzed the effects of diatomite (DI) as natural reference particle at the three temperature regimes and compared those to the MP effects.

2. Materials and methods

2.1. Mussel culture

About 1000 *D. polymorpha* individuals were collected in October 2017 at the Oberwald Lake in Mörfelden-Walldorf, Germany (49° 59′ 0.242″ N, 8° 35′ 48.666″ E). In the laboratory, bivalves were maintained in a 50 L tank at approximately 14 °C water temperature and a 16:8 h light:dark cycle for 4 weeks prior to the experiments. First, individuals were cultured in aerated lake water which was substituted stepwisely with aerated OECD medium (OECD, 2016, guideline no. 242) within the first week. From week two, 50% of the medium was renewed twice a week. Mussels were fed with algae (*Desmodesmus subspicatus*) ad libitum thrice a week.

2.2. Particle preparation

DI powder was purchased from Sigma-Aldrich (product no: 18514, Taufkirchen, Germany). Irregular MP were prepared from orange fluorescent PS drinking cups by cryomilling (for details see chapter S1.1). The polymer type of the cups was verified by attenuated total reflection-Fourier-transform infrared spectroscopy (ATR-FTIR spectroscopy, Spectrum Two, PerkinElmer, Waltham, MA, USA). Further, we characterized the chemicals in the polymer using pyrolysis-GC–MS analysis with a Multi-Shot Pyrolyzer and Auto-Shot sampler (Frontier-Laboratories, Saikon, Japan) attached to an Agilent 7890B gas chromatograph and an Agilent 5977B Mass Selective Detector (Agilent, Santa Clara, CA, USA). The PS cups had low but detectable levels of chemicals. However, their thermodesorption products could not be matched to compounds commonly used in plastics (for details see Chapter S1.2).

The DI and MP fractions ≤63 μm were isolated by sieving (see S1.1). Particle abundance and size distribution in the 2–60 μm fraction were determined with a Coulter Counter (Multisizer 3, Beckman Coulter, USA). For both the DI and MP powder, particle abundance increased exponentially with decreasing particle size. 90% of the MP and the DI particles were smaller than 12.4 μm and 11.8 μm, respectively (for details see chapter S1.3).

2.3. Mussel exposure with microplastics and diatomite

We exposed *D. polymorpha* at three temperatures (14, 23 or 27 °C) either to MP (6.4, 160, 4000 or 100,000 p mL^{-1}) or DI (100,000 p mL^{-1}) for 14 d. At each exposure temperature, one additional treatment without particles served as control group. In total, our study design included 18 treatments each consisting of 52 *D. polymorpha* individuals (20–23 mm) kept in a tank (14 × 20 × 20 cm) with 3 L OECD medium. Prior to the exposure, we accustomed mussels to be exposed at elevated temperature (23 and 27 °C) slowly over 1–3 d to the new culturing temperature to prevent high mortality rates due to the sudden increase of water temperature.

We constantly aerated the tanks through glass pipettes to ensure algae and particle distribution in the water column. Because we still observed a partial sedimentation of the particles a few hours after particle addition, we also mixed the water column manually at least once a day with a stainless-steel spoon in each treatment. The top of the tank was covered with aluminum foil to prevent particle loss as well as cross-contamination between the different treatments. Water temperature was monitored in 15 min intervals over the whole exposure period in each treatment using submersed temperature loggers (HOBOWare, Onset Computer Corporation, Bourne, USA). The average temperature (± standard deviation) of the respective treatments were 13.77 ± 0.80, 23.42 ± 0.41 and 26.76 ± 0.47 °C.

The bivalves were fed daily with live algae (*D. subspicatus*) at a concentration of 0.25 mg TOC individual^{-1} (equivalent to 4.3 mg TOC L^{-1}) based on Walz (1978), recited by Clarke (1999), who reported that one to two-year old *D. polymorpha* require up to 2 mg L^{-1} TOC of algae. Mortality was recorded daily and dead individuals were removed from the tanks. On days 5 and 12 of the experiment, we additionally examined the mussels' activity by determining the percentage of individuals with opened valves in each treatment (three separate observations per day). Evaporation from the treatments was checked daily and, if necessary, accounted for by refilling with distilled water. Every 3–4 d, the medium was completely renewed by transferring the mussels to tanks filled with new medium and the corresponding particle concentrations. The required MP and DI mass was weighed in on a precision scale for each treatment, except for the 6.4 p mL^{-1} treatment. For the latter, we prepared a 100-fold concentrated stock solution in OECD medium and substituted 30 mL of the medium in the 3 L tank with stock solution to reach a final concentration of 6.4 p mL^{-1}.

After 14 d, hemolymph from five individuals per treatment (approximately 200–300 μL individual^{-1}) was withdrawn from the posterior adductor muscle with a sterile syringe (0.5 mL, 29Gx1/2 in.) and the phagocytic activity of the hemocytes was determined. The other mussels were directly frozen in liquid nitrogen and stored at −80 °C until examined for energy content and oxidative stress metabolites in the midgut gland (MGG).

2.4. Energy content and oxidative stress in the midgut gland

For energy content and oxidative stress analysis, MGGs from ten individuals per treatment were dissected, weighed (wet weight), homogenized (see S1.4) and frozen at −80 °C until analyzed. The MGG masses varied between the individuals but the mean MGG mass in the treatment groups did not differ significantly (Fig. S3). The protein content in the homogenates was analyzed based on Bradford (1976) using a serial dilution of bovine serum albumin (0.1%, m/v) as standard. The glycogen (anthrone assay) and the total lipid content (sulfo-phospho-vanillin assay) were determined according to Benedict (2014) with slight modifications. For glycogen and lipid analysis, a serial dilution of a glucose solution (0.1%, m/v) and a canola oil-chloroform-mixture (0.1%, *v*/v) were used as standards, respectively. Based on the standards, protein, glycogen and total lipid contents in the homogenates were calculated and are given as energy content per MGG mass (J mg^{-1}). The total energy content in the MGGs (J mg^{-1}) was estimated as the sum of the protein, glycogen and total lipid content.

Lipid peroxidation in the homogenates was assayed using the thiobarbituric reactive substances assay (TBARS), following the protocol from Furuhagen et al. (2014). The TBARS assay quantifies malondialdehyde (MDA, a major decomposition product from lipid peroxidation) through the reaction of MDA with thiobarbituric acid which produces a fluorescent product. Based on a serial dilution of an 80 μM MDA stock solution, MDA concentrations in the homogenates were calculated. Results on the MDA content in the homogenates are expressed as MDA equivalents per MGG mass (μmol mg^{-1}).

The antioxidative capacity in the homogenates was determined by the ORAC assay (oxygen radical absorbance capacity, Ou et al., 2001, Furuhagen et al., 2014). The assay is based on the ability of the antioxidants in the homogenates to delay the degradation of fluorescein in presence of a radical forming chemical. As antioxidant standard, we used a serial dilution of a 200 μM Trolox solution (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid, vitamin E derivate). The antioxidative capacity of the homogenates is presented as Trolox equivalents per MGG mass (μmol mg^{-1}). Further details on energy and oxidative stress assays are included in chapters S1.5 and S1.6.

2.5. Phagocytic activity of D. polymorpha hemocytes

Effects on the immune function of *D. polymorpha* were assessed through the phagocytic activity of the hemocytes in response to foreign stimuli. Hemocytes (500 μL hemolymph with 150,000 cells) of six mussels per treatment were exposed individually to 1 μm PS microbeads (50 beads per cell, Fluoresbrite YG microspheres, PolyScience, Hirschberg an der Bergstraße, Germany) for 3 h at room temperature. 250 μL of each cell suspension was separated and incubated with microbeads on ice. This control sample was analyzed in the same way as the remaining sample to correct the phagocytosis rates for particles which only adsorbed to the cell surface but were not phagocytized. We excluded dead cells from the FACS analysis by staining with propidium iodide (Sigma-Aldrich, Taufkirchen, Germany, details in S1.7).

For all samples, we determined the fraction of hemocytes with ≥3 phagocytized microbeads compared to the total number of living cells with fluorescence-activated cell sorting (FACS) on a BD FACSVerse (BD Biosciences, Heidelberg, Germany). Results of hemocyte samples with <5000 FACS counts were excluded, for which reason replicate numbers in the different treatments range between 4 and 6.

2.6. Clearance rate of D. polymorpha

To determine the clearance rates, we used three additional tanks (1.5 L OECD medium) with 26 individuals (20–23 mm) for each temperature (14, 23 or 27 °C) under same conditions as described in Section 2.3. One tank per temperature served as a control, while the other two included 100,000 p mL^{-1} MP or DI, respectively. On day 3 and 10 of the exposure, we quantified the clearance rate of ten individuals per tank as the difference in the chlorophyll fluorescence of an algae suspension (*Pseudokirchneriella subcapitata*) in the medium prior to and after an individual exposure of the bivalves to the algae suspension for 45 min (for details see S1.8). After the clearance experiments, individuals were returned into their tanks.

2.7. Statistics

We determined the effects of the factors "temperature", "MP "as well as their interaction (temperature × MP) on each of the endpoints using general linear (mixed) models (GL(M)M, temperature: fixed variable, MP: covariable) with IBM SPSS Statistics (version 25). Data for each endpoint was used as dependent variable and was either integrated untransformed, log-transformed, square root transformed or logit-transformed into the model. Prior to each GLM, normality (Shapiro-Wilks test), variance homogeneity (Levene test) and heteroskedasticity (White test) requirements were tested. Statistical analysis of particle type effects (MP vs. DI) were performed in the same way by running GL(M)M with the variables "temperature", "particle type" (both fixed variables) and its interaction (temperature × particle type).

Data for each endpoint was visualized using GraphPad Prism 7.04 (GraphPad Software Inc., USA) and linear regression between MP concentrations and the dependent variable were calculated for each temperature (for details see S1.9).

3. Results

3.1. Effects of thermal stress

Throughout the 14-d exposure of *D. polymorpha* individuals to 0–100,000 p mL^{-1} irregular PS MP at 14, 23 and 27 °C, mortality only occurred at 27 °C. Here, 21 out of a total of 260 animals died (8.1%) without relationship to particle exposure. Furthermore, thermal stress significantly affected the mussel activity, the protein, glycogen, lipid peroxidation (TBARS assay) and the remaining antioxidant capacity (ORAC assay) in the midgut gland as well as the phagocytic activity of the mussel hemocytes (immune assay, Table 1, Fig. 1, $p < .05$). In

contrast, an increase in temperature did neither affect the clearance rate (Fig. 2) nor the total lipid and energy content in the MGG (Fig. S5).

The mussel activity was highest at 14 °C (average of all MP exposure groups (including the control): 43.1%), decreased to 39.1% at 27 °C and was lowest at 23 °C (30.7%). Mussel activity did therefore not decrease proportionally with increasing exposure temperature, but rather had its minimum at 23 °C (Fig. 1). The same relation was observed for the glycogen and the antioxidant capacity which were highest at 14 °C (glycogen: 0.139 J mg^{-1} MGG, antioxidant capacity: 495 μmol Trolox mg^{-1} MGG) and lowest at 23 °C (glycogen: 0.122 J mg^{-1} MGG, antioxidant capacity: 307 μmol Trolox mg^{-1} MGG). The opposite trend was observed for the protein content being highest in the MGG of mussels exposed at 27 °C (0.465 J mg^{-1} MGG) and lowest in individuals at 23 °C (0.339 J mg^{-1} MGG).

The lipid peroxidation in the MGG and the phagocytic activity of the hemocytes decreased proportionally with increasing exposure temperature. For both endpoints, the strongest effects were observed at 14 °C (MDA content: 14.3 μmol mg^{-1} MGG, phagocytic activity: 10.1%) that decreased at 27 °C to 11.4 μmol mg^{-1} MGG and 5.85%, respectively.

3.2. Effects of microplastics exposure

Exposure to increasing MP concentrations (6.4–100,000 p mL^{-1}) did not result in a significant effect on mortality. Mortality occurred in none of the exposures at 14 and 23 °C. At 27 °C, mortality rates were 9.62% in the control, 13.46% in the 6.4 p mL^{-1} and 5.77% in the 160, 4000 and 100,000 p mL^{-1} PS exposures. Therefore, mortality was not dose dependent.

Furthermore, the exposure of *D. polymorpha* to MP had no significant effects on the mussel activity, energy reserves, lipid peroxidation, immune function or the clearance rate (Figs. 1, 2, S4, Table 1, $p > .05$). However, the remaining antioxidant capacity in the MGG was significantly affected by the MP exposure (Table 1, $p < .01$). At the three temperatures, the antioxidant capacity decreased with increased MP concentrations, especially at high MP concentrations (4000 and 100,000 p mL^{-1}). This effect was more pronounced at 14 °C compared to mussels exposed at 23 and 27 °C.

3.3. Comparison of microplastics and diatomite effects

We additionally compared the effects of MP with DI as a representative of naturally occurring particles. For each temperature, we therefore performed a separate treatment with 100,000 p mL^{-1} DI particles and compared the results to the corresponding MP treatment (100,000 p mL^{-1}).

Mortality in the DI and the MP treatment were identical at 14 °C (0%) and 27 °C (5.77%) and very similar at 23 °C (DI: 1.9%, MP: 0%). There was no significant difference between the effects of DI and MP regarding the mussel activity, energy reserves, lipid peroxidation, immune function and the clearance rate (Figs. 1, 2, S4, Table S2). However, the remaining antioxidant capacity was significantly higher in the DI compared to the PS MP treatments at all temperatures ($p < .05$, Table S2).

3.4. Interaction of temperature and particle exposure

Throughout the exposure of *D. polymorpha* to MP at 14, 23 and 27 °C, no significant interaction between the two stressors (MP, temperature) was observed (Table 1, $p > .05$). Similarly, the statistical comparison of the MP and DI exposure did not indicate any significant interaction between the two variables temperature and particle type (Table S2, $p > .05$).

4. Discussion

4.1. Effects of thermal stress

The exposure of *D. polymorpha* at three different water temperatures (14, 23 and 27 °C) causes major effects on the behavior, metabolism and immune function. 14 °C was chosen as a reference temperature as Walz (1978) reported the highest ingestion rate for *D. polymorpha* at this temperature. *D. polymorpha* individuals are more active at 14 °C compared to animals held at 23 and 27 °C and have the highest glycogen levels, remaining antioxidative capacity and phagocytic activity of the hemocyte cells. These results suggest that 14 °C represents the optimal out of the three temperatures allowing for high activity rates, energy storage and a functional immune response.

In response to increasing water temperatures, *D. polymorpha* changes its behavior and metabolism to enhance its thermotolerance. Previous studies with *D. polymorpha* have shown that these adaptative mechanisms enable the species to survive temperatures of up to 30 °C, while the exposure to higher temperatures over several days often increases mortality (reviewed by Karatayev et al., 1998 and McMahon, 1996). We, therefore, selected 23 and 27 °C as temperatures inducing thermotolerance mechanisms of *D. polymorpha* in a medium (23 °C) and extreme scenario (27 °C). These temperatures are not supposed to fully mimic climate change conditions. Still, water temperature measurements by Quednow and Püttmann (2008) in freshwater systems of southern Hesse (the region from which our dreissenids originated) show that average surface water temperatures can range between 4 °C in winter and >20 °C in summer. In the light of increasing freshwater temperatures due to climate change (IPCC, 2014) and a predicted strong increase in frequency and intensity of heatwave events (IPCC, 2019), rapidly increasing water temperatures up to 23 °C may be reached in these freshwater systems now or in future.

With regard to behavior, increasing water temperatures in our experiments reduces the valve opening of *D. polymorpha*, especially at 23 °C. Valve closure is a common strategy of mussels to outlive unfavorable conditions for a short time. Over an extended period, however, the

Table 1
Results of the general linear model and general linear mixed model (only between-subject) for the effects of temperature and microplastics exposure on mussel activity, clearance rate (on days 3 and 10), energy reserves (total energy, proteins, glycogen, total lipids) and oxidative stress (TBARS, ORAC) in the midgut gland of *D. polymorpha* as well as the phagocytic activity of hemocytes.

Variable		Mussel activity	Clearance rate (3 d)	Clearance rate (10 d)	Total energy	Proteins	Glycogen	Total lipids	TBARS	ORAC	Phagocytic activity
Temperature	*df*	2	2	2	2	2	2	2	2	2	2
	F	10.852	0.066	2.412	0.275	12.302	5.081	0.049	7.331	11.225	9.553
	p	**0.004**	0.936	0.099	0.760	**<0.001**	**0.007**	0.953	**0.001**	**<0.001**	**<0.001**
Microplastics	*df*	1	1	1	1	1	1	1	1	1	1
	F	0.041	0.055	1.850	0.185	2.349	2.725	0.080	0.005	8.336	0.882
	p	0.844	0.816	0.179	0.668	0.128	0.101	0.778	0.943	**0.004**	0.351
Temperature × Microplastics	*df*	2	2	2	2	2	–[a]	2	2	2	2
	F	0.140	0.129	1.593	1.455	0.123	–[a]	1.449	0.636	2.203	0.959
	p	0.871	0.879	0.213	0.237	0.884	–[a]	0.238	0.531	0.114	0.388

[a] Interaction term not included in the model.

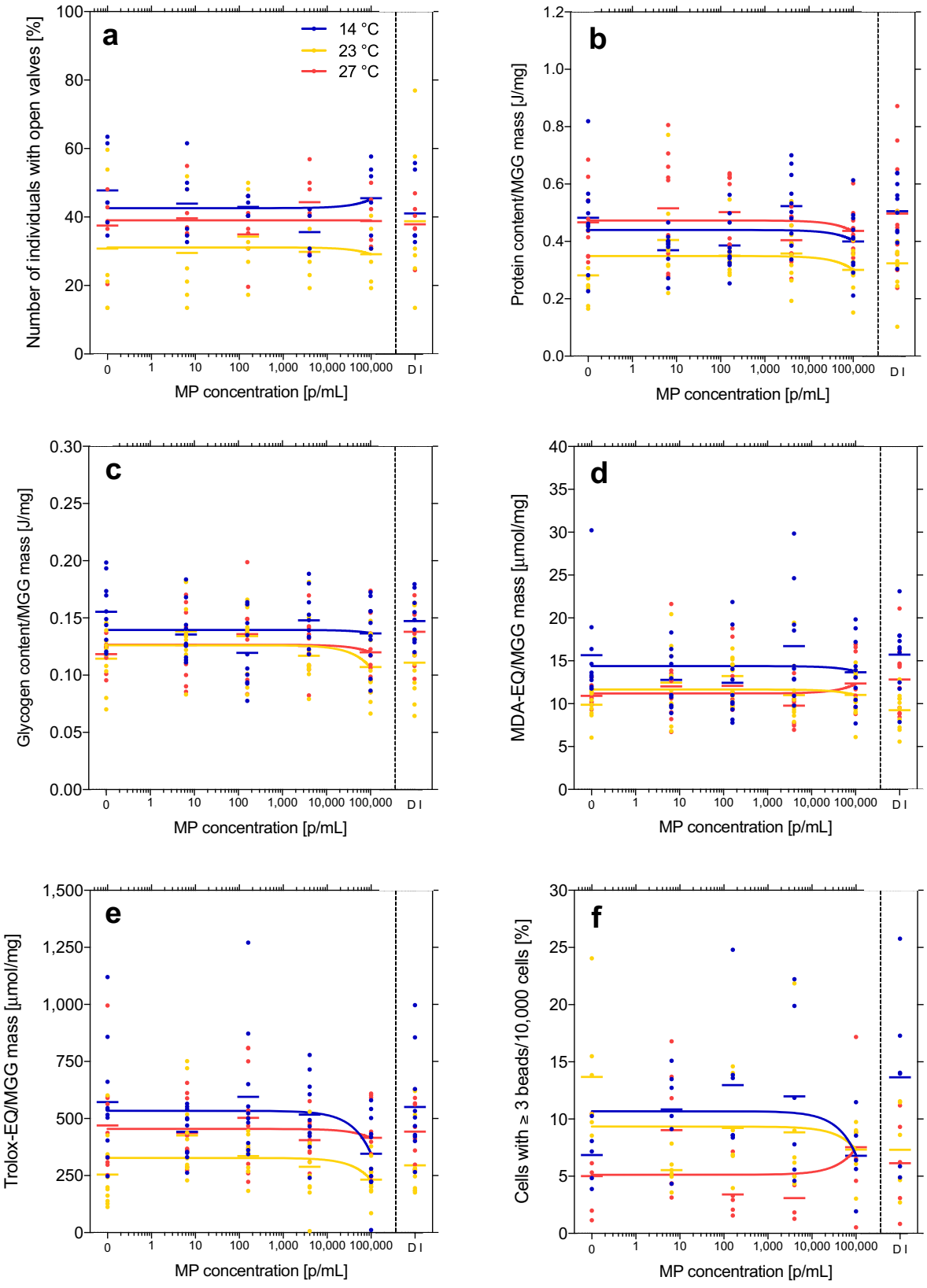

Fig. 1. Effect of polystyrene microplastics (MP, 0–100,000 p mL^{-1}) or diatomite (DI, 100,000 p mL^{-1}) on *D. polymorpha* exposed at 14, 23 and 27 °C for 14 d. (a) Mussel activity, (b) protein content, (c) glycogen content, (d) malondialdehyde content (MDA, TBARS assay) and (e) the remaining antioxidant capacity (Trolox equivalents, ORAC assay) in the midgut gland and (f) the phagocytic activity of hemocytes. Dots = results from the replicates, short lines = mean, regression = linear regression of the results in the microplastics exposures. (a) $n = 6$ (2 d with three observations each), (b–e) $n = 10$ individuals, (f) $n = 4$–6 individuals.

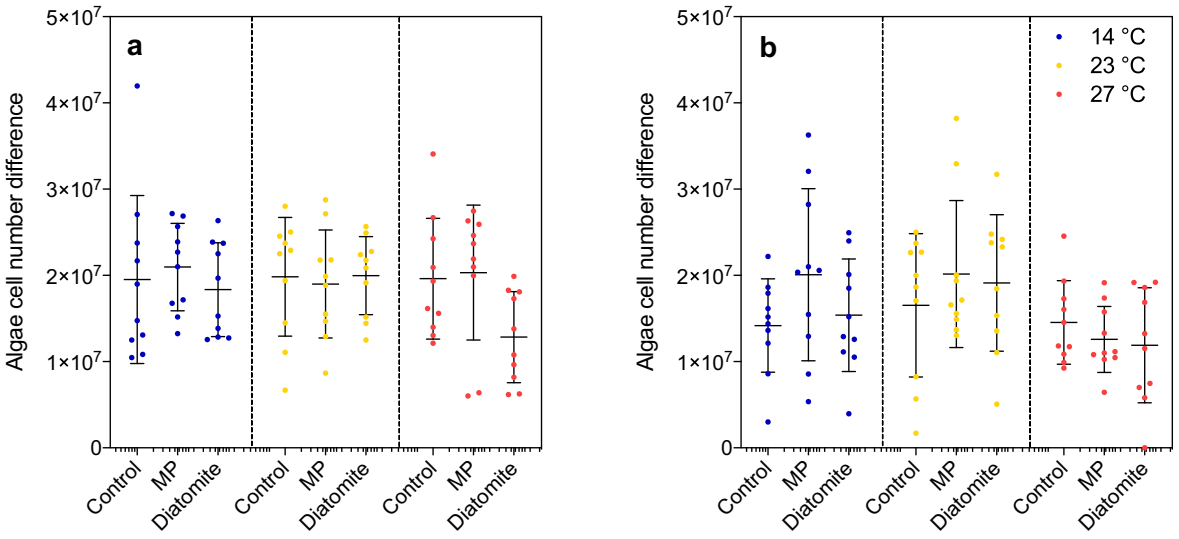

Fig. 2. Clearance rate (mean ± standard deviation) of *D. polymorpha* after an exposure to particle-free medium (Control), polystyrene microplastics (MP, 100,000 p mL^{-1}) or diatomite (100,000 p mL^{-1}) for (a) 3 d and (b) 10 d at 14, 23 and 27 °C. n = 10 for each group.

reduced gas exchange causes metabolic depression, a shift from an aerobic to an anaerobic metabolism and a depletion of the energy reserves (Anestis et al., 2007). Furthermore, elevated water temperatures also enhance the energy metabolic of mussels, while the clearance rate for food particles usually decreases (Anacleto et al., 2014; Anestis et al., 2010; Hornstein et al., 2018; Juárez et al., 2018). Consequently, we expected the heat-stressed mussels to reduce their clearance rate and deplete their energy reserves to maintain their metabolism.

We did not observe a reduction of clearance rates in our experiments with increasing water temperatures, probably due to the high interindividual variation. Instead, the glycogen content is reduced in individuals held at 23 and 27 °C, indicating an increased mobilization of energy reserves in these individuals. Under stress conditions, mussels mobilize both lipid and carbohydrate reserves first before they change towards protein catabolism (Aldridge et al., 1995). Surprisingly, however, we did not find a significant temperature effect on the total lipid storage and the total energy storage of *D. polymorpha*. We assume this may be related to the high interindividual variation of the total lipid content in the MGG as well as by the limited sensitivity of our lipid assay.

Besides a change in the metabolism, thermal stress also depleted the antioxidant capacity in *D. polymorpha*. This is an indicator that the mussels experienced oxidative stress; a common response towards chemical or physical stressors (such as thermal stress) that triggers the production of reactive oxygen species (ROS) (reviewed by Verlecar et al., 2007). In case of oxidative stress, mussels express high amounts of antioxidant enzymes which scavenge ROS (Verlecar et al., 2007). The high protein levels in the MGG of animals held at 27 °C may be a consequence of the activated antioxidant system but could have also resulted from a generally enhanced metabolic activity.

Excess ROS can react with biomolecules and cause oxidative damage, for instance lipid peroxidation. A typical reaction production of ROS with polyunsaturated fatty acids is MDA (Vlahogianni et al., 2007). Therefore, based on the reduced antioxidant capacities in mussels held at 23 and 27 °C, we expected MDA concentrations to increase. A temperature-induced MDA increase has already been shown in studies with the freshwater mussel *Anodonta anatina* (Falfushynska et al., 2014) as well as the marine mussels *Mytilus galloprovincialis* (Coppola et al., 2018) and *Perna viridis* (Verlecar et al., 2007) which were exposed to thermal stress for ≥14 d. Contrasting our expectation, *D. polymorpha* held at 14 °C water temperature had the highest MDA concentrations. A very similar observation has been made for the marine clam *Chamelea gallina* (Matozzo et al., 2013). The authors assume that the induction of antioxidant enzymes was at least partially responsible for the lower MDA content in the clams under thermal stress. Furthermore, the high MDA levels in the control group may also be a result of the TBARS method which can overestimate MDA levels due to a cross-reaction of thiobarbituric acid with cyclic peroxides, β-unsaturated aldehydes and other contaminants (Oakes and Van Der Kraak, 2003). Nevertheless, we assume that the reduced lipid peroxidation in *D. polymorpha* at higher temperatures is related to an induction of the antioxidant defense system. The depleted antioxidant capacity indicates that the mussels experienced thermal stress.

Thermal stress also affects the immune function of *D. polymorpha* by reducing the phagocytic activity of the hemocytes in our study. These results are in accordance with earlier studies that reported a similar depression in immunology and hemocyte phagocytosis due to thermal stress (Hégaret et al., 2003; Hornstein et al., 2018; Monari et al., 2007). A suppression of immune function resulting from thermal stress may render mussel more susceptible to infections.

Interestingly, thermal stress has stronger effects on individuals held at 23 °C compared to the more extreme 27 °C, for instance regarding the valve opening rates, glycogen reserves and the antioxidant capacity. Interestingly, mussels held at 23 °C had the lowest protein content compared to the 14 and 27 °C treatments. As described earlier, reduced mussel activity may have led to the depletion of the glycogen reserves and a switch to protein catabolism as well as to enhanced oxidative stress. The reasons for these unexpected effects at the intermediate temperature, however, remain unknown.

Nevertheless, *D. polymorpha* held at 23 and 27 °C show changes in behavior, metabolism and immune function due to thermal stress. In a second step, we were further interested how the combined exposure with MP alters the temperature-induced effects and whether a combination of both stressors enhances the overall toxicity.

4.2. Effects of microplastics exposure

MP cause much lower effects in *D. polymorpha* compared to thermal stress (with no interaction between the two stressors). The antioxidative capacity in the MGG is the only endpoint affected by the additional MP exposure, while no effect was determined on behavior, metabolism or immune function. The depletion of the antioxidant capacity was most prominent in mussels exposed to the highest MP concentration (100,000 p mL^{-1}). Further, the effect is more pronounced at 14 °C water temperature and is rather minor at 23 and 27 °C. This is consistent with the reduced antioxidative capacity in mussels experiencing thermal stress. Here, the additional effect of the MP on an already depleted

antioxidative system will be less pronounced compared to unstressed control individuals. As a consequence, MP effects seem to be more relevant in unstressed mussels, while in combination with more potent stressors, such as higher temperatures, their effects may be less relevant.

Lenz (2016) made a very similar observation when analyzing the combined effects of thermal stress and PVC MP in six marine mussel species. In *Mytilus trossulus* and *Crassostrea gigas*, they observed both thermal and MP-induced effects on the respiration, but, similar to our results, MP effects were only present in the control animals. In a third species, *Perna viridis*, however, MP induced effects at all temperatures (Lenz, 2016). The authors discuss that thermal stress masks other stressors especially in species which are adapted to low and stable water temperatures (e.g., *Mytilus trossulus* and *Crassostrea gigas*). Those species are especially susceptible to thermal stress compared to species which are adapted to temperature variations and occasional thermal stress situations (*Perna viridis*).

As discussed in Section 4.1, surface water temperature in Hessian freshwater systems can vary intensively over the course of the year and, thus, *D. polymorpha* is adapted to marked changes in water temperature. However, these changes are slow allowing sufficient time for adaptation, while the increase in water temperature in the experiments occurred rapidly within 1–3 d. Such rapid temperature increase may have disrupted an adaptation causing thermal stress and, thus, masked the oxidative stress caused by MP (similar to *M. trossulus* and *C. gigas* in the study by Lenz, 2016).

Besides mussels, the joint effects of thermal stress and MP have previously been studied in fish. Ferreira et al. (2016) and Fonte et al. (2016) exposed *Pomatoschistus microps* juveniles to elevated water temperatures, PE MP and a chemical stressor. In both studies, MP effects were limited to a change in lipid peroxidation, while temperature, similarly to mussels, affected the behavior and induced the oxidative stress response. In contrast, Wen et al. (2018) reported greater impacts of PE compared to thermal stress on the predatory performance, digestion and energy production of the freshwater fish *Symphysodon aequifasciatus*. Moreover, PE MP induced mortality in daphnids which were more sensitive at higher water temperatures (Jaikumar et al., 2018). Accordingly, the results of the available studies are inconsistent when taking into account other than mussel species. From this, we hypothesize that the intensity of the applied stressors as well as the adaption mechanism of freshwater species towards the applied stressors may influence the impact of the tested stressors. Hence, in different species or under divergent exposure conditions, MP can have different effects from the ones we observed.

Furthermore, when interpreting multiple stressor toxicity of thermal stress and MP, it has to be considered that most studies applied MP concentrations higher than currently found in the environment. MP concentrations in rivers are still rather low ranging up to 0.5 p mL^{-1} (Lahens et al., 2018). Effects at MP concentrations of up to 100,000 p mL^{-1} therefore seem to be currently not environmentally relevant. In our study, we aimed at including both environmentally relevant concentrations (6.4 p mL^{-1}) which may be reached in the near future due to increasing plastic pollution in aquatic ecosystems (Lebreton et al., 2019) as well as high MP concentrations as a proof-of-principle. Limited MP toxicity even at high MP concentrations, thus, indicates that PS fragments (≤63 μm) currently represent a rather low hazard for dreissenids in the environment.

4.3. Comparison to natural particles

Both, in our experiment and in the study by Lenz (2016), MP were a minor stressor compared to changes in water temperature. A reason for why mussels are resistant to MP exposures may be the evolutionary adaption of many filter-feeding organisms to high turbidity and suspended solids. Hence, we compared the effects of natural DI particles and MP and observed very little differences. Significant particle type effects were only detected for the antioxidative capacity in *D. polymorpha*. Further, we observed a non-significantly enhanced phagocytic activity of the hemocytes in the DI compared to the MP exposure at 14 °C. This suggests that the particle type had some, but rather limited influence on the response of *D. polymorpha* to high concentrations of suspended particles and that under the conditions used in this study, MP are not much more toxic than natural particles.

5. Conclusion

Our multiple stressor study illustrates that thermal stress has a stronger impact on *D. polymorpha* compared to PS MP (≤63 μm). Increasing the water temperatures from 14 to 23 and 27 °C significantly affects the activity of the mussels, their energy reserves (protein, glycogen content) and oxidative stress markers (lipid peroxidation, antioxidative capacity) as well as the immune function. In contrast, MP exposure to very high concentrations (up to 100,000 p mL^{-1}) only affects the antioxidative capacity without significant interactive effects of MP and thermal stress.

Our results imply that dreissenid mussels are more sensitive to thermal stress than to MP exposure because they are evolutionary adapted to high loads of suspended particles. The latter is supported by the results from an exposure to natural DI particles which resulted in largely similar effects like MP.

While we did not observe an interaction of thermal stress and MP exposures in dreissenid mussels, MP represents just one out of numerous anthropogenic stressors in aquatic ecosystems. They, thus, may contribute to effects in a complex, multi-stressor environment which cannot be predicted based on simplified laboratory experiments. In the light of increasing plastic pollution in aquatic environments, future MP concentrations will likely increase (Lebreton et al., 2019) making joint effects more probable. Therefore, the integration of our data into the broader context of future multi-stressor studies will enable a comprehensive assessment of risks resulting from plastic pollution in a changing aquatic environment.

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.

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Author contributions

AW and NJ conceived and performed the study and analyzed the data. All authors wrote the manuscript and agreed on the final version.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.scitotenv.2020.137253.

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