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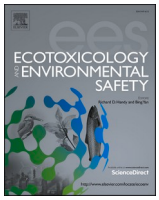

Microplastics and low tide warming: Metabolic disorders in intertidal Pacific oysters (*Crassostrea gigas*)

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ABSTRACT

Sessile intertidal organisms live in a harsh environment with challenging environmental conditions and increasing anthropogenic pressure such as microplastic (MP) pollution. This study focused on effects of environmentally relevant MP concentrations on the metabolism of intertidal Pacific oyster *Crassostrea gigas*, and its potential MP-induced vulnerability to warming during midday low tide. Oysters experienced a simulated semidiurnal tidal cycle based on their natural habitat, and were exposed to a mixture of polystyrene microbeads (4, 7.5 and 10 μm) at two environmentally relevant concentrations (0.025 μg L^{-1} and 25 μg L^{-1}) for 16 days, with tissue samplings after 3 and 12 days to address dose-dependent effects over time. On the last day of exposure, the remaining oysters were additionally exposed to low tide warming (3 °C h^{-1}) to investigate possible MP-induced susceptibility to aerial warming. Metabolites of digestive gland and gill tissues were analysed by using untargeted ^{1}H nuclear magnetic resonance (NMR) based metabolomics. For the digestive gland metabolite profiles were comparable to each other independent of MP concentration, exposure time, or warming. In contrast, gill metabolites were significantly affected by high MP exposure and warming irrespective of MP, initiating the same cellular stress response to counteract induced oxidative stress. The activated cascade of antioxidant defence mechanisms required energy on top of the general energy turnover to keep up homeostasis, which in turn may lead to subtle, and likely sub-lethal, effects within intertidal oyster populations. Present results underline the importance of examining the effects of environmentally relevant MP concentrations not only alone but in combination with other environmental stressors.

1. Introduction

Microplastic (MP) pollution has become an emerging threat in marine environments (Andrady, 2011, Avio et al., 2017, Cole et al., 2011, Galloway and Lewis, 2016) and MP have been found in several taxa, e.g., marine mammals (Nelms et al., 2019, Zantis et al., 2021, for review), birds (Zhu et al., 2019), fish (Wang et al., 2020, for review), and invertebrates (Wright et al., 2013). MP (plastic particles < 5 mm) have the same size range like planktonic food particles and sediments, which makes MP potentially bioavailable for filter feeders at the bottom of the food web. Filter-feeding bivalves transport MP via microvilli along their gills towards their mouth and ingest them (Ward, Rosa et al., 2019, Ward, Zhao et al., 2019, Woods et al., 2018). In addition to causing tissue irritations such as inflammation (von Moos et al., 2012), blockages of the digestive tract, or false satiation followed by reduced food intake (Avio et al., 2017), MP may also enter the circulatory system (Browne et al., 2008) or cells via endocytosis (Gaspar et al., 2018). The potential implications of MP exposure are disrupted cellular metabolism (Capolupo et al., 2018, Gardon et al., 2018, Gardon et al., 2020, Cappello et al., 2021) that comprise increased oxidative stress (Paul-Pont et al., 2016), immune alterations (Pittura et al., 2018), shifts of energy balance, and reproductive disorders with negative effects for offspring (Sussarellu et al., 2016, Gardon et al., 2018), all together leading to potentially negative consequences at the population level.

Within the last decade, more emphasis was given towards environmentally realistic MP concentrations in laboratory studies (Lenz et al., 2016). Dose-dependent MP effects on oysters have been investigated by using relevant MP concentrations ranging from 0.25 μg L^{-1}, 2.5 μg L^{-1} up to 25 μg L^{-1} (Gardon et al., 2018) and 0.008 μg L^{-1}, 10 μg L^{-1} up to 100 μg L^{-1} (Revel et al., 2020), the latter reporting no biological effects

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for the organisms. Most laboratory studies investigating the effects of MP on intertidal organisms such as mussels and oysters however used fully submerged experimental setups, mimicking the subtidal habitat. Depuration has been reported to be an effective method to reduce MP content in oysters (Graham et al., 2019, Choi et al., 2022), as bivalves can egest MP both via faeces and pseudofaeces (Ward, Rosa et al., 2019, Ward, Zhao et al., 2019, Woods et al., 2018). Sessile filter-feeding bivalves inhabiting the upper intertidal zone, however, regularly experience aerial exposure during low tide, in which they cannot get rid off any egested MP or MP-containing pseudofaeces from their shell cavity as they keep their valves closed to avoid desiccation. In addition to subtidal setups, investigations of MP effects on both ecological and economic key species in intertidal setups are of high importance for the evaluation of potential consequences of MP pollution in intertidal ecosystems. The Pacific oyster, *Crassostrea gigas* (Thunberg 1793), is a highly tolerant, fast growing species, which lives preferably on hard substrates from the intertidal zone up to 40 m depth (FAO, 2024). Their robustness allows them to be cultivated all over the world with their production being of large economic value. Aquaculture related translocations led to a wide global distribution of oyster species (Bromley et al., 2016), and *C. gigas* has become an invasive species in the German Wadden Sea, often outcompeting native bivalves due to their fast feeding and growing regime (Pogoda et al., 2011, Bayne, 2017).

The upper intertidal zone nevertheless is a harsh environment in which sessile inhabitants like *C. gigas* are exposed to extreme abiotic fluctuations which leads them to often live close to their physiological limits (Helmuth et al., 2002, Sokolova and Boulding, 2004, Helmuth, 2009, Bruhns et al., 2023). Apart from desiccation or salinity stress, increasing air temperatures affect intertidal communities e.g., during midday low tides in summer. The fluctuating temperature regime generated by low tide warming and returning cold water at high tide has recently been reported to induce energetically costly metabolic rearrangements in *C. gigas* (Bruhns et al., 2023). In addition, intertidal oysters seem to be susceptible to MP pollution, as high tide lines as well as intertidal mudflats and mussel banks are generally higher in MP concentrations than the open ocean (Liebezeit and Dubaish, 2012, Mathalon and Hill, 2014). During an assessment of MP abundance in organisms from the intertidal mudflats of Sylt, Germany, MP was found in 91.3 % of the investigated intertidal *C. gigas*, with median 13.1 MP counts per individual (i.e., 0.86 MP g^{-1} WW, Fischer, 2019; for comparison: global average MP content in oysters: 1.41 ± 0.33 MP g^{-1} WW, Wootton et al., 2022). Since combinations of multiple drivers may induce synergistic or antagonistic effects in organisms (Moberg, 2000, Sokolova and Lannig, 2008), subtidal experimental setups may neglect potential metabolic implications of MP on intertidal bivalves. However, to the best of our knowledge, we are not aware of any laboratory-based assessment to date that has addressed MP effects on the metabolism of *C. gigas* inhabiting the intertidal.

The objective of this study was to investigate potential effects of polystyrene MP on the metabolism of intertidal Pacific oysters using a ^{1}H nuclear magnetic resonance (NMR) based metabolomics approach. ^{1}H NMR metabolomics is an accepted tool in aquatic ecotoxicology to measure changes in metabolite levels after exposure to pollutants (e.g., Viant, 2007, Viant, 2008, Cappello, 2020) and has already been applied to address MP effects e.g., on Mediterranean mussels (Cappello et al., 2021) and zebrafish (Lu et al., 2016, Qiao et al., 2019). In particular, we investigated the following hypotheses: (I) there are dose- and time-dependent effects of polystyrene MP on the metabolite levels of intertidal *C. gigas*, (II) MP-exposed intertidal oysters are more vulnerable towards warming during midday low tide. To address these hypotheses, specimens were exposed to a simulated semidiurnal tidal cycle based on their natural habitat, and to two environmentally relevant MP levels. The MP levels were chosen to represent tidal mudflats, and especially mussel and oyster banks, in which MP concentrations can be much higher than in other coastal regions such as e.g., sandy beaches (Liebezeit and Dubaish, 2012, Lo et al., 2018). In addition, although a first screening of polymer composition revealed that polyethylene, polyethylene terephthalate and polypropylene were more abundant than polystyrene (Fischer, 2019), the latter was omnipresent in sediment and surface water samples analysed from the Southern North Sea (Lorenz et al., 2019) as well as in several invertebrates at the Sylt sampling site (Fischer, 2019). This study aimed at filling the existing knowledge gaps regarding MP-induced effects on intertidal oysters. Since oysters, with respect to MP uptake, perform size selection criteria rather than selection based on polymer type (Mladinich et al., 2022), the MP type and morphology used in this study were based on previous MP exposure studies on subtidal oysters (e.g., Gardon et al., 2018, Gardon et al., 2020, Sussarellu et al., 2016). Within our defined laboratory conditions, this approach aimed to simulate intertidal habitat conditions as realistically as possible and by using ^{1}H NMR spectroscopy, to unravel potential effects of MP exposure on the metabolite levels of intertidal Pacific oysters.

2. Materials and methods

2.1. Target organism

Adult oysters (90–120 mm) were collected during low tide from intertidal mudflats along the Northern coast of Sylt, Germany (N 55° 01' 37", E 8° 25' 59"; part of the Schleswig-Holstein Wadden Sea National Park) in September 2019 (16 °C water temperature; 30.7 PSU), and transported to the aquarium holding systems of the Alfred Wegener Institute (AWI) in Bremerhaven. The oysters were mechanically cleansed from epifauna and depurated for two weeks in aerated tanks within a recirculating natural seawater system (16 ± 1 °C; 32 PSU). Water parameters were regularly monitored and oysters were fed twice a week with a commercially available algal solution (SA Premium Blend Live Marine Phytoplankton) following the product recommendations. All animals used for this research were treated in accordance with the respective national and institutional animal welfare guidelines and permits.

2.2. Polystyrene microplastic particles

Groups of oysters were exposed to different concentrations of polystyrene MP beads to investigate potential dose-dependent effects: no MP (control; CTR), low MP at a concentration of 0.025 μg L^{-1} (LMP), and high MP at a concentration of 25 μg L^{-1} (HMP). The MP concentrations were selected based on environmentally relevant MP concentrations in coastal regions with fine-grained sediments such as mudflats (e.g., Liebezeit and Dubaish, 2012, Dubaish and Liebezeit, 2013, Mathalon and Hill, 2014, Lo et al., 2018), from which the HMP concentration has been reported to cause biological effects in oysters (e.g., Sussarellu et al., 2016, Gardon et al., 2018). Based on the particle retention rates of oysters (Ward and Shumway, 2004), a mixture of MP diameters of 4 μm, 7.5 μm and 10 μm was used for MP exposure (Bangs Laboratories Inc, Polysciences Europe). The MP were supplied by the manufacturer in aqueous suspensions (1 % w/v) at concentrations of 2.377 × 10^8 MP mL^{-1} (4 μm), 4.596 ×10^7 MP mL^{-1} (7.5 μm), and 1.832 × 10^7 MP mL^{-1} (10 μm), respectively. The suspensions were mixed for preparing an initial stock suspension of 25000 μg L^{-1} in milliQ water, containing all MP diameters at equal weight. This stock served as the HMP working suspension. To obtain the LMP working suspension, the HMP working suspension was further diluted by a factor of 1000. During the experiment (see Section 2.3), MP treatments were prepared as follows: 1 mL of the respective LMP or HMP working suspension was added to every LMP or HMP incubation jar containing 1 L natural seawater to finally achieve the LMP and HMP treatment exposure concentrations (i.e., 0.025 μg L^{-1} and 25 μg L^{-1}, respectively).

2.3. Experimental setup and tissue sampling

For the experimental exposure, 135 oysters (104.5 ± 22.5 g; 10.2 ± 0.7 cm) were placed individually into single 1.5 L glass incubation jars (i.e., one oyster per jar) filled with 1 L natural seawater (originated from open North Sea; pre-filtered with protein skimmer to remove bacteria, proteins, and fine particles; salinity: 32 PSU; temperature 16 °C). Glass lids on top of the jars prevented cross-contamination and a glass tube placed through the lid provided continuous aeration and water movement. In order to simulate their natural habitat, a daily tidal cycle was mimicked by manually filling and draining the incubation jars twice a day, following a 9 h immersion: 3 h emersion rhythm (Fig. 1). During the high tide phase of the scenario, the oysters were completely immersed in 1 L natural seawater (11:00 – 20:00 and 23:00 – 08:00, respectively). During the low tide phase, water was slowly sucked out of the jars and the oysters were exposed to air (08:00 – 11:00 and 20:00 – 23:00, respectively). Each oyster was fed twice a day during high tide (11:00 and 23:00, respectively) with a mixed diet of *Nannochloropsis oculata*, *Phaeodactylum tricornutum* and *Chlorella sp.* (cell size range of 2 – 20 μm) at a concentration of 10000 cells mL^{-1} (SA Premium Blend Live Marine Phytoplankton). The respective water and air temperatures (16 ± 1 °C) during high and low tide scenarios were continuously monitored via HOBO loggers (Onset, USA). After seven days of acclimation to the experimental setup conditions, 12 oysters were sacrificed to represent the oysters' physiological conditions prior to the start of the MP exposure. The remaining oysters were evenly divided into three treatment groups (n = 41 each): control (CTR; 0 μg L^{-1}), low MP (LMP; 0.025 μg L^{-1}), high MP (HMP; 25 μg L^{-1}). The respective MP concentrations were achieved by adding 1 mL of the LMP or HMP stock suspensions (see Section 2.2) to the LMP or HMP treatment jars twice a day during feeding (Fig. 1). Over the course of the experiment (16 days), room temperature was kept constant (16 ± 1 °C) and a photoperiod of 12 h:12 h was applied. To address the hypotheses, the experiment was divided into two parts. For examining potential dose-dependent MP effects on metabolites over time, oyster tissue samplings (n = 12 per treatment) occurred after 3 days (3d) and 12 days (12d) of exposure. For addressing potential MP-induced vulnerability towards low tide warming, the remaining oysters (n = 10 per treatment) experienced aerial warming during the final low tide simulation at the last day of the exposure period (from 16 to 26 °C, at a rate of 3 °C h^{-1} in a temperature-controlled cabinet). After 3 h of aerial warming, the oysters were again fully immersed in 1 L of natural seawater at 16 °C (i.e., simulating the return of high tide) and provided with food and MP for the respective treatments prior to sampling. At all sampling points, the sampling procedures started 4 h after high tide induction (i.e., immersion of the oysters in natural seawater including food and respective MP treatment) to ensure that the oysters had time to metabolically recover from air exposure (see Fig. 1). At each sampling point, 10–12 oysters of each treatment group were randomly selected for tissue analyses, weighed, measured, and dissected on ice. Tissues (digestive glands and gills) were shock-frozen in liquid nitrogen and stored at – 80 °C until further analyses.

2.4. Metabolic profiling using NMR spectroscopy

Metabolites of digestive glands and gills were extracted following the methanol-chloroform extraction method for small samples as applied in Tripp-Valdez et al. (2017), with minor modifications for *C. gigas*. Briefly, 50–60 mg frozen tissue was homogenised in an ice-cold mix of 450 μL methanol and 140 μL milliQ water via Precellys 24 (Bertin Technologies, France) for 20 s at 6000 rpm and 4 °C. An ice-cold mix of 450 μL chloroform and 450 μL milliQ water was added to the homogenate, vortexed, incubated on ice for 10 min, and centrifuged for 10 min at 3000 g and 4 °C. The resulting upper layer was dried overnight using a SpeedVac (RVC 2–33 IR, Christ GmbH, Germany) and the dried pellets were resuspended in 1-fold of tissue weight deuterated water (D_2O) containing 3-(trimethylsilyl)propionic-2,2,3,3-d_4 acid sodium salt (TSP; 0.05 wt%; Sigma Aldrich, St. Louis, USA) as internal standard and chemical shift reference. Untargeted metabolic profiling based on ^1H NMR spectroscopy was performed on a 400 MHz ultra-shielded vertical 9.4 T NMR wide bore spectrometer equipped with Advance III HD electronics (Bruker-BioSpin GmbH, Germany) using a 1.7 mm triple tuned ^1H-^{13}C-^{15}N NMR probe. 1D-CPMG (Carr-Purcell-Meiboom-Gill)--spectra with water presaturation (protocol cpmgpr1d) were acquired by using TopSpin 3.2 software (Bruker-BioSpin GmbH, Germany) with acquisition parameters as published in Georgoulis et al. (2022). Chenomx NMR suite 8.1 software (Chenomx Inc., Canada) was further used for phase, baseline, and shim correction as well as calibration to the TSP signal (TSP at 0.0 ppm as internal reference). Specific metabolites were identified in the ^1H NMR spectra from the tissue samples using the internal Chenomx database, in combination with databases taken from the literature (Tikunov et al., 2010, Cappello et al., 2018, Götze et al., 2020). Metabolite quantification was further assessed by using the Chenomx integration routine, with the TSP signal integral being calibrated to the given concentration of 3.2 mM.

2.5. Statistics

Metabolite analyses result from 10–12 individually analyzed biological replicates sampled at each time point and treatment. Nalimov outlier test was used to detect outliers with a significance level of P = 99.9 % (Noack, 1980). Results are presented in millimolar (mM) concentrations as means ± standard deviations (SD). Multivariate statistical analyses were performed using the online platform MetaboAnalyst (MetaboAnalyst 6.0; Xia and Wishart, 2016). Briefly, the metabolite concentration data of identified metabolites were *log10* transformed to obtain stabilised variance across the detected concentrations. Principal Component Analysis (PCA) was performed to visualise the multivariate metabolomics data in scores plots, clustering together samples that have similar metabolic profiles. Univariate statistical analyses were performed using PRISM Graph Pad software (version 10). Prior to statistical analysis, the metabolite data were tested for normality and were *ln*

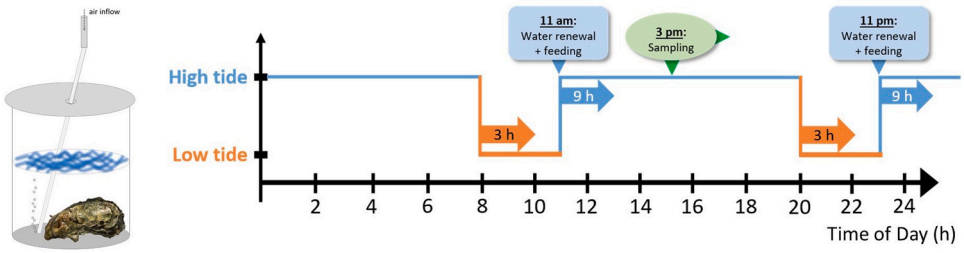

Fig. 1. Schematic presentation of the tidal cycle and sampling time used during the incubation experiment. 135 oysters were individually placed into single 1.5 L glass incubation jars prepared with glass tubes providing continuous aeration on a shelf in a temperature controlled room (room temperature: 16 ± 1 °C). During the high tide period, the incubation jars are filled with 1 L natural seawater, enriched with phytoplankton and different doses of MP. On sampling days, tissue samplings took place at 3 pm.

transformed if needed. Parametric two-way ANOVA was used to address effects of the MP treatments on relevant metabolites within each sampling point, within treatments over time (3d vs. 12d), as well as before and after the warming scenario. These were followed by Tukey's post-hoc tests for multiple comparisons of group means to identify significant differences between treatments, respectively. If assumptions were not met, a non-parametric Kruskal-Wallis test followed by a Dunn's post-hoc test was used to identify significant effects between treatments. For all statistical tests, the significance level was set to $P \leq 0.05$.

3. Results

3.1. Tissue-specific dose-dependent MP effects over time

Both digestive gland and gill ^{1}H NMR spectra were dominated by two organic osmolytes, namely taurine and betaine. These osmolytes are typically accumulated in bivalve tissues to counteract changes in salinity via osmoregulatory processes. Other prominent classes included amino acids and its derivates (e.g., homocysteine, threonine, glutamate, glutamine), metabolites related to the energy metabolism (e.g., fumarate, UDP-glucose, lactate), and osmolytes (e.g., hypotaurine, betaine). The metabolite profiles of oysters exposed to different MP

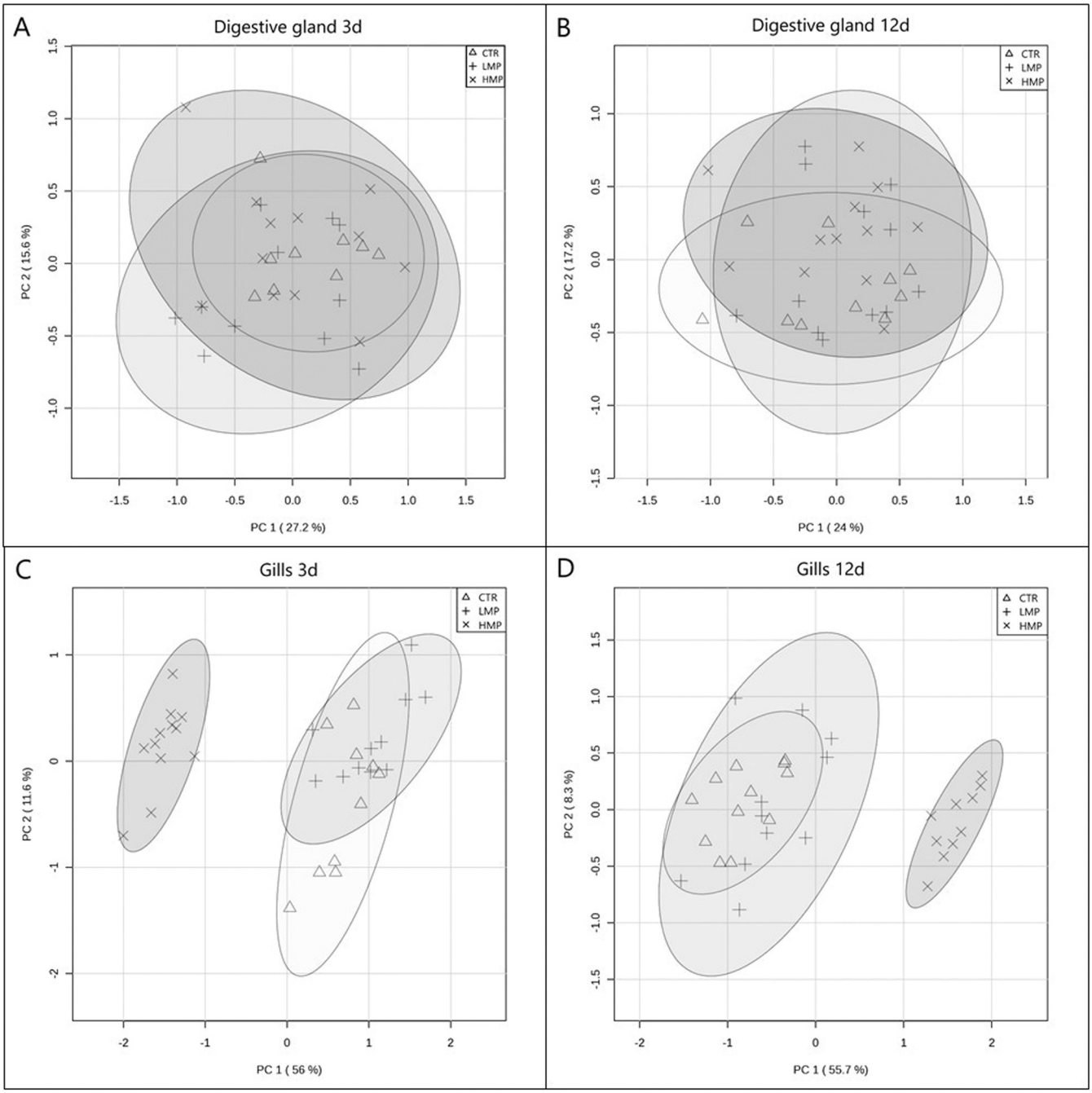

Fig. 2. PCA scores plots of the ^{1}H NMR spectra from all samples (n = 9–12 per treatment), showing the impact of MP exposure on the metabolic profile in digestive gland (**A**, **B**) and gill (**C**, **D**) tissues of *C. gigas* dependent on MP concentration and exposure time. 3d: Sampling after 3 days of exposure. 12d: Sampling after 12 days of exposure. CTR: Control; LMP: low MP exposure (0.025 μg L^{-1}); HMP: high MP exposure (25 μg L^{-1}). Ellipses correspond to a confidence interval of 95 %.

concentrations over time determined by the ^{1}H NMR spectra showed tissue-specific differences. In digestive gland tissue, 31 metabolites were identified. The metabolic profiles of oysters' digestive glands were comparable to each other independent of MP concentration or time, indicated by overlapping of all treatment groups in the PCA scores plots (Fig. 2 A, B). In gills, 26 metabolites were identified, and the metabolic profiles were significantly affected by MP exposure. Therefore, further analysis focused on examining the differences in gill metabolites in greater detail. Gill metabolic profiles of control oysters and oysters exposed to low MP were comparable to each other over time, represented by overlapping PCA scores plots at both sampling points (Fig. 2 C, D). In contrast to low MP exposed oysters, the gill metabolic profiles of oysters exposed to high MP showed a clear clustering from profiles of control oysters along the PC1 axis (explaining 56 % and 55.7 % of variance at 3d and 12d, respectively). The metabolite profiles of control oyster gills remained comparable over time since all control groups overlapped in the corresponding PCA scores plot (data not shown).

Following the results of the ANOVA, the most affected metabolite levels were grouped into three major classes (amino acids and derivatives, metabolites related to energy metabolism, osmolytes). These were plotted against time, and metabolite levels at each time point were compared to control levels (Fig. 3). Statistics revealed that MP induced significant changes in all tested metabolite levels ($P < 0.0001$), while they remained independent from time, as already indicated by the PCA (except for fumarate, $P = 0.0140$). Both homocysteine (Tukey's, 3d & 12d: $P < 0.0001$) and threonine (Dunn's, 3d & 12d: $P < 0.0001$) levels significantly increased in oysters exposed to high MP, while levels in low MP exposed oysters remained constant (Fig. 3A). A similar pattern was recognized for glutamate levels (Tukey's, 3d & 12d: $P < 0.0001$), whereas the counterpart amino acid glutamine exhibited a significant decrease in concentration in high MP exposed oysters (Dunn's, 3d: $P = 0.0002$, 12d: $P = 0.0208$). In the group of energy metabolism related

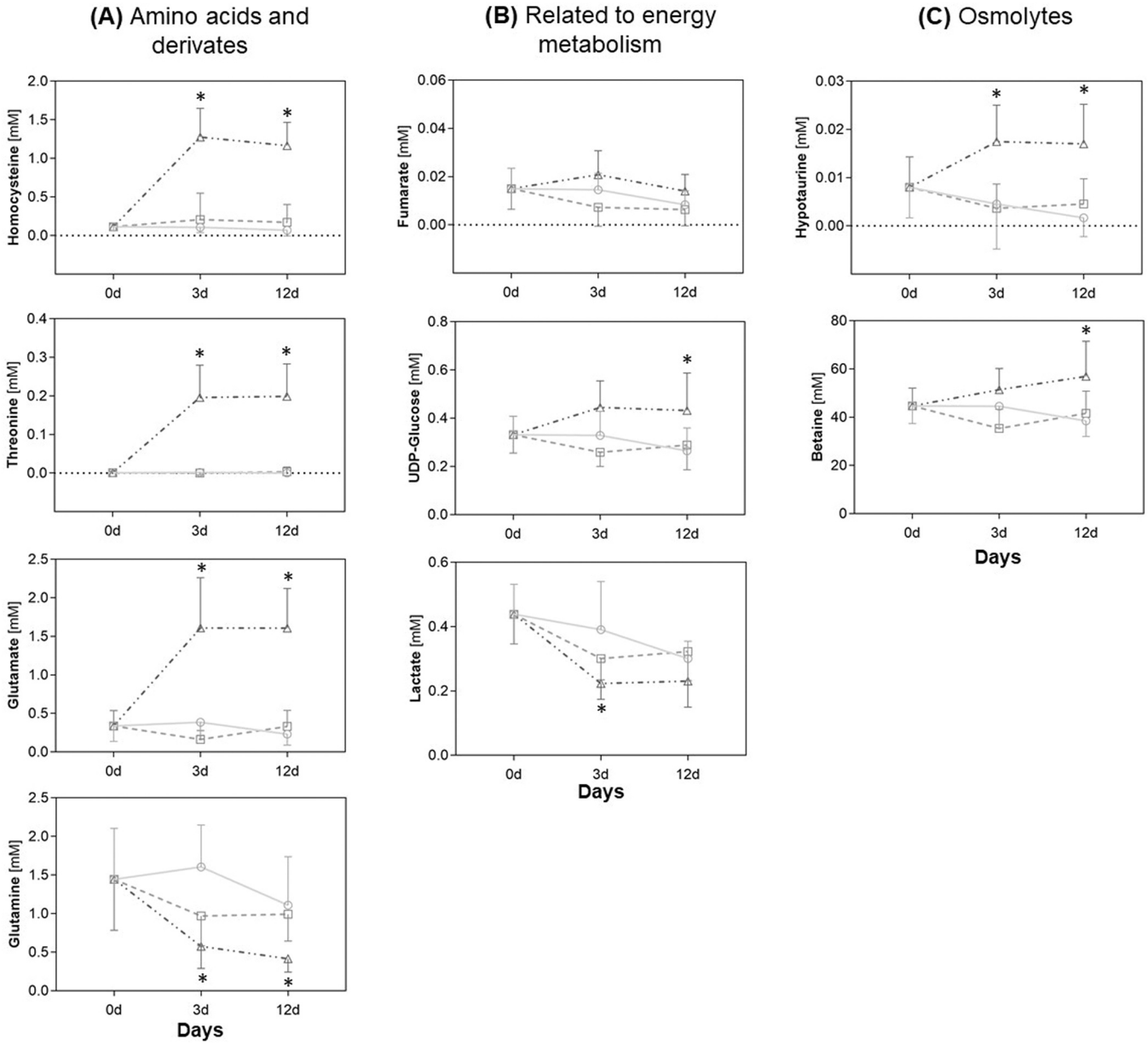

Fig. 3. Metabolite concentrations in gill tissue of *C. gigas* over exposure time to different MP concentrations. (**A**) Amino acids and derivates; (**B**) metabolites related to the energy metabolism; (**C**) osmolytes. Control: solid line (circles); low MP exposure (0.025 μg L^{-1}): dashed line (squares); high MP exposure (25 μg L^{-1}): dashed-dotted line (triangles). Data are given in means ± SD (n = 9–12). Asterisks indicate significant differences in metabolite levels of MP exposed oysters relative to control at same time points (Tukey's or Dunn's; $P \leq 0.05$).

metabolites, UDP-glucose levels increased in high MP exposed oysters, but were significantly higher than those of control oysters only after 12d of exposure (Tukey's, 12d: $P = 0.0065$). In contrast, lactate levels in high MP exposed oysters were significantly lower than in control oysters after 3d of exposure (Tukey's, 3d: $P = 0.0003$) (Fig. 3B). Osmolyte concentrations showed a clear trend towards an increase in high MP exposed oysters (Fig. 3C). Hypotaurine levels increased significantly at 3d and remained stable thereafter (Dunn's, 3d: $P = 0.0034$, 12d: $P = 0.0007$), while betaine levels steadily increased over time, with the highest concentration being significantly higher than control oysters at 12d (Tukey's, 12d: $P = 0.0004$).

3.2. MP-induced vulnerability towards warming

To unravel potential effects of MP exposure on the vulnerability of oysters towards a low tide warming event, corresponding metabolite data were analysed by using PCA. Since the metabolic profiles of oysters' digestive glands were comparable to each other independent of warming (data not shown), the focus was placed on gills. The PCA scores plot of gill metabolites revealed a clear separation of oysters after low tide warming (16–26 °C) from oysters without warming (16 °C), independent from MP concentration (PC1: 59.9 %, PC2: 6.3 %; Fig. 4). In addition, the group of oysters without warming exposed to high MP overlapped with the groups of oysters exposed to warming, indicating that high MP exposure induced a similar metabolic response in oyster gills as warming (Fig. 4).

The corresponding ANOVA revealed that the most affected metabolites from the warming scenario were grouped into the same three major classes as described above for MP treatment over time (i.e., amino acids and derivates, metabolites related to energy metabolism, osmolytes; see Section 3.1). Except for glutamine and lactate, all other depicted metabolite levels were clearly increased in oysters exposed to warming, regardless of MP exposure. In addition, the group of oysters without warming but exposed to high MP followed the same trend, while

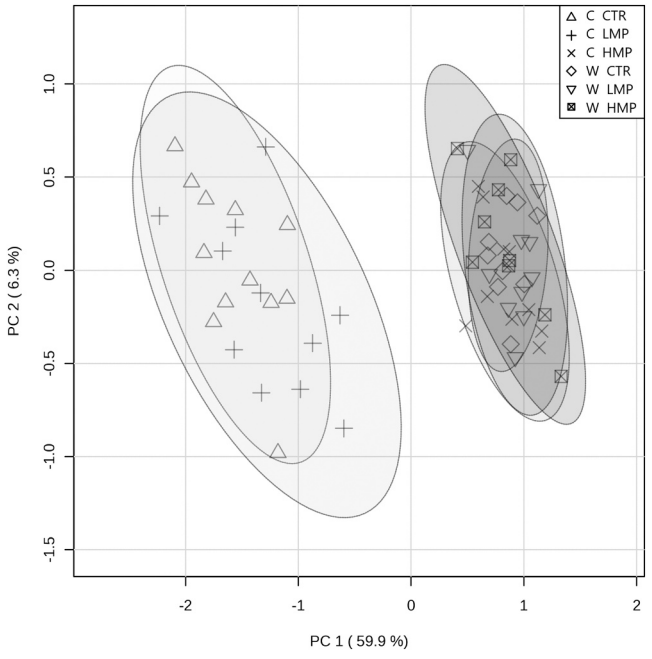

Fig. 4. PCA scores plot of ^{1}H NMR spectra of the gill metabolic profile of *C. gigas* from different MP treatments before and after a single low tide warming event. C: Before warming, represented by the 12d treatment groups at ambient 16 °C; W: After low tide aerial warming scenario from 16 to 26 °C at a rate of 3 °C h^{-1}; CTR: Control; LMP: low MP exposure (0.025 μg L^{-1}); HMP: high MP exposure (25 μg L^{-1}). N = 9–12 per treatment. Ellipses correspond to a confidence interval of 95 %.

control oysters and low MP exposed oysters at ambient temperature shared very similar metabolite levels (Fig. 5). Warming appeared to be the stronger factor compared to MP. MP alone only had significant effects on homocysteine, glutamate, glutamine, and lactate levels ($P < 0.0001$, $P = 0.0009$, $P = 0.0020$, $P = 0.0478$, respectively), whereas warming induced significant changes in all metabolite levels ($P = 0.0038$ for Betaine; $P < 0.0001$ for others). Although MP alone did not significantly change the levels of betaine and fumarate, its interaction with warming induced significant changes ($P = 0.0002$, $P = 0.0202$, respectively), as was detected for homocysteine, glutamate, and glutamine ($P < 0.0001$, $P = 0.0009$, $P = 0.0182$, respectively).

4. Discussion

This study sought to provide new information about dose- and time-dependent effects of polystyrene MP beads on digestive gland and gill metabolite levels in intertidal *Crassostrea gigas*, and tested whether MP exposure may increase the oysters' vulnerability towards low tide warming. For doing so, untargeted ^{1}H NMR-based metabolic profiling was used, which is an applied approach in aquatic ecotoxicology for addressing metabolic effects of environmental drivers as well as pollution on intertidal bivalves (Lannig et al., 2010, Tikunov et al., 2010, Cappello et al., 2020, Cappello et al., 2021, Matoo et al., 2021, Georgoulis et al., 2022).

The metabolic profiles of the digestive glands were comparable regardless of MP concentration and exposure time. The selected MP size classes were defined within the preferred particle selection range of *C. gigas*, which has a selection efficiency of up to 100 % for 5–6 μm particles (Ward and Shumway, 2004). Larger particles have a longer gut retention time than smaller particles (Van Cauwenberghe and Janssen, 2014), however, particles of this size range are not thought to enter cells in oysters (Sussarellu et al., 2016, Gaspar et al., 2018), but rather pass through the digestive system. Though the current study lacks histological tissue analyses for MP tracking, histological slides from gut samples of *C. gigas* from a previous study showed no MP accumulation after two, five, and eight weeks of exposure to a mixture of 2 μm and 6 μm polystyrene MP beads (23 μg L^{-1}; Sussarellu et al., 2016). The authors therefore assumed a high egestion potential of spherical polystyrene MP of this size class. Similar observations were made in the same species (*C. gigas*) when exposed to a mixture of polyethylene and polypropylene particles (0.4 – 500 μm; 0.008 μg L^{-1}, 10 μg L^{-1}, 100 μg L^{-1}; Revel et al., 2020). Since the exposure conditions in our study were comparable to those mentioned, we can assume that *C. gigas* exhibits the same egestion efficiency in the intertidal zone. Given that there was no detectable shift in the metabolite levels of the digestive glands, we further conclude that an emersion time of three hours does not exacerbate intestinal toxicity of potentially ingested MP. Future investigations using e.g., *in vivo* nuclear magnetic resonance imaging (MRI) may provide further information about the fate of ingested MP in intertidal *C. gigas* during prolonged emersion periods.

In contrast to the digestive gland, the metabolite profiles of gills provided a strong dose-dependent MP effect. Gill tissue is often more affected and responsive to stress than the digestive gland (e.g., Almeida et al., 2005). Since oysters retain seawater in their mantle cavity during shell closure at low tide and thus presumably continue to be exposed to either external MP in seawater or egested and accumulating MP, or both, it might be worth to clarify if and to what extent the period of emergence could exacerbate the effect of MP on the gills but not digestive gland. The PCA showed a clear separation of metabolic profiles of high MP exposed oyster gills from both control oysters and low MP exposed oysters. These findings suggest that control and low MP exposed oyster gills maintain similar metabolomic pathways whereas the metabolic profile of high MP exposed oysters changed significantly. The MP-induced alterations consisted of shifts in amino acid metabolism, energy metabolism and osmoregulatory processes. The simulated low tide warming scenario induced the same metabolic responses as the high

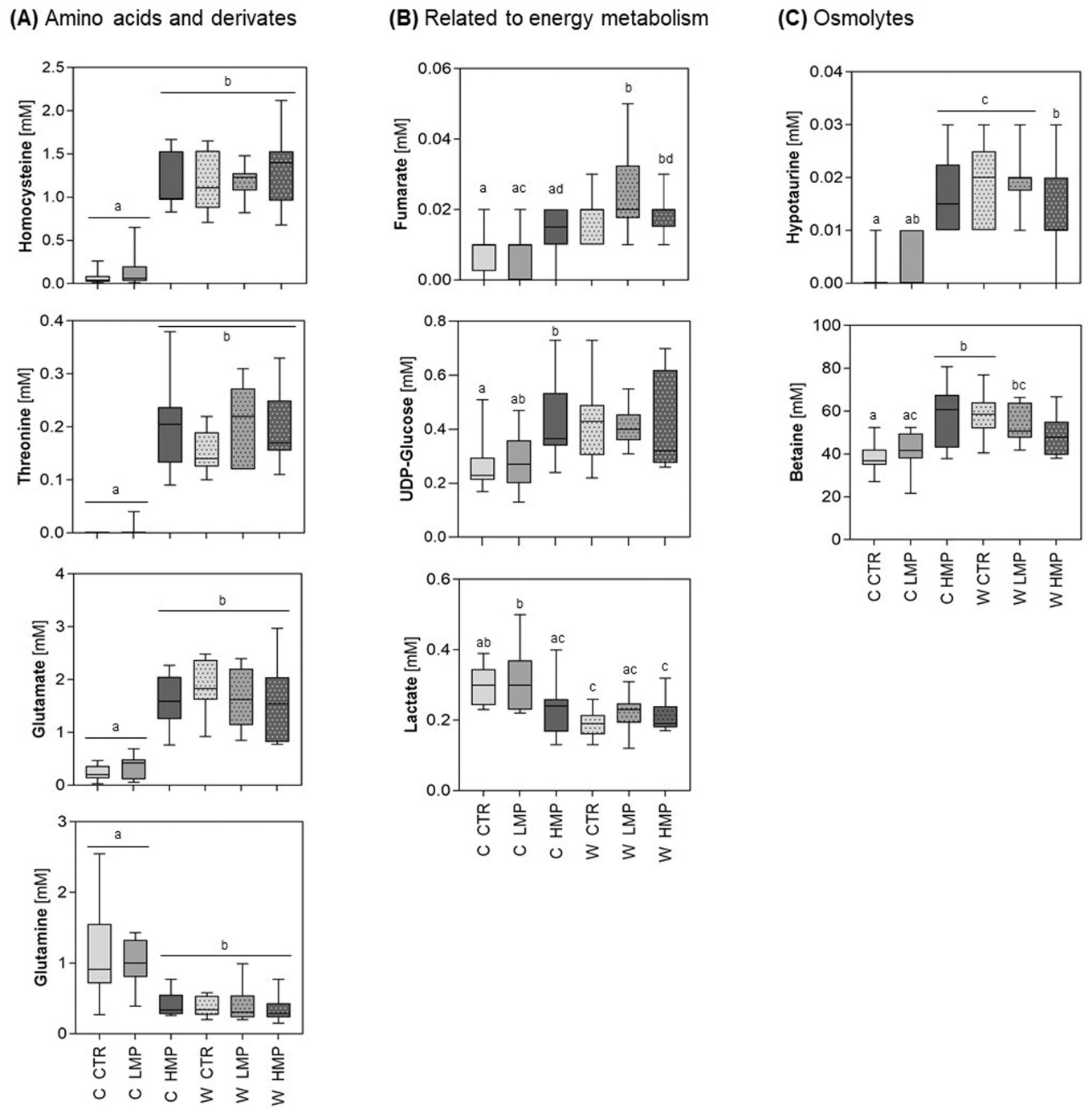

Fig. 5. Significant changes in the gill metabolic profile of *C. gigas* from different MP treatments before and after a low tide warming event. (**A**) Amino acids and derivates; (**B**) metabolites related to the energy metabolism; (**C**) osmolytes. C: Before warming, represented by the 12d treatment groups at ambient 16 °C (no filled pattern); W: After low tide aerial warming scenario from 16 to 26 °C at a rate of 3 °C h^{-1} (filled pattern); CTR: Control; LMP: low MP exposure (0.025 μg L^{-1}); HMP: high MP exposure (25 μg L^{-1}). Data are given in medians ± min/max (n = 9–12). Different letters indicate statistically significant differences between treatment groups (Tukey's or Dunn's; $P \leq 0.05$).

MP exposure at ambient temperature whereby, not only the type of metabolites, but also the corresponding metabolite levels were comparable. We thus conclude that both emersive warming and high MP concentration triggered a very similar cellular stress response. This suggests that it may not be a gradual response, but rather a non-specific on-off response to counteract the induced cellular stress. The reduction of reactive oxygen species (ROS) accumulation in gill tissue of *C. gigas* for example has been described as a conserved response mechanism to different stressors such as high temperature, low salinity, and air exposure (Zhang et al., 2015). The present results imply that the combined effects of MP and warming on metabolites seem not synergistic or antagonistic due to the similar stress response. The cellular stress response is a complex mechanism of different levels from macromolecular repair and stabilisation, energy repartitioning and cell cycle arrest to programmed cell death (Kültz, 2005). To further evaluate potential MP-induced susceptibility of intertidal *C. gigas* to low tide warming events, we propose to perform an integrative multi-biomarker analysis to decipher the underlying mechanisms and the extent of the overall

stress response.

Considering the consistent metabolic changes of high MP exposure at ambient temperature and of warming irrespective of MP load, the results are discussed in a combined approach. Current literature states that both heat stress (Wang, Dong et al., 2018, Wang, Ren et al., 2018) and MP exposure (Trestrail et al., 2020, Li et al., 2022) may induce oxidative stress in invertebrates such as bivalves. Previous studies reported that MP exposure induced the formation of intracellular ROS in e.g., marine copepods (*Paracyclopina nana*) (Jeong et al., 2017) and haemocytes of blood clams (*Tegillarca granosa*) (Tang et al., 2020). The accumulation of ROS may result in inflammatory reactions as has been reported for blue mussels (*Mytilus edulis*) (von Moos et al., 2012) and Asian clams (*Corbicula fluminea*) (Fu et al., 2022). Even low MP exposure concentrations (10 μg L^{-1}) led to inflammatory tissue alterations such as slight cilia degeneration of branchial epithelial cells of *C. fluminea* (Fu et al., 2022). Our study lacks histological examinations of gill tissue, however, for our study organism *C. gigas*, no evidence of significant tissue irritation was observed for both short-term (10 days; Revel et al., 2020) and long-term (2 months; Sussarellu et al., 2016) MP exposure using a similar range of MP concentrations as in this study.

Apart from inflammatory tissue alterations, the MP-induced cellular oxidative stress leads to the activation of the antioxidant system (Trestrail et al., 2020), represented by e.g., elevated glutathione levels in marine bivalves (Cappello et al., 2021, Li et al., 2022). Glutathione is involved in numerous protective detoxification reactions and in the maintenance of cellular redox status and works as a non-enzymatic intracellular antioxidant to protect cells from oxidative stress by reducing the formation of free oxidative radicals (Meister and Anderson, 1983, Lushchak, 2012). Glutamate is an important metabolite involved in the production of glutathione. Levels of glutamate, betaine and homocysteine were significantly increased, which is in line with previous findings of increased glutamate and homocysteine levels in heat-stressed Mediterranean mussels (*Mytilus galloprovincialis*) (Georgoulis et al., 2023). Betaine, an important osmolyte in marine bivalves, supports cellular stability in several ways. Besides its role in maintaining the osmotic balance, betaine is an important donor for methyl-groups to synthesise homocysteine, another important metabolite of the transmethylation pathway. The increased glutamate levels together with the affected (homo-)cysteine-methionine metabolism represented by the significant increase in homocysteine and betaine levels may therefore indicate upregulated glutathione synthesis to maintain the cellular antioxidant status (Lushchak, 2012) and counteract ongoing oxidative stress in oyster gills exposed to high MP and low tide warming.

Increased threonine and hypotaurine levels found in our study support this line of thought. Threonine acts as an endogenous antioxidant to protect cells against various toxic xenobiotics. Hypotaurine serves as a cytoprotective antioxidant that scavenges harmful radicals through the formation of taurine, which has been reported to accumulate in heat-stressed mussels (Georgoulis et al., 2022). Among others, glutamate, taurine, and threonine also act as neurotransmitter substances. MP exposure has been shown to induce neurotoxic disruptions in bivalves (Tang et al., 2020, Tlili et al., 2020) and other aquatic animals (Xiong et al., 2023, for review). Glutamate-mediated neurotoxicity appears through excessive synaptic glutamate accumulation which results in neuronal overactivation and has been discussed for marine bivalves exposed to different pollutants (green mussels, Wu and Wang, 2010; Manila clams, Liu et al., 2011). In addition, the authors of a recent study on oysters *Pinctada fucata martensii* suggested potential glutamate neurotoxicity mediated by the exposure to polyvinyl chloride MP (Lu et al., 2024), so that the detected shifts in the glutamate-glutamine metabolism together with the high levels of threonine may indicate possible neurotoxic effects of high MP exposure and warming.

The detected shifts in glutamine and glutamate levels together with the shifts in metabolites directly related to energy metabolism further suggest changes in the gill energy metabolism. Glutamate levels strongly increased while glutamine levels significantly decreased, thus effects on ammonia homeostasis become evident. Glutamate is an anaplerotic amino acid for α-ketoglutarate, an intermediate of the tricarboxic acid (TCA) cycle, so that the elevated glutamate over glutamine level may indicate an increased catabolic use of amino acids filling up the TCA cycle. Given that the decreased glutamine/glutamate ratio suggests a shift to increased protein catabolism over protein anabolism and thus cell proliferation, the data indicate an increased energy demand probably provoked by the activation of cellular stress defence mechanisms. Although they were not significant for all groups due to high intraspecific variation, there is a trend of rising levels of UDP-glucose and fumarate in oyster gills exposed to high MP and to warming irrespective of MP load while lactate levels decreased (Fig. 5B). An increase of fumarate and UDP-glucose levels were also reported in a recent study on the effect of heat hardening on metabolite driven thermoprotection in *M. galloprovincialis* (Georgoulis et al., 2023). Fumarate is an intermediate of the TCA cycle and a key metabolite for late anaerobiosis. Anaerobiosis and metabolic depression are common tools for oysters facing periods of hypoxia during low tide, however, tissue sampling took place after four hours recovery time from low tide to avoid measuring metabolic shifts induced by just the low tide scenario instead of MP exposure and warming. Since lactate did not accumulate accordingly, the increasing levels of fumarate may indicate up-regulated work of the complex II of the respiratory chain, while the high glutamate levels fuel the TCA cycle via α-ketoglutarate. This is in line with a recent study reporting strongly up-regulated aerobic metabolism during recovery from cyclic hypoxia in soft shell clams (*Mya arenaria*) (Ouillon et al., 2023).

Given that the gills of control and low MP exposed oysters did not show the same metabolic shifts, the increased glutamate levels together with the altered fumarate, UDP-glucose, and lactate levels indicate changes in the energy budget of high MP and of warming exposed oysters. The energy budget comprises all expenditures required for the general maintenance of an organism to maintain homeostasis, growth and reproduction. Under stress, some energy resources are shifted to general stress reactions in order to keep up homeostasis, which may lead to a lack of energy available for growth and/or reproduction (e.g., Sokolova et al., 2012, Sokolova, 2013). Under pollutant stress, *M. galloprovincialis* increased its cellular levels of glucose and the intermediates of the TCA cycle which may indicate either potential mobilisation of energy reserves or metabolic compensatory mechanisms to counteract the metabolic needs under unfavourable conditions (Cappello et al., 2013). UDP-glucose is formed via glycogenesis and serves, among other things, as an important intermediate product for the biosynthesis of glycogen, which is primarily used in bivalves as the main energy source under stress rather than fatty acids (Erk et al., 2011). The decreasing lactate levels together with increasing levels of UDP-glucose therefore may imply the stimulation of gluconeogenesis in order to assist replenishing glycogen stores, as has been suggested for hypoxia-tolerant *M. arenaria* (Ouillon et al., 2023) and *C. gigas* (Bruhns et al., 2023). Two months exposure to 0.25 μg L^{-1} and 25 μg L^{-1} polystyrene MP resulted in a clear dose-dependent decrease of individual energy budget of Pearl oysters (*Pinctada margaritifera*) (Gardon et al., 2018). While both ingestion rate and respiration rate remained unaffected, the assimilation efficiency significantly decreased with increasing MP concentrations, leading to a negative Scope For Growth in *P. margaritifera* exposed to 25 μg L^{-1} MP, reflecting a loss of energy reserves and as such a potential loss of available energy for somatic growth. This represents a clear sub-lethal impact at the individual level, which may even have negative consequences at the population level due to the potential impairment of reproduction (Gardon et al., 2018).

The metabolic shifts detected in the present study indicate that the intertidal oysters were stressed in a similar manner, both from high MP exposure, i.e., 25 μg L^{-1}, and from low tide warming irrespective of MP load. Based on these results, we propose that the toxicity of MP is mainly raised from the induction of ROS generation (Prinz and Korez, 2020), as typically known for heat stress, respectively (e.g., Wang, Ren et al.,

2018, Wang, Dong et al., 2018). Oysters inhabiting intertidal mudflats are prone to be regularly exposed to higher MP concentrations than in open waters (Liebezeit and Dubaish, 2012, Mathalon and Hill, 2014), which may result in negative consequences on the energy budget, as indicated by the detected metabolic shifts. In addition, intertidal oysters are regularly exposed to warming during midday low tide at a rate of 3 $^{\circ}C\,h^{-1}$, which induces the same metabolic shifts that may finally lead to energy budget deficiency. Present results underline the importance of examining the effects of environmentally relevant MP concentrations not only alone but in combination with other environmental stressors of both abiotic (e.g., salinity stress, desiccation, warming) and biotic (e.g., predation) natures. A multi-stressor, multi-biomarker analysis is therefore essential to assess a potential synergistic increase in energetically costly metabolic pathways such as antioxidant defence, that may lead to subtle, and likely sub-lethal, toxicological effects within intertidal oyster populations.

5. Conclusion

The present study provides valuable insight into the dose-dependent effects of environmentally relevant MP concentrations on the digestive gland and gill metabolite levels of intertidal *Crassostrea gigas*. The ^{1}H NMR-based metabolomics approach was proven to be a valid method in unravelling potential MP-induced metabolic disorders in different tissues of *C. gigas* revealing a stronger response in the gills than the digestive gland. Taking into account all the metabolic disturbances identified, we conclude that both high MP exposure and low tide warming irrespective of MP load lead to the same stress response in the oyster gills. The resulting oxidative stress activates a cascade of antioxidant defence mechanisms that in turn requires an adaptation of the energy system to maintain homeostasis. Future studies using integrative multi-biomarker analyses will help to determine the extent of underlying cellular stress responses and to clarify potential synergistic effects of combined MP exposure and warming on the metabolism of *C. gigas* in the intertidal zone, with the ultimate aim of assessing potential consequences on population and ecosystem level.

CRediT authorship contribution statement

Nina Paul: Writing – original draft, Methodology, Investigation, Formal analysis, Conceptualization. **Anette Tillmann:** Writing – review & editing, Formal analysis. **Gisela Lannig:** Writing – review & editing, Supervision, Funding acquisition, Conceptualization. **Bernadette Pogoda:** Writing – review & editing, Supervision, Funding acquisition, Conceptualization. **Magnus Lucassen:** Writing – review & editing, Supervision, Funding acquisition, Conceptualization. **Nicholas Mackay-Roberts:** Writing – review & editing. **Gunnar Gerdts:** Writing – review & editing, Project administration, Funding acquisition. **Christian Bock:** Writing – review & editing, Validation, Supervision, Resources, Project administration, Methodology, Funding acquisition, Conceptualization.

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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