



The Effect of Microplastics on the Bioenergetics of the Mussel *Mytilus coruscus* Assessed by Cellular Energy Allocation Approach

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Marine microplastics pollution is a major environmental concern in marine ecosystems worldwide, yet the biological impacts of microplastics on the coastal biota are not yet fully understood. We investigated the impact of suspended microplastics on the energy budget of the mussels *Mytilus coruscus* using the Cellular Energy Allocation (CEA) approach. The mussels were exposed to control conditions (no microplastics) or to one of the three concentrations of 2 μm polystyrene microspheres (10 , 10^4 , and 10^6 particles/L) for 14 days, followed by 7 days of recovery. Exposure to high concentrations of microplastics (10^4 or 10^6 particles/L) increased cellular energy demand (measured as the activity of the mitochondrial electron transport system, ETS) and depleted cellular energy stores (carbohydrates, lipids, and proteins) in the mussels whereas exposure to 10 particles/L had no effect. Carbohydrate levels decreased already after 7 days of microplastics exposure and were restored after 7 days of recovery. In contrast, the tissue levels of lipids and proteins declined more slowly (after 14 days of exposure) and did not fully recover after 7 days following the removal of microplastics. Therefore, the total energy content and the CEA declined after 7–14 days of exposure to high microplastics concentrations, and remained suppressed during 7 days of subsequent recovery. These findings demonstrate a negative impact of microplastics on energy metabolism at the cellular level that cannot be restored during a short time recovery. Given a close link of CEA with the organismal energy balance, suppression of CEA by microplastics exposure suggests that bioenergetics disturbances might lead to decreases in growth and productivity of mussels' populations in environments with heavy microplastics loads.

Keywords: microplastic, energy status, cellular energy allocation, energy metabolism, mussel

INTRODUCTION

An exponential increase in production and use of plastic materials leads to a rise in the amount of plastic waste in aquatic habitats worldwide (Andrady and Neal, 2009; Wang C. et al., 2020). Ocean is a major sink for plastic waste with estimated >150 million tons of plastics present in marine ecosystems (MacArthur, 2017). Over time, the plastic waste breaks down into microplastics (0.1 μm –5 mm in diameter) and nanoplastics (diameter < 0.1 μm) through microbial degradation, prolonged UV exposure or physical abrasion (Weis et al., 2015). Microplastics have been globally detected in aquatic environments, including beaches, sediments and water column (Zhao et al., 2018; Teng et al., 2019; Li et al., 2020; Wang C. et al., 2020) and are considered ubiquitous emergent contaminants in marine ecosystems (Rochman et al., 2019). One of the major concerns related to the environmental impacts of microplastics is their interference with organisms' feeding (Botterell et al., 2019). Due to their small size, microplastics are easily ingestible by marine organisms and can accumulate in their digestive tract and other tissues potentially interfering with the digestion and nutrient uptake (Wright et al., 2013a,b). Furthermore, accumulated microplastics might induce indirect energetic costs due to the toxicity and tissue damage (Sokolova, 2021), and can also be transferred and enriched through the food chain, thus posing both ecological and human health threats (Brandts et al., 2018; Yu et al., 2018; Larue et al., 2021). The ingestion of microplastics by marine biota has been extensively documented in the laboratory experiments and in the field (Khan and Prezant, 2018; Pinheiro et al., 2020; Xu et al., 2020; Müller, 2021), but its implications for nutrition and energy metabolism remain insufficiently well-studied in marine organisms, including suspension feeders (such as bivalves) that are considered one of the most susceptible trophic guilds for microplastics (Wright et al., 2013a,b).

An emerging paradigm in the environmental risk assessment of microplastics considers that ingestion of microplastics might alter the food consumption and energy allocation in marine species (Galloway and Lewis, 2016). Microplastic might prevent the adequate intake of food and incur high energetic cost due to impaired internal homeostasis and upregulation of energetically costly protective mechanisms, such as immune system and cellular stress response (Détrée and Gallardo-Escárate, 2018). This implies that organisms must draw from their energy reserves to satisfy the metabolic demand and/or reallocate energy from fitness-related processes, such as growth or reproduction toward maintenance to compensate for energy deficiency caused by microplastics exposure (Wright et al., 2013a; Gardon et al., 2018). Earlier studies in aquatic organisms (including bivalves, polychaetes, and crustaceans) exposed to microplastics reported suppression of the feeding rates, decrease in growth, and depletion of energy stores (Wright et al., 2013a; Watts et al., 2015; Yin et al., 2018; Pedersen et al., 2020). These changes indicate elevated energy costs of dealing with stress in suboptimal environments and imply negative shifts in the energy budget of the organism limiting the amount of energy allocated into growth under by microplastic exposures. Energy

budget of an organism can be assessed at the organismal level by measuring the amount of assimilated energy and energy lost to respiration and excretion to determine the energetic Scope for Growth (SFG) (Widdows and Johnson, 1988) or biochemically by assessing the amount of energy reserves and energy consumption at the cellular level using the Cellular Energy Allocation (CEA) approach (De Coen and Janssen, 1997, 2003; Verslycke et al., 2003, 2004a; Smolders et al., 2004). The CEA approach provides an ecologically relevant tool to assess the energy budget of an organism linking changes at the cellular level with higher levels of biological organization (De Coen and Janssen, 1997; Smolders et al., 2004) and is commonly used in ecotoxicological assessments as an early indicator of pollutant-induced stress (De Coen and Janssen, 2003; Smolders et al., 2004; Van Dievel et al., 2019; Noor et al., 2021; Wu et al., 2021). Given the direct impact of microplastics on feeding behavior and maintenance costs of animals (Wright et al., 2013a; Watts et al., 2015; Galloway and Lewis, 2016; Yin et al., 2018; Pedersen et al., 2020), determination of CEA could provide important insights into the physiological and ecological consequences of exposures to these important emerging pollutants in marine organisms.

The aim of our present study was to determine the impacts of microplastics exposures on bioenergetics of the mussels (*M. coruscus*) using the CEA approach. Mussels are excellent bioindicators for environmental impacts of microplastics due to their wide distributing, key ecological functions as ecosystem engineers, and significant roles in marine food webs and human food security (Li et al., 2019). As filter-feedings, marine mussels can take up and accumulate microplastics (Hamm and Lenz, 2021). The energy metabolism of mussels is sensitive to environmental stressors including pollutants so that shifts in the energy budget provide a sensitive measure of stress in these organisms (Sokolova et al., 2012). Earlier studies reported that microplastics can increase the respiration rate (Green, 2016) and reduce the feeding rate of the mussels (Pedersen et al., 2020), which can have direct impact on their energy budget. Therefore, we hypothesized that microplastics could cause disturbances in the energy status of the mussel resulting in higher energy demand for basal maintenance and decreased energy reserves and reflected in low CEA. We also hypothesized that the severity of the negative bioenergetic effects of microplastics and the ability of mussels for subsequent recovery will depend on the concentration of microplastics. To test these hypotheses, we exposed the mussels *M. coruscus* to different concentrations of suspended polystyrene microplastics (0, 10, 10^4 , and 10^6 particles/L) for 14 days followed by 7 days of recovery, and measured CEA indicators including the available energy (E_a) by determining the protein, lipid, and carbohydrate content of the tissues, the energy consumption (E_c) assessed as the activity of mitochondrial electron transport system (ETS) activity, and CEA as the ratio of E_a and E_c . This study provides insights into the bioenergetics consequences of microplastic exposure and subsequent recovery in the mussels and helps to close an important gap in our knowledge of physiological and ecological effects of microplastics in marine organisms (Smolders et al., 2004; De Sá et al., 2018).

MATERIALS AND METHODS

Preparation and Identification of Microplastics

The stock solution of 2 μm polystyrene microspheres (5.69×10^9 particles/mL, 2.5% w/v, 10 mL) used in the experiment was purchased from BaseLine Chromtech Research Center (Tianjin, China). Fourier transform infrared spectroscopy (Nicolet iN10 MX, Thermo Fisher Scientific) was used to characterize and verify the polystyrene microspheres, and the surface morphology and particle size of polystyrene microspheres were analyzed by scanning electron microscope (SEM, Hitachi JSM-7500F). The physical characterization data of microplastics was reported in our earlier paper (Wang X. et al., 2020).

Sample Collection

The thick shell mussels (dry weight 1.5 ± 0.90 g; shell length 7.95 ± 0.32 cm) were collected from Gouqi Island, Zhejiang Province, China ($30^\circ 43' 14.268''$ N, $122^\circ 47' 0.3696''$ E). Only undamaged healthy mussels of similar size were used in the experiment. The shell surface was cleaned from biofouling, and the mussels were placed in laboratory breeding systems equipped with circulating filter devices for 2 weeks (temperature: $25 \pm 0.4^\circ\text{C}$; pH: 8.1 ± 0.05 ; salinity: $25 \pm 0.8\text{‰}$; photoperiod: LD 12: 12 h). The mussels were fed with *Chlorella vulgaris* (5×10^4 cells/mL) twice a day.

Experimental System and Design

Experimental mussels were randomly divided into four treatment groups and exposed to clean seawater (0 particles/L) or to one of the three test concentrations of polystyrene microspheres (10 , 10^4 , and 10^6 particles/L). The concentration of 10 particles/L simulated the microplastic pollution in the Yangtze River estuary offshore (Zhao et al., 2014), and 10^4 and 10^6 particles/L were the concentrations set based on previous toxicology studies of microplastics (Capolupo et al., 2018; Magni et al., 2018). The experiment was conducted in triplicates with a density of 20 mussels per tank (30 L). Coulter counter (Multisizer 3, Beckman, Irvine, CA, United States) was used daily to ensure the stability of the concentration and particle size distribution of microplastics in each treatment group. The mussels were fed with *C. vulgaris* (5×10^4 cells/mL) every day, and half of the seawater (15 L) was changed in all tanks every day to ensure high water quality. After changing the water, add polystyrene microspheres to maintain concentration to meet the exposure test requirements. After 14 days of microplastics exposures, the seawater in all tanks was completely replaced, and the recovery experiment was carried out for 7 days in the clean seawater without microplastics. The digestive glands of mussels were sampled on day 7, 14 of microplastics exposures and on day 21 (corresponding to 7 days of recovery). To minimize the influence of individual differences, the digestive glands of three mussels from the same tank were pooled. The pooled samples were placed in a cryotube, immediately frozen in liquid nitrogen and stored at -80°C until further analyses. The digestive gland was chosen as one of the most metabolically active organs and the main site of

energy storage in mussels (Gosling, 1992). For each experimental treatment, three biological replicates (each consisting of the pooled tissues of three mussels) were used.

Energy Reserve and Electron Transfer System Activity Measurement in Digestive Glands

The chloroform-methanol extraction method was used to determine the lipid content of mussel digestive glands (Van Handel, 1985). The protein content was determined using a commercial kit (Nanjing Jiancheng Bioengineering Institute, China) according to the Coomassie Brilliant Blue colorimetric method (Bradford, 1976). The total carbohydrate content was measured according to the method of De Coen and Janssen using glucose as the standard (De Coen and Janssen, 1997). All absorbances were determined by a microplate reader (Flexstation[®]3, Molecular Devices, San Jose, CA, United States). The available energy (E_a) was calculated by summing the energetic values for the different reserves, using an enthalpy of combustion of 17 kJ g^{-1} for glycogen, 39.5 kJ g^{-1} for lipids and 24 kJ g^{-1} for proteins, respectively (Gnaiger, 1983). The ETS activity of mussel digestive glands was measured by the reduction assay of 2-(4-iodophenyl)-3-(4-nitrophenyl)-5-phenyl tetrazolium chloride (INT) and was converted into energy equivalents using the oxyenthalpic equivalents of 484 kJ/mol O_2 for the average mixture of protein, lipid and carbohydrate to represent energy consumption (E_c) (Haider et al., 2018). The ratio of E_a to E_c was used as a measure for the CEA (Verslycke et al., 2004b).

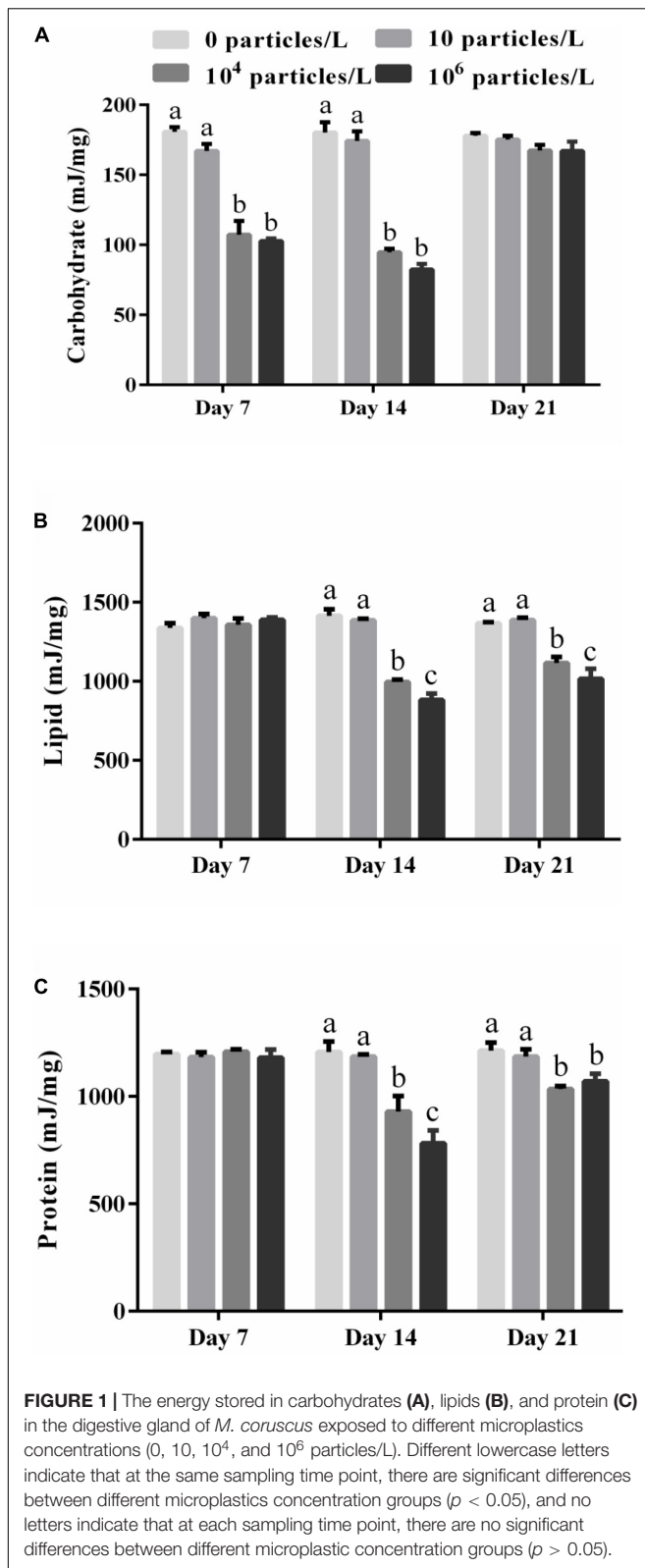
Statistical Analyses

SPSS 25 (SPSS Inc., Chicago, IL, United States) software was used for statistical analysis. The data were tested for the homogeneity of variance by Levene's Test, and the normal distribution of the data was tested by Shapiro-Wilk. One-way ANOVA followed by Tukey's HSD test was used to determine the significant differences between different microplastics treatment groups at each time point, and the effects were considered significant if $p < 0.05$.

RESULTS

The energy stored in carbohydrates decreased in the digestive gland of the mussels exposed for 7–14 days to 10^4 or 10^6 particles/L compared with the control group ($p < 0.05$) (Figure 1A). Exposure to 10 particles/L of polystyrene microplastics had no effect on the tissue content of carbohydrates ($p > 0.05$). There was a tendency for concentration-dependent decrease in the carbohydrate content caused by microplastics, but the difference between the mussels exposed to the two highest tested microplastics concentrations was not statistically significant ($p > 0.05$). The tissue levels of carbohydrates returned to the control baseline after 7 days of recovery in all experimental groups (Figure 1A).

The lipid energy content of the digestive gland remained stable at the baseline (control) levels after 7 days of microplastics



exposure, but decreased significantly in a concentration-dependent manner after 14 days of exposure (Figure 1B). This decline was significant in the mussels exposed to 10⁴ or

10⁶ particles/L ($p < 0.05$). The lipid energy content in the mussels exposed to 10⁴ or 10⁶ particles/L was not restored after 7 days of recovery compared with the control group ($p < 0.05$) (Figure 1B).

Similar to lipids, the protein energy content of the digestive gland decreased in a concentration-dependent manner after 14 days of exposure to 10⁴ or 10⁶ particles/L ($p < 0.05$) (Figure 1C). The protein energy content in the mussels exposed to 10⁴ or 10⁶ particles/L was not restored after 7 days of recovery compared with the control group ($p < 0.05$) (Figure 1C).

The total energy reserve (E_a) of the digestive glands of mussels was not significantly affected by the microplastics after 7 days of exposure but was suppressed in the mussels exposed to 10⁴ or 10⁶ particles/L after 14 days ($p > 0.05$) (Figure 2A). Recovery for 7 days partially restored the E_a of the digestive glands of mussels exposed to 10⁴ or 10⁶ particles/L ($p < 0.05$) (Figure 2A).

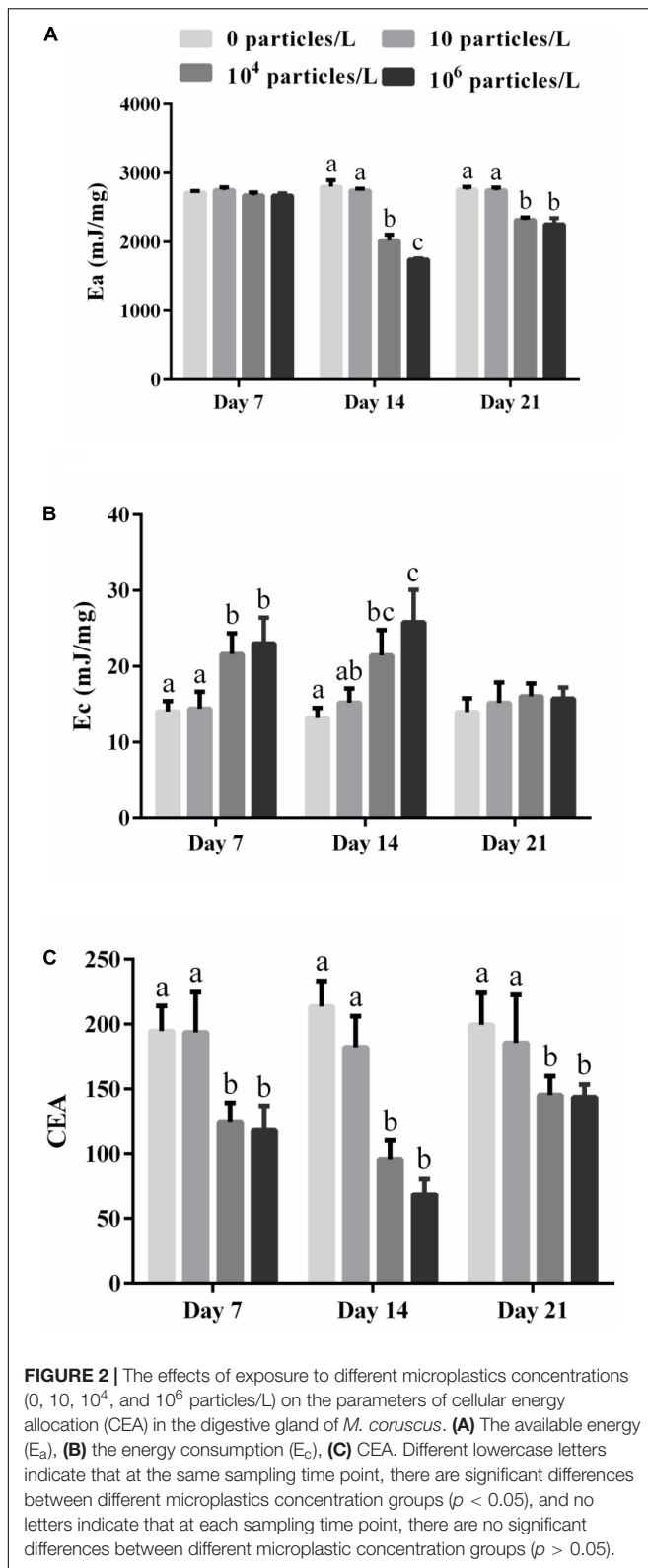
The cellular energy consumption (E_c) indicative of the energy demand of the mitochondrial ETS increased in the digestive gland of the mussels exposed to 10⁴ or 10⁶ particles/L after 7 and 14 days of exposure ($p < 0.05$) (Figure 2B). The lowest tested microplastics concentration (10 particles/L) had no significant effect on E_c ($p > 0.05$) (Figure 2B). After 7 days of recovery, the E_c values returned to the control baseline in all experimental groups ($p > 0.05$) (Figure 2B).

The CEA of the mussels was significantly decreased in the digestive gland of the mussels after 7–14 days of exposure and 7 days of recovery in the mussels exposed to 10⁴ or 10⁶ particles/L ($p < 0.05$) (Figure 2C). Exposure to 10 particles/L of polystyrene microplastics and no effect on the CEA of the mussels ($p > 0.05$) (Figure 2C).

DISCUSSION

Shifts in the cellular energy budget are considered an early warning sign of sublethal toxicity of pollutants in marine organisms with a direct link to organismal fitness (Giesy and Graney, 1989; Smolders et al., 2003). Our present study shows that exposure to high microplastic concentrations (10⁴ or 10⁶ particles/L) negatively affected the energy balance of the mussels *M. coruscus* with significant carry-over effects after 7 days of recovery. However, the lowest tested microplastics concentration (10 particles/L) had no effect on mussels' bioenergetics. These findings indicate that heavy microplastics pollution presents a health hazard to the mussels due to interference with energy metabolism, yet based on the reported concentrations of microplastics in the water column (ranging from 10⁻⁴ to 10² particles/L (Burns and Boxall, 2018; Ma et al., 2020), the environmental risk to the mussels from microplastics exposure is relatively low under the present-day pollution scenarios.

Our present study indicates that elevated energy demand is an important mechanism of the health hazard posed to the mussels by microplastics exposures. This is reflected in an increase in the mitochondrial ETS activity that serves as a marker of cellular energy requirements of mussels (Fanslow et al., 2001; Ivanina et al., 2012) and depletion of tissue



energy reserves. A significant increase in ETS activity of the mussels exposed to high concentrations of microplastics indicates activation of mitochondrial metabolism and an increase

in the cellular ATP demand. The data available to date show variable effects of microplastics on the respiration of bivalves. In oysters *Ostrea edulis* the respiration rate was not significantly affected by exposures to different types of microplastics for 60 days, despite some variability among different microplastics treatments (Green, 2016). No effects of microplastics exposures (100 or 1,000 particles/L) were reported for the mussels *Choromytilus chorus* (Opitz et al., 2021). This variability likely reflects differences in the exposure conditions including the differences in the concentrations and types of used microplastics. In our exposures, only high concentrations (10^4 or 10^6 particles/L) elicited the respiratory response whereas (the lowest test concentrations) had no effect. Elevated cellular oxygen consumption in the mussels exposed to high levels of microplastics indicates high basal maintenance costs, likely due to the costs of the stress protection and damage repair. High concentrations of microplastics can induce oxidative stress and inflammation in marine bivalves (Hu and Palić, 2020; Sendra et al., 2021) requiring expression of energetically costly protective mechanisms, such as antioxidants, molecular chaperones, protein quality control, and immune responses (Détrée and Gallardo-Escárate, 2017, 2018; Cole et al., 2020).

The decrease in energy reserves of *M. coruscus* exposed to high concentrations of microplastics (10^4 or 10^6 particles/L) indicates that the elevated energy demand is not compensated by an increase in energy uptake. This notion is supported by earlier reports that microplastics significantly suppress the filtration rate and energy assimilation efficiency of mussels (Gardon et al., 2018; Pedersen et al., 2020). Different energy reserves were engaged at different times of exposure to support energy demand of microplastics-exposed mussels. Carbohydrates (glycogen) reserves in the digestive gland were depleted already after 7 days of exposure to high concentration of microplastics, whereas the tissue levels of lipids and proteins decreased only after 14 days of exposure. The early depletion of glycogen is consistent with its role as a rapidly mobilizable metabolic fuel used to cover a rapid increase in energy demand in bivalves, for example during environmental stress or onset of gametogenesis (Smolders et al., 2004). Glycogen is also rapidly resynthesized after the microplastics exposure ceased in the mussels reaching the baseline levels after 7 days of recovery. In contrast, depletion of lipids and proteins occurs later during the microplastic exposure and takes longer to recover after the exposure ceases. A similar decrease in the levels of triglycerides, one of the main components of lipid storage, was found in the digestive glands of oyster *Crassostrea gigas* (Teng et al., 2021). These findings show an important role of lipids in supporting elevated metabolism during long-term exposures to microplastics.

Interestingly, the protein content of the digestive gland has shown a significant decline after 14 days of exposure to high concentrations of microplastics (10^4 or 10^6 particles/L) in *M. coruscus*. This finding was unexpected because protein breakdown is considered a last resort used only during prolonged starvation or periods of extreme energy demands (Albentosa et al., 2007; Haider et al., 2018). A decrease in the protein content in microplastics-exposed mussels might therefore indicate severe energy deficiency. Alternatively, lower protein content might

indicated suppressed protein synthesis or elevated protein turnover in microplastics-exposed *M. coruscus*. Our previous research found that microplastics can reduce the activity of digestive proteases, thereby affecting the absorption of amino acids by mussels (Wang X. et al., 2020). Therefore, amino acid limitation might negatively affect the protein synthesis capacity of the mussels exposed to high concentrations of microplastics. This hypothesis requires further testing, but regardless of the underlying mechanism, protein loss in the mussels' tissues is expected to have negative effect on the metabolic performance and structural integrity of the cells that are critically dependent on the proteins. Overall, the total amount of energy (E_a) was decreased in the mussels exposed to high concentrations of microplastics after 14 days reflecting lower content of all three major types of metabolic fuels. The CEA was also suppressed during the entire period of microplastics exposure demonstrating mismatch between the cellular energy demand and energy provision. An incomplete recovery of E_a and CEA after 7 days indicate that high concentrations of microplastics have long-term negative effects of the energy budget of the mussels, and further research is needed to determine whether these effects are reversible.

Overall, our present study demonstrates the utility of the CEA approach to detect negative physiological effects of microplastics in marine animals and differentiate between the concentrations that might be relatively benign to the mussels (10 particles/L) and those that show strong negative effects (10^4 or 10^6 particles/L). However, high concentrations (1.1×10^5 particles/L) of microplastic uptaken showed no significant effects on the CEA of *Mytilus edulis* in polluted habitats (Van Cauwenberghé et al., 2015), which may be due to species specific response of mussels to microplastics. The CEA might be useful in environmental risk assessment of microplastics, since a decrease in CEA has

immediate implications for growth, reproduction, immunity or stress response (Erk et al., 2011) and can thus help identifying habitats where mussels performance and sustainable growth would be negatively impacted by environmental stressors (Gomes et al., 2015; Wu et al., 2021) including microplastics pollution.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/supplementary material, further inquiries can be directed to the corresponding author/s.

AUTHOR CONTRIBUTIONS

YS, XW, WH, and YW contributed to the conception and design of the study. YS, XW, MH, and XC performed the statistical analysis. YS, XW, XC, IS, SW, and MH wrote the first draft of the manuscript. WL and JF wrote sections of the manuscript. All authors contributed to the manuscript revision, read, and approved the submitted version.

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