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

Supplementary Information

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S1 Supplementary materials and methods

S1.1 Particle preparation

Microplastics (MP) were produced by cryomilling in a swing mill (Retsch, MM400, Haan, Germany) with a stainless-steel milling chamber (volume: 50 mL, Retsch Technology, Haan, Germany) and a Ø 25 mm stainless steel ball. The polystyrene (PS) cup was crushed into pieces of up to 3 cm length and 5 g of these pieces were ground three times for 4 min at 30 Hz. Before and between the runs, the chamber was cooled with liquid nitrogen for 2 min.
300 g of MP and diatomite (DI) powder were separately sieved through a 63 µm woven wire mesh sieve (Retsch, product no.: 60.131.000063, Haan, Germany) on a sieving tower (Retsch Technology, AS200basic, Haan, Germany) for 4 h (amplitude: 20 Hz).

S1.2 Particle characterization

PS as polymer type was verified by Fourier-transform infrared spectroscopy in Attenuated Total Reflection mode (ATR-FTIR spectroscopy, Spectrum 2, Perkin Elmer, Waltham, MA, USA). The polymer spectrum was measured with the range set to 4,000–450 cm^{-1} (4 scans per wave number, resolution: 4 cm^{-1}, suppression of CO_2 and H_2O peaks). The major peaks in the FTIR spectrum (Fig. S1a) were in accordance with the data published by Jung et al. (2018) and confirmed that the material was PS.

The chemical content in the PS sample was determined by pyrolysis-GC-MS (py-GC-MS, 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)). PS powder (100–300 mg per pyrolysis cup) was heated to 280 °C for 5 min. Thermodesorption products were detected for 30 min in selected ion monitoring mode.

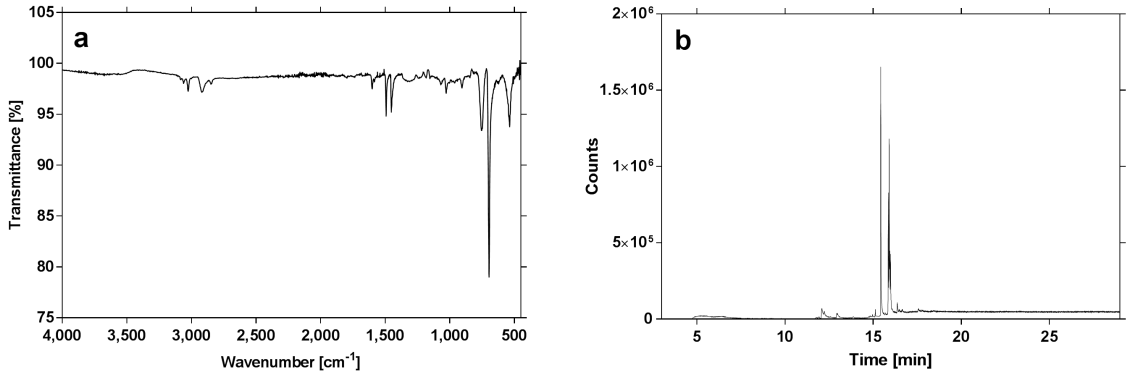

Fig. S1: (a) ATR-FTIR spectrum and (b) py-GC-MS spectrum of the polystyrene drinking cups.

The py-GC-MS spectrum (Fig. S1b) was analyzed with the Agilent MassHunter Workstation Software (version B.05.00, Agilent Technologies, Santa Clara, CA, USA) using "Chromatogram Deconvolution". We only analyzed peaks with an absolute height of at least 2 % of the highest peak and with a relative height of at least 5,000 counts. Additional settings were: RT window size factor = 100, peak filter = excluded m/z: 28, extraction window: left m/z delta = 0.3 and right m/z delta = 0.7. The mass spectra of the resulting peaks were compared to the NIST 2011 Mass Spectral Library (National Institute of Standards and Technology, Gaithersburg, MD, USA).

We detected the fluorophore 1,4-diphenyl-,(E,E)-1,3-butadiene, (Tab. S1) as well as m-phenethyl-benzonitrile, and (2,3-Diphenylcyclopropyl)methyl phenyl sulfoxide as thermodesorption products. The fluorophore might originate from the dye of the drinking cup. The other two desorption products, however, are not commonly used in polymers and their origin, therefore, remains unknown.

Tab. S1: Detected peaks in the py-GC-MS mass spectrum and corresponding tentative identification based on the NIST database (highest match scores).

	Polymer	Base Peak	RT	Height	Area	Compound name	Score
1	PS	90.99	12.089	21,601	245,656	not identified	none
2	PS	104.01	12.223	14,719	85,223	not identified	none
3	PS	204.02	12.947	6,699	66,710	not identified	none
4	PS	90.99	15.430	622,777	2,067,796	Benzonitrile, m-phenethyl-	57.03
5	PS	90.99	15.856	156,943	1,009,078	1,3-Butadiene, 1,4-diphenyl-, (E,E)-	73.09
6	PS	90.99	15.904	233,782	1,639,405	(2,3-Diphenylcyclopropyl)methyl phenyl sulfoxide, trans-	75.13
7	PS	90.99	15.942	83,831	477,232	(2,3-Diphenylcyclopropyl)methyl phenyl sulfoxide, trans-	59.17
8	PS	90.99	15.983	84,599	781,256	(2,3-Diphenylcyclopropyl)methyl phenyl sulfoxide, trans-	67.09
9	PS	90.99	16.364	14,536	65,237	not identified	none
10	PS	90.99	16.485	6,457	98,021	not identified	none

S1.3 Particle concentrations and size distribution

The concentrations of MP and DI particles (≤ 63 µm) were determined using a Coulter Counter (Beckman Coulter, Multisizer 3, Krefeld, Germany) by suspending 2 mg MP or DI in 50 mL electrolytic solution (0.9 % NaCl solution, < 0.2 µm sterile-filtered) and adding 5 mL of these suspensions to 147 mL electrolytic solution. The suspension was directly measured three times with a 100 µm capillary (Beckman Coulter, Krefeld, Germany, detection range: 2–60 µm, aperture: -1,600, gain: 2, analytical volume: 1 mL). The measurements were repeated three times for both particle types. Additionally, background measurements without MP or DI were performed to quantify contamination in the electrolyte solution. The MP and DI concentrations were corrected, accordingly. The MP and the DI powder contained 287,526 particles per mg^{-1} and 4,632,990 particles mg^{-1}, respectively.

Results on particle size distributions from Coulter Counter measurements were averaged and fitted with GraphPad-Prism Software (Version 7.04, San Diego, CA) using a “One phase decay” function (relative particle size, Fig. S2) or a “One phase association” function (cumulative particle size of MP) and a “Cumulative Gaussian-Percentage” function (cumulative particle size of DI). From the cumulative size distributions, we determined the maximum size of 50, 75, 90, 95 and 99 % of the particles (insets in Fig. S2).

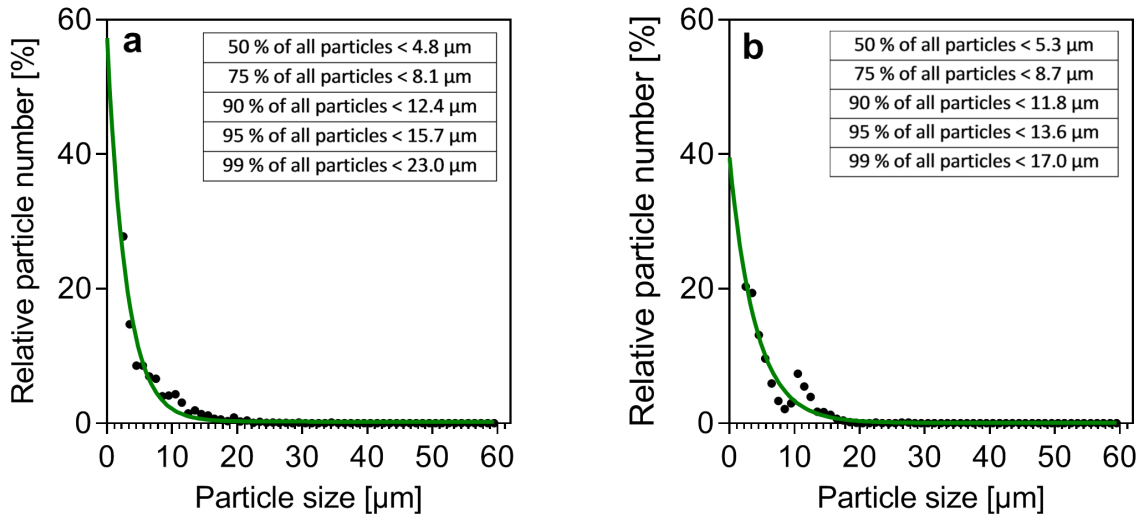

Fig. S2: Relative size distribution of (a) microplastics and (b) diatomite particles. Size distribution was summarized from three separate measurements and approximated with a One phase decay function using GraphPad Prism. The tables in the insets present the results derived from cumulative particle distributions.

S1.4 Preparation of midgut gland homogenates

Midgut glands (MGG) from ten individuals per treatment were dissected and weighed. Fig. S3 indicates that MGG mass varied intensively within each exposure group, while MGG mass did not differ significantly between any of the exposure groups (Kruskal-Wallis test, $p > 0.05$).

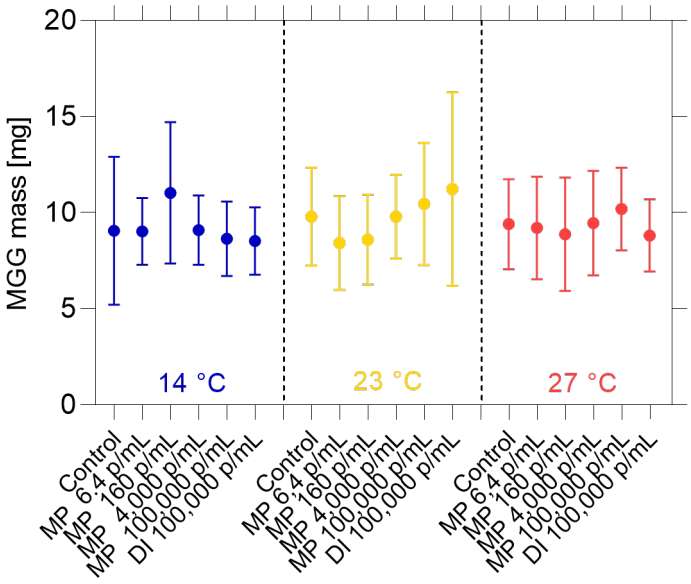

Fig. S3: Migut gland mass (MGG, mean ± standard deviation) of *D. polymorpha* after an exposure to particle-free medium (Control, 0 p mL^{-1}), polystyrene microplastics (MP, 6.4–100,000 p mL^{-1}) or diatomite (DI, 100,000 p mL^{-1}) for 14 d at 14, 23 and 27 °C.

Midgut glands (MGG) were homogenized in 360 µL of potassium phosphate buffer (PPB; 10 mM, pH 7,4) with two stainless steel balls (∅ 2–3 mm) in a swinging mill for 30 min (15×2 min). The tissue as well as its homogenate was constantly placed on ice between the processing steps to avoid degradation.

For the glycogen and total lipid assay, 150 µL of midgut gland homogenate was mixed with 50 µL 2 % (m/v) Na_2SO_4 solution (Sigma-Aldrich, Munich, Germany). 25 µL of this mixture was further diluted with 6.25 µL 2 % Na_2SO_4 solution and 18.75 µL PPB to obtain the required dilution for the protein assay.

For the thiobarbituric reactive substances assay (TBARS), 160 µL of each tissue homogenate were mixed with 160 µL PPB (10 mM, pH 7.4). The dilution for the oxygen radical absorbance capacity assay (ORAC) was produced by mixing 10 µL of the TBARS dilution with 90 µL PPB.

S1.5 Protein, glycogen and total lipid assay

S1.5.1 Protein assay

The protein assay was performed according to Bradford (1976). As standard, 1, 3, 6, 12.5, 25, 37.5 and 50 µL of a 0.1 % (m/v) bovine serum albumin solution (BSA; Sigma-Aldrich, Darmstadt, Germany) were mixed with 2 % Na_2SO_4 solution to obtain a total volume of 50 µL. 50 µL of 2 % Na_2SO_4 solution were used as negative control. The negative control, the standards as well as 50 µL of the homogenate dilution (see S1.4) were mixed with 1.5 mL Bradford reagent (A6932, AppliChem GmbH, Darmstadt, Germany) and incubated for 5 min at room temperature. Subsequently, 2×200 µL were pipetted into transparent 96-well plates and optical density was determined spectrometrically at 595 nm (Spark 10, Tecan, Switzerland).

S1.5.2 Glycogen and total lipid assay

100 µL of homogenate dilution for the glycogen and total lipid analysis (S1.8) were mixed with 1.6 mL of a 1:1 chloroform-methanol solution (chloroform: VWR International, Darmstadt, Germany; methanol: Carl Roth, Karlsruhe, Germany). After 1 h of incubation on ice, homogenates were centrifuged at 845 g for 2 min (Centrifuge 5702, Eppendorf, Hamburg, Germany). The pellet was analysed for its glycogen content, while the supernatant (which includes the lipid fraction) was separated, mixed with 600 µL distilled water and centrifuged at 845 g for 2 min. Subsequently, the resulting upper phase was removed, and the lower phase was used for total lipid analysis.

For glycogen analysis, the pellet was dissolved in 5 mL of anthrone-sulphuric acid reagent (750 mg anthrone, Merck, Darmstadt, Germany; 385 mL 98 % H_2SO_4, VWR, Darmstadt, Germany; 150 mL demineralized water) and incubated in a water bath at 95 °C for 17 min. As standard, 1, 3, 6, 12.5, 25, 50, 100, 200, 400 and 800 µL of a 0.1 % (m/v) D-(+)-glucose solution (VWR, Darmstadt, Germany) was processed in the same way. As negative control, 5 mL of anthrone-sulphuric acid reagent were incubated alone. 2×200 µL from each replicate were pipetted into transparent 96-well plates and optical density was determined spectrometrically at 625 nm.

Lipid fractions were reduced to a volume of approximately 20 µL by evaporation in a water bath at 70 °C. As standard, 1, 3, 6, 12.5, 25, 50, 100, 200, 400 and 800 µL of a canola oil solution (0.1 % v/v in chloroform, REWE, Cologne, Germany) were concentrated in the same way. Then, 200 µL of sulphuric acid (95–98 %, CAS: 7664-93-9, VWR, Darmstadt, Germany) were added and samples were incubated in the water bath at 70 °C for 13 min. As negative control, 200 µL of sulphuric acid were incubated alone. After the incubation, 5 mL of vanillin-phosphoric acid reagent (600 mg vanillin, Sigma-Aldrich, Munich, Germany; 400 mL 85 % H_3PO_4, VWR, Darmstadt, Germany, 100 mL demineralized water) were added to all samples

and incubated at room temperature for 5 min. Finally, 2×200 µL from each tube were transferred into a transparent 96-well plate and the absorbance was recorded at 560 nm using a Tecan Spark 10 photospectrometer.

S1.5.3 Calculation of the energy content

Protein, glycogen and total lipid contents were determined by plotting the optical densities of the BSA, glucose and canola oil standard solutions as a function of its energy content [µg]. Energy values were estimated as 17.2 J mg^{-1} for proteins and glucose (Higgs et al. 1995) as well as 34.04 J mg^{-1} for lipids (REWE, product information) and recalculated for each standard dilution. The energy contents of the MGG samples were interpolated from a quadratic regression of the results from the standard measurements and normalized to the MGG wet weight [J mg^{-1}]. Finally, we calculated individual protein, glycogen and total lipid contents in the MGG as well as the total energy content (= energy content of proteins+glycogen+total lipids).

S1.6 Oxidative stress assays

S1.6.1 Thiobarbituric acid assay (TBARS)

Malondialdehyde (MDA) as standard substance was produced by hydrolysis of 1,1,3,3-Tetramethoxypropane (TMP, Sigma-Aldrich, Munich, Germany). 41.2 µL of TMP were added to 250 mL ultrapure water and 0.25 mL hydrochloric acid (1 M, CAS: 7647-01-0, VWR, Darmstadt, Germany) and incubated at 52–55 °C in a heating cabinet for at least 1 h through which a 1 mM aqueous solution was produced. A serial dilution (80, 40, 20, 10, 5, 2.5 µM) of the MDA solution was prepared as standard. Ultrapure water was used as negative control.

100 µL of the homogenate dilutions (S1.4, two replicates per homogenate), of the negative control (three replicates) as well as of the MDA standard (three replicates per concentration) were mixed with 100 µL of an ice-cold 10 % (m/v) trichloroacetic acid solution and 120 µL of a 2 mM thiobarbituric acid solution (Sigma-Aldrich, Munich, Germany). The mixture was incubated at 95 °C for 1 h and, after cooling down to room temperature, mixed with 180 µL butanol-pyridine-mixture (14:1 ratio; 1-butanol: AppliChem, Darmstadt, Germany; pyridine: Sigma-Aldrich, Munich, Germany). Samples were centrifuged at 5,200 g and 0 °C for 5 min for phase separation. Subsequently, 80 µL of the upper phase were pipetted into black 96-well plates (Nunc F96 MicroWell, Thermo Fisher Scientific, Waltham, USA) and fluorescence was measured (extinction: 540 nm, emission: 590 nm, gain: 48) using a Tecan Spark 10 spectrophotometer. Fluorescence of the replicate samples (see above) was averaged.

We determined lipid peroxidation in the MGG by comparing the fluorescence of samples with the fluorescence of the MDA standard. For this, results of the MDA standard and the negative control were plotted as a quadratic function of the concentration and MDA equivalents in the MGG samples were interpolated from this function. Due to high variability of total lipid contents in the different treatments, we chose to normalize the resulting MDA equivalents to MGG wet weight (µmol mg^{-1}) and not to the total lipid content.

S1.6.2 Oxygen radical absorbance capacity assay (ORAC)

For each ORAC assay, a standard was produced from a 200 µM 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox) solution (Sigma-Aldrich, Munich, Germany) by dilution (200, 100, 50, 25, 12.5, 6.25 µmol). PPB was used as negative control.

20 µL of the control (three replicates), of the homogenate dilutions (S1.4, two replicates per homogenate) as well as of the Trolox standard (three replicates per concentration) were pipetted into a black 96-well plates. 150 µL of a 0.106 µM fluorescein solution (Sigma-Aldrich, Munich, Germany) was added to each well and plates were incubated at 37 °C for 20 min. Finally, 30 µL of a 152.66 mM 2,2′-azobis (2-methylpropionamidine) dihydrochloride solution (AAPH, Sigma-Aldrich, Munich, Germany) were quickly added to each well and fluorescence (extinction: 485 nm, emission: 520 nm, gain: 43) was recorded at 37 °C in 1 min intervals for 12 h on a Tecan Spark 10 instrument.

For data analysis, fluorescence values of the Trolox standard and the analysed samples were plotted as a function of the measurement time (12 h) and the area under each curve (AUC) was determined for each sample. AUC results of replicates were expressed as means. AUC values of the Trolox standard were plotted as a function of the concentration and the antioxidative capacity of the homogenates were interpolated as Trolox equivalents. Correspondingly to the TBARS assay, we normalised the antioxidative capacity to the MGG wet weight (µmol mg^{-1}).

S1.7 Phagocytic activity of D. polymorpha *hemocytes*

For analysis of hemocyte phagocytic activity, we extracted 250–300 µL hemolymph from five mussels per treatment and immediately stored it in separate reaction tubes on ice. Hemocyte concentrations in the hemolymph were determined with a Neubauer improved counting chamber using trypan blue staining. Then, each hemolymph sample was diluted with *D. polymorpha* serum to a total volume of 500 µL and a concentration of 300,000 cells mL^{-1}. *D. polymorpha* serum was produced by pooling hemolymph extracted from non-exposed

individuals and directly heating it to 56 °C for at least 30 min. After centrifugation at 21,130 g for 15 min, the supernatant was directly frozen and later used as serum. 1 µm PS spheres (Fluoresbrite YG microspheres, PolyScience, Hirschberg an der Bergstraße, Germany, excitation: 441 nm, emission: 486 nm) suspended in *D. polymorpha* serum were added at a ratio of 50 spheres per hemocyte cell (7.5 µL of a 10^9 spheres mL^{-1} stock solution) to the hemolymph. Each sample was gently vortexed and directly split in two subsamples with 250 µL each, one being incubated at room temperature and the other one on ice.

After 3 h of incubation, 10 µg mL^{-1} propidium iodide (PI, Sigma-Aldrich/Merck, Taufkirchen, Germany, excitation: 482 nm, emission: 608 nm) were added and samples were directly analysed using a BD FACSVerse (BD Biosciences, Heidelberg, Germany). PI penetrates the membrane of dead cells and intercalates with nucleic acids. After excluding dead cells from the main cell population according to PI fluorescence (Fig. S4a–b, Gate P2: living hemocytes in the main cell population; Gate P3: dead hemocytes in the main cell population), we determined the number of living hemocytes with ≥ 3 spheres (Gate P6 in Fig. S4c, Gate P6 is a subpopulation from Gate P2). Only FACS analyses with ≥ 5,000 living cell counts were used. We extrapolated data from all FACS measurements to 10,000 living cells to allow data comparability between the different samples. Results from the sample exposed at room temperature were corrected for the number of hemocytes with ≥ 3 spheres from sample exposed on ice to account for particles which were adsorbed on the hemocytes cell surface, but not phagocytized. Based on the corrected data, we determined the fraction of living hemocytes with ≥ 3 spheres compared to all analyzed living hemocytes.

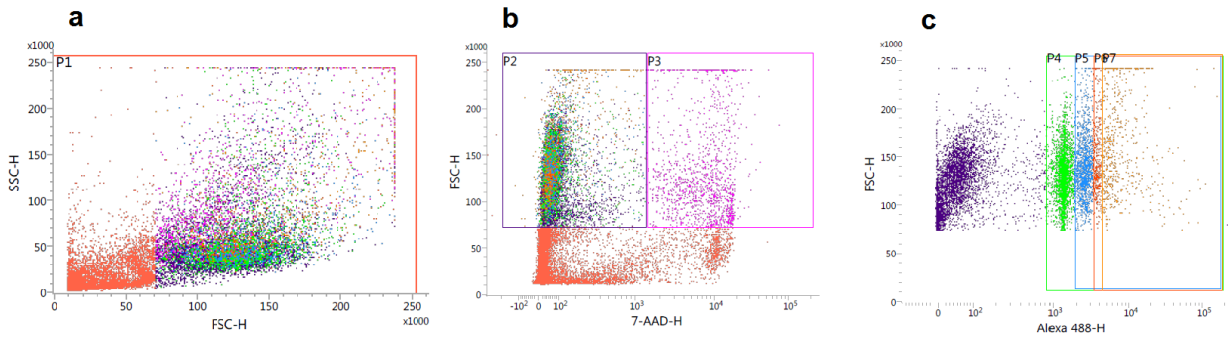

Fig. S4: Characterization of hemocytes exposed to 1 µm polystyrene spheres with FACSVerse. (a) Size (FSC) vs. granularity (SSC) of analyzed hemocytes (488 nm laser, FSC: 254.6 Voltage, SSC (filter: 481-496 nm): 324.5 Voltage), (b) Gating of living (gate P2) and dead (gate P3) hemocytes due to PI fluorescence (488 nm laser, 7-AAD (filter: 673-727, mirror: 665 LP), 300.3 Voltage), (c) Gating of living hemocytes (from Gate 2) with ≥ 1 (P4), ≥ 2 (P5), ≥ 3 (P6) or ≥ 4 spheres (P7) (488 nm laser, Alexa 488 (filter: 511-543, mirror: 507 LP), 304.5 Voltage).

S1.8 Clearance rate of D. polymorpha

Clearance activity of *D. polymorpha* was assessed by exposing mussels individually to an algae suspension (*Pseudokirchneriella subcapitata*, 5,000–5,500 relative fluorescence units (RFUs), approximately 1.48–1.64×10^6 cells mL^{-1}) and measuring chlorophyll fluorescence prior to and after the exposure (emission: 440 nm, extinction: 680 nm, GENios, Tecan, Männedorf, Switzerland). 250 mL glass jars were filled with 50 mL algae suspension and gently stirred with a magnetic stirrer. Ten *D. polymorpha* individuals were randomly selected from each of the treatments and individually exposed to the algae suspension for 45 min (beginning from the time when the mussel opened its valves). Before the introduction of the mussels as well as after 45 min, 3×200 µL of algae suspension were removed from each exposure vessel and transferred into black 96-well plates. Algae concentrations were determined using chlorophyll fluorescence (RFU). OECD medium (OECD 2016, guideline no. 242) was used as blank sample to account for background fluorescence. Replicate results were expressed as means and the clearance rate of each *D. polymorpha* individual was expressed as ΔRFU = RFU (prior to exposure) – RFU (after exposure).

S1.9 Statistics

For clearance rate, energy reserves, oxidative stress and immunological results, we ran separate general linear models (GLM) with IBM SPSS (version 25) to determine the contribution of the variables “temperature“, “MP “ as well as their interaction on the overall effect. We included temperature (14, 23, 27 °C) as fixed variable and MP (0–100,000 p mL^{-1}) as covariable in the model. In the GLM for the clearance rate, the variable MP obtained only two states (Control, 100,000 p mL^{-1} MP) and MP was, therefore, also considered a fixed variable. For mussel activity, we analyzed the data from the six consecutive observation with a general linear mixed model (GLMM) with “time“ as inner-subject variable and “temperature“ and “MP“ as between-subject variables.

Data for each endpoint was used as dependent variable and was either integrated untransformed (glycogen, clearance rate), log-transformed (protein, TBARS, ORAC), square-root transformed (total energy, total lipids) or logit-transformed (immunity, mussel activity) into the model. Normality (Shapiro-Wilks test), variance homogeneity (Levene test) and heteroskedasticity (White test) requirements were met for most data sets. In case of the GLMs for the ORAC and the immune assay results, outliers caused non-Gausian distribution and heterogenic variances, respectively. In case of the GLM for the ORAC results, outlier removal did not lead to different results indicating robustness of the GLM, while for the immune function GLM the interaction term turned significant. In the results section, however, we present the more conservative results including the outlier to avoid overinterpretation of data.

We initally included the temperature × MP interaction term in all GLMs. In case of a non-significant interaction term, we refined GLMs excluding the interaction terms. Results, however, did not differ and we, therefore, present the final results including the interaction term (except for the glycogen GLM where the interaction term is left out).

Statistic analysis of particle type effects (MP vs. DI) were performed in the same way as for MP effects. We ran GLM and GLMM with the variables “temperature“, “particle type“ (both fixed variables) and its interaction (temperature × particle type) as well as “time“ as inner-subject variable in case of the GLMM for mussel activity. In case of the GLMM, the number of degrees of freedom were too low to integrate the interaction term into the model.

Dependent variable data was included untransformed (total energy, glycogen, total lipids, TBARS, ORAC, clearance rate), square root-transformed (protein) or logit-transformed (immunity, mussel activity) into the separate models. Normality, variance homogeneity and heteroskedasticity criteria were met for all data sets except for the clearance rate at day 3. Data on clearance rates was non-normally distributed according Shapiro-Wilks test, but visual analysis did not indicate a severe violation of normality. A further exclusion of the non-significant interaction term removed a former significant effect of particle type. Again, we here present the more conservative results excluding the interaction term.

2 Results

2.1 Effects on the energy reserves

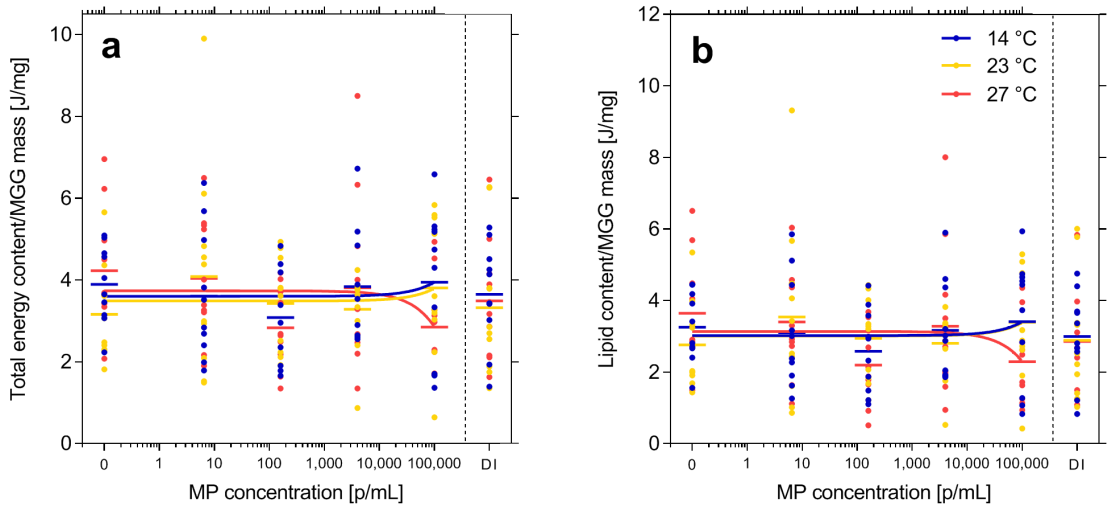

Fig. S5: Relative (a) total energy content and (b) total lipid content in the midgut gland (MGG) of *D. polymorpha* after an exposure to particle-free medium (Control, 0 p mL^{-1}), polystyrene microplastics (MP, 6.4–100,000 p mL^{-1}) or diatomite (DI, 100,000 p mL^{-1}) for 14 d at 14, 23 and 27 °C. Dots = results of each replicate, short lines = mean, regression = linear regression of the results in the MP treatment. n = 10 for each temperature and particle concentration.

2.2 Effects of microplastics versus natural particles

Tab. S2: Results of the general linear model and general linear mixed model (only between-subject results) analyzing the effects of temperature and particle type (microplastics vs. diatomite) on mussel activity, clearance activity (on day 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. * = interaction term not included in the model

Variable		Endpoints									
		Mussel activity	Clearance (3 d)	Clearance (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	2.165	1.654	5.276	0.839	10.524	7.090	1.027	8.665	8.140	1.337
	p	0.316	0.201	**0.008**	0.438	**< 0.001**	**0.002**	0.365	**0.01**	**0.001**	0.280
Particle type	*df*	1	1	1	1	1	1	1	1	1	1
	F	0.101	3.868	1.221	0.013	3.228	2.279	0.092	0.077	5.688	0.447
	p	0.781	0.054	0.274	0.909	0.078	0.137	0.763	0.782	**0.021**	0.510
Temperature × Particle type	*df*	- *	- *	2	2	2	2	2	2	2	2
	F	- *	- *	0.442	0.758	0.628	0.322	0.751	1.523	1.734	0.820
	p	- *	- *	0.645	0.474	0.537	0.726	0.477	0.227	0.186	0.452

3 References

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