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Continuous Exposure to Microplastics Does Not Cause Physiological Effects in the Cultivated Mussel *Perna perna*

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Abstract

The environmental impact of microplastics is a challenging theme, especially under realistic experimental conditions. We investigated physiological responses to 0.1–1.0 µm PVC particles intake by the mussel *Perna perna* after a relative long-term exposure (90 days) at a less extreme concentration compared with previous studies (0.125 g/L). Microplastic intake was inferred by the presence of PVC in the feces of mussels, and physiological damages were assessed through ingestion rate, assimilation efficiency, growth rate, cellular and molecular biomarkers (lysosomal integrity, lipid peroxidation, and DNA damage), and condition index. All physiological responses showed nonsignificant effects of the microplastics on the exposed mussels. We suggest that, despite the experimental concentration of microplastics, mussels were able to acclimate to the exposure through their abilities for long-term recovery and tolerance to stresses. These data have positive implications for environmental health and in terms of human food resource because mussel farming is a worldwide practice that heavily relies on plastic materials, increasing the chances of microplastic exposure and mussels contamination.

Microplastic pollution is an emerging and worldwide threat for marine ecosystems (Boerger et al. 2010), originating from land-base and maritime activities (Barnes et al. 2009). These small particles (< 5 mm) (Arthur et al. 2009) can be produced by industries for direct applications (i.e., medicine and cosmetics, named as primary microplastics) or be a consequence of the degradation of large items (secondary microplastics) (Ye and Andrady 1991; Erikson and Burton 2003). Microplastics in marine environments have been reported in both urban and remote locations (Cole et al. 2011), contaminated with other pollutants (Hirai et al. 2011; Fisner et al. 2013) and associated with biological impacts, such as uptake by wild and cultured species (Murray and Cowie 2011; Foekma et al. 2013; Van Cauwenberghe and Janssen 2014). Because plastic degradation in marine environment tends to be slow (Gregory and Andrady 2003), microplastics' persistence and impacts will unquestionably rise with time.

Approximately 80% of marine plastics come from land-based activities (Andrady 2011), which makes coastal urbanized areas constantly susceptible to inputs of microplastics from domestic and industrial origins (Browne et al. 2011; Fisner et al. 2013; Mathalon and Hill 2014; Gallagher et al. 2015). As a consequence, coastal organisms may be commonly in contact with microplastics (Mathalon and Hill 2014; Santana et al. 2016), creating long-term exposures that involve the intake of this pollutant. This impactful scenario can be especially risky for coastal organisms that are sessile or sedentary and filter feeder, such as mussels. The lack or low mobility of these animals hinders them to move to microplastic-free environments and their constant filtering habit make them always prone to uptake plastic particles floating on the water column. Microplastic ingestion by mussels has been described in nature in different parts of the

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world (Van Cauwenberghe and Janssen 2014; Mathalon and Hill 2014; Santana et al. 2016; Li et al. 2016). In laboratory experiments, mussels were susceptible to ingest, retain, and assimilate microplastics within tissues after acute exposures, suffering from physiological effects as a consequence of the intake (Browne et al. 2008; Von Moos et al. 2012; Avio et al. 2015).

Microplastic ingestion by marine biota can cause impacts in all levels of biological organization (see GESAMP 2016 for examples). For individuals, the effects range from molecular and cellular stress and tissue damage to alteration of physiological rates (Von Moos et al. 2012; Wegner et al. 2012; Cole et al. 2013; Browne et al. 2013; Besseling et al. 2013; Avio et al. 2015; Sussarellu et al. 2016; Lu et al. 2016). To illustrate, oxidative stresses, such as reduction of antioxidant capacity, lysosomal membrane desistability, and DNA strand breaks, were observed in lugworms and mussels after microplastic exposure and uptake (Browne et al. 2013; Avio et al. 2015). Impacts at low biological level, such as those mentioned, can indicate metabolic pathways related to pollutant reactions on organisms and are usually analyzed by cellular and molecular biomarkers that offer rapid responses to the stressors before ultimate effects (i.e., early warning). In a higher level of organization, mollusks, copepods, and polychaete had their feeding rate, feces production, and assimilation efficiency altered by microplastic ingestion (Besseling et al. 2013; Cole et al. 2015; Sussarellu et al. 2016; Lu et al. 2016). Indeed and regardless biological level of organization, all these impacts can cause a shift on organisms' energy allocation, which with time might affect ecological relevant features such as growth, reproduction and survival. This has already been observed in pacific oysters exposed to PS microspheres (Sussarellu et al. 2016) and should be investigated for other marine taxa. According to Helmuth (2009), very different patterns of effects can exist among different biological levels, especially when analyzed at the individual level. Therefore, investigating both physiological rates and biomarkers can be complementary for risk assessments.

Most studies on microplastic uptake and impacts report responses due to short periods of microplastics exposure (maximum of 28 days; Besseling et al. 2013), whereas information about longer exposure is still limited (Van Cauwenberghe et al. 2013). In scenarios of long exposure, toxic agents are released periodically and for long periods, which can change toxicant effects on biota compared to short-term studies (Moreira 2011). Organisms, when long-exposed to a stressor, can show (i) changes in ecologically important sublethal endpoints (e.g., growth and reproduction) (Peterson et al. 2003); (ii) increase in susceptibility to disease and other pressures (Blakley 1985; Di Giulio and Scanlon 1985); or (iii) acclimation and/or adaptation to the stressing conditions (Hamdoun et al. 2003). In this context, it is important to test the consistency of biological responses to microplastics, considering longer and continuous exposure scenarios. This will support a more complete risk assessment for microplastic pollution in the marine environment by adding different time-scales (Underwood and Peterson 1988).

Studying the effects of longer microplastic exposure can also be an issue of human interest if the exposed organisms are of commercial interest. Mussels, for instance, are worldwide eaten by humans as seafood, and part of mussels supply originates from farm systems heavily reliant upon plastic materials (i.e., plastic sock nets and polypropylene long lines) (Mathalon and Hill 2014). Data from the United Nations Food and Agriculture Organization show that mussels comprised 12.4% of the total cultured mollusks in the world (FAO 2014), reaching almost 2 million tons in 2010 (FAO 2011). Both wild and farmed mussels are susceptible to microplastics contamination (Van Cauwenberghe and Janssen 2014; Mathalon and Hill 2014), with cultured mussels being more severely contaminated relative to wild mussels (Mathalon and Hill 2014). This suggests that farm systems can be a constant source of microplastics to mussels, highlighting the importance of the biological risks associated with microplastic long-term exposures to seafood industry and its management.

Environmentally relevant concentrations of exposure should also be a concern for studies with laboratory experiments on microplastic uptake (GESAMP 2016). This is because by simulating the field scenario of exposures we would obtain more accurate data to discuss current risks of microplastics pollution. However, experimental works on microplastic uptake and impacts have so far considered relatively high loads of microplastics that do not represent the pollution present in natural sea environments (Browne et al. 2008; Von Moos et al. 2012; Avio et al. 2015; Sussarellu et al. 2016). Such discrepancy, in turn, could be seen as a precautionary strategy of early stages of research (i.e., to guarantee microplastic exposure), especially considering small microplastic particles (< 100-µm size range), for which experimental designs do not have baseline information to rely on. Studies assessing microplastic presence in the water column have focused on microplastics of hundreds of micrometers or more in diameter size (Gallagher et al. 2015), whereas experimental studies on uptake and effects commonly use as plastic model particles varying from < 1- to 80-µm diameter in size (the present study and Von Moos et al. 2012, respectively). Thus, the most important types of microplastics with regard to ecological risks (the smallest ones) are not being evaluated in field (Syberg et al. 2015) and experimental procedures keep having concentrations of microplastics far exceeded from environmental relevance.

We investigated the effects of exposing brown mussels *Perna perna* to a relatively long-term and less extreme

contamination condition as compared to previous studies (Von Moos et al. 2012; Avio et al. 2015). We exposed the mussels to 0.125 g/L of commercial microparticles of E/M PVC during 90 days. Our hypothesis was that microplastic uptake under a less extreme but constant scenario of exposure induces health impacts on different biological levels of mussels' organization. To test the hypothesis, we evaluated a suite of cytological and physiological responses that could indicate negative effects of microplastics on mussels' overall health. They are: (i) physiological rates (clearance rate, absorption efficiency, growth rate); (ii) signals of cellular and molecular stress (lysosomal integrity, lipid peroxidation, and DNA damage); and (iii) health condition (condition index and mortality). Due to the lack of field information on the quantity of plastic within the size range of our model (0.1–1.0 µm of diameter), we based the choice of microplastic concentration on a preliminary experiment that tested PVC ingestion under different concentrations of exposure (see details on Online Supplementary Material). The 0.125 g/L was the lowest concentration of PVC tested with intake records by mussels. Although it corresponds to a much higher number of microplastic particles than registered by field data (Eriksen et al. 2014), this concentration also is 4–20 times lower than concentrations previously studied (Browne et al. 2008; Von Moos et al. 2012; Avio et al. 2015). The 90 days of constant exposure to PVC was 10–720 times longer than the periods of microplastic contamination so far tested with mussels (Browne et al. 2008; Von Moos et al. 2012; Avio et al. 2015), allowing us to study growth rates, which under optimal field conditions can be close to 0.8 cm/month (Ferreira and Magalhães 2010).

Methods

Experimental Models of Microplastic and Organism

Among the many types of polymers, PVC is the third most used thermoplastic in the world (ABIPLAST 2012) and is present in tubes to civil industry, children's toys, and hospital supplies (Rodolfo et al. 2006). In addition, the intake of PVC by marine benthic invertebrates has already been reported (Graham and Thompson 2009; Browne et al. 2013; Avio et al. 2015). The industrial grade of E/M PVC used in this study is sold for manufactories as a nano/micrometric sized powder (0.1–1.0 µm diameter). Such size range might increase the risks associated to E/M PVC exposure and intake by marine biota, as suggested for other small sized plastics (Browne et al. 2008; Lu et al. 2016). However, this is the first study on microplastic ingestion and impacts using a plastic model of 0.1–1 µm in size range. This specific PVC is also a solid particle, without porosity, which increases its bulk density (varying from 1.38 to 1.40 g/cm^3; Rodolfo et al. 2006). Thus, E/M PVC tends to spread easily throughout the water column and eventually reach the sea bottom, which facilitates further dispersion in the marine environment. The particles used in this work were donated by a polymer's industry, so supplier's intellectual property policy restricted the access to information about their composition and concentration of additives. Nevertheless, it is known that PVC is a plastic that necessarily contains chemical additives, especially plasticizers as phthalates (Rodolfo et al. 2006). More than 90% of plasticizers are used in PVC and phthalates can compose up to 50% of PVC weight, which might be hazardous if leached from microplastics to marine environment (OECD 2004; Oehlmann et al. 2009; Lithner et al. 2009, 2011).

Perna perna is a relevant model to examine effects of microplastic ingestion. It is a coastal mussel of commercial importance (Fernandes et al. 2008) and is widely distributed along tropical and subtropical regions of the Atlantic Ocean (Henriques 2004). As with other bivalves, it is easily cultivated and extracted from natural beds for human consumption (Fernandes et al. 2008). Also, microplastic uptake by *P. perna* has been confirmed in wild populations commonly used for human consumption (Santana et al. 2016).

Experimental Setup and Analysis of Effects

One hundred mussels (2.5 cm average shell length) were purchased from a farm located at Lagoinha Beach, Ubatuba (23°31′S 45°12′O). Epifauna was removed, and the mussels were left for 5 days to acclimate in an aerated tank with natural seawater and no sediment, under salinity and temperature-monitored conditions (35 g/kg; 21 ± 2 °C). Seawater was taken from the Ubatuba research field station of the Oceanographic Institute of the University of São Paulo (IOUSP). During this period, the organisms were fed only by the organic particles present in the natural seawater. The reproductive peaks of the test mussels for the region of Ubatuba (sampling site) occur in February and September (Marques 1988). Thus, to avoid increased variability among replicates related to biotic factors, such as reproductive status, the experiment was run from October to December. The reproductive status of mussels was verified by the observation of the gonadal maturation of ten organisms at the beginning and the end of the experiment. Mussels were immature at both times.

The experimental procedure was performed at IOUSP main laboratory (São Paulo, Brazil). The setup consisted of 80 mussels (2.5 ± 0.43 cm of shell length) randomly collected among the acclimatized mussels and assigned to individual glass aquaria (2 L; without sediment) of natural seawater. Natural seawater was originated from Ubatuba research station. During 90 days, 40 mussels were exposed to 0.125 g/L (equivalent to 1.115×10^{10} particles/L) of E/M

PVC powder and the others were maintained as control. The amount of microplastic needed for each aquarium was weighed, mixed in a small volume of seawater, and added by using a syringe without needle. Such seawater volume was part of the 2 L of seawater present in each aquarium. During the experiment, all aquaria were aerated. The turbulence produced from aeration suspended PVC particles throughout the water column. Aquaria were maintained with 12-h light–dark illumination regime, at 21 ± 2 °C, pH 7.5–8.0, salinity = 35 g/kg, ammonia (0.00 ppm), nitrite (0.05 ppm), and nitrate (1.00–2.00 ppm), all monitored three times per week. Nutrients were monitored using nutrient kits for aquarium water quality (Red Sea©). Considering the long exposure period of this experiment, to avoid the exacerbated use of PVC microspheres, the water in each aquarium was partially replaced (50%) three times per week. First, the feces were carefully siphoned using a silicon rose to maximum avoid any collection of suspended microplastics. Thereafter, the water was homogenized and removed (to ensure a maximum of equal distribution of microplastics to preserve the initial concentration). In each water replacement, the amount of PVC was replaced to maintain the initial concentration of microplastics (0.125 g/L) by using the syringe method as described previously. Mussels were fed with 3×10^4 cells/mL (dosed with Neubauer Chamber) of a 7:3 mixture of *Chaetoceros muelleri* to *Isochrysis* sp. algae (Microalgae Bank of IOUSP). The mixture of algae was inserted in one pulse every 2 days (i.e., after every water change and 1 day more) by using a syringe without needle. Microplastic intake by mussels was visually inferred by the presence of the absolutely white PVC in their siphoned feces, whereas control bivalves had feces with a regular brown color of organic matter and no signs of white material (i.e., plastic). Ingested microplastics were, therefore, not quantified. Also, although extra care was taken when the feces were siphoned, suspended PVC present in the water were eventually sampled together. To avoid any confusion between the defecated PVC and the noneaten particles siphoned, feces were placed in a Petri dish and carefully rinsed with Milli-q water. Free PVC (i.e., noningested ones) was then suspended in the water of the petri dish, whereas PVC on the feces remained on the feces. Mussels' feces are encapsulated by mucus and have a very distinctive appearance, facilitating this visual inspection.

After the 90 days of exposure to microplastics, possible effects of this relatively long-term exposure were quantified. Potential physiological effects were evaluated by measuring key physiological rates (clearance rate, absorption efficiency, and growth rate), signals of cellular and molecular stress, and health condition (mortality, condition index, and three biomarkers: lysosomal integrity, lipid peroxidation, and DNA damage). All investigated parameters can be disturbed due to microplastic exposure and intake, either because plastics are nonnutritive particles and might interfere on the quality of the seston that mussels are filtering or because of additives and monomers' toxic properties. Some parameters have already been assessed for microplastic effect on marine biota, showing responses to this pollutant (Von Moos et al. 2012; Wegner et al. 2012; Cole et al. 2013; Browne et al. 2013; Besseling et al. 2013; Avio et al. 2015; Sussarellu et al. 2016; Lu et al. 2016). To optimize the use of these indicators, every mussel was used in more than one type of analysis. The number of mussels analyzed for each physiological parameter is shown below.

Clearance Rate

The clearance rate (CR) (volume of filtered water per hour) was estimated by measuring the decrease in algal cell density of the experimental aquaria over one hour, using the following equation (Omory and Ikeda 1984):

$$\mathrm{CR}\,((\mathrm{L})/(\mathrm{h}) = V/N)\left\{\left(Ln\left(C_\mathrm{i}\right) - Ln\left(C_\mathrm{f}\right)\right)/\Delta t - f\right\}$$

V = volume of test flask (L), N = number of organisms per test flask, C_i = initial concentration of phytoplankton, C_f = final concentration of phytoplankton, Δt = time of incubation (h), f = factor of correction (obtained by the same equation applied for control flasks).

After 24 h of starvation in filtered seawater (0.7 µm), 30 individuals exposed to microplastics and 30 from the control group were individually arranged in aquaria with filtered seawater and 140 cells/mL of *C. muelleri*. The quantities of phytoplankton present in the water at the beginning and end of the assay were measured by the Welschmeyer fluorometric method (excitation 665 nm and emission at 665 and 673 nm) (Welschmeyer 1994). The assay was conducted in the dark in order to avoid planktonic growth and under controlled conditions of salinity and temperature (35 g/kg and 23 °C).

Absorption Efficiency

Absorption efficiency (AE) was determined from the relative content of organic matter (OM) present in the food (seston) given to mussels (I) compared with the OM of their feces collected at the end of the assay (F) using the following equation (Conover 1996):

$$\mathrm{AE}\,(\%) = \{((I - F)/(1 - F))I\} \times 100$$

This test was performed with the same 60 mussels and under the same conditions as the CR measurements, but OM of feces was measured 24 h after food was administered. The OM content of the phytoplankton and the feces was determined by combustion in a muffle furnace for two hours, following the method described in Resgalla Jr et al. (2007).

Growth Rate

The growth rate (GR) for each of the 80 mussels used in the experiment was estimated as proposed by Resgalla Jr et al. (1999). The method considers the variation of weight during the months experiment (i.e., three months). Wi and Wf are the initial and final wet weights, respectively, and change in time was equal to 90 days. Length and width are not considered by this method.

$$\mathrm{GR}\left(\mathrm{g/(month)}\right) = \left[Ln\left(W_{\mathrm{f}}\right) - Ln\left(W_{\mathrm{i}}\right)\right]/\Delta t$$

Biomarkers

To identify signs of microplastic damage at lower levels of biological organization, three biomarkers of effect were analyzed in 30 randomly selected mussels, 15 from each treatment. The analyzed biomarkers were lysosomal integrity, lipid peroxidation, and DNA damage. All of them can be related to oxygen reactive molecules (or species, ROS) over produced in organisms' body under stressed situations (i.e., oxidative stress). ROS are chemical species containing reactive oxygen (O_2^-) that interact with different cellular compounds, basically disturbing their molecular stability and generating nonfunctionalities (Held 2015). Biomarkers were assessed in samples of both gills and hemolymph due to possible interaction with microplastics (Browne et al. 2008; Von Moos et al. 2012; Avio et al. 2015). The contact with the gills is expected to occur quickly and more intensely, because all microplastics in the aquaria are susceptible of being filtered by the mussels (due to the mussels' filter-feeding habit). The contact between microplastics and hemolymph is more persistent but requires a longer time period to occur as it depends on the translocation process from gut within organisms (Browne et al. 2008). This last also involves smaller quantities of particles compared with gills exposure (Browne et al. 2008). The mode of action of the analyzed biomarkers and their techniques of detection are detailed below.

Lysosomal Integrity: Neutral Red Retention Time Assay

Lysosomal integrity was evaluated in hemolymph using the neutral red retention time assay (NRRT), which relates the hemocytes viability to the capacity of lysosomes to retain the neutral red dye over time (Lowe et al. 1995). Neutral red is a toxic dye uptaken by the lysosomes when cells are exposed to it. The toxicity of the dye destabilizes lysosome's membrane with time, enabling the dye to leak into the cytosol (Lowe et al. 1995). Healthy and nonstressed cells have stronger lysosome membranes that retain the neutral red dye for longer periods within the lysosome compared with stressed cells (Lowe et al. 1995). Stressed cells will have weaker lysosome membranes due to lipid peroxidation or membranes with disturbances on enzymes responsible for regulating membrane transport (Moore et al. 2006). Therefore, in this assay, the health of the cells is associated to the time that the dye takes to destabilize the lysosomes' membranes. The NRRT (expressed in minutes) was measured following Lowe et al. (1995), and the data were interpreted according to Pereira et al. (2011). The NRRT was obtained when 50% or more of the cells showed a leakage of neutral red dye into the cytosol and/or abnormalities in the color, shape, and size of hemocytes.

Lipid Peroxidation

Lipid peroxidation (LPO) was analyzed in samples of gills using the thiobarbituric acid method (TBAR) described by Wills (1987). This method indicates the status of cellular membranes' lipid oxidation, mainly caused by the increased amount of ROS generated under stress (Pereira et al. 2011). The technique correlates the lipid oxidation to one of its products: the organic molecule malondialdehyde (MDA). Data needs to be normalized by total content of proteins, which is a proxy of total cells analyzed. Total protein was obtained by using the Bradford method (kit for analysis: Sigma Aldrich). Results were expressed as µg TBAR/mg of total protein.

DNA Damage

DNA damage indicates weakness on nucleotide chains, which can be caused by different disturbances on the DNA, including its increased oxidation by ROS or any other electrophile (Jena 2012). DNA damage was quantified in gill samples using the alkaline precipitation assay (Olive 1998) followed by examination of DNA strand breaks via fluorescence (Gagné et al. 1995). For this method, fluorescence is related to the amount of DNA strand breaks, which means DNA damages. As for lipid peroxidation, data needs to be normalized by total proteins. Total protein was obtained by the Bradford method (kit for analysis: Sigma Aldrich). The results were expressed as µg of damage DNA/µg of total protein.

Condition Index

The condition index (CI) was calculated for all 80 mussels by using the method suggested by Baird (1958), expressed by the ratio between soft body wet weight and the total weight. Values were compared to Marques' index, by which a good CI will be between 0.15 and 0.25 (Marques 1988). Both soft body wet weight and total weight was measured in a digital weighting scale of 0.01 g of precision.

Data Analysis

Data from the analyzed parameters were compared between control and exposed mussels using Student's *t* test (significance level: $\alpha = 0.05$). Before the statistical analyses, data were tested for normality and, when necessary, were transformed applying the most suitable transformations (Figs. 1, 2). To aid in the discussion of statistics results, the coefficient of variability (CV) of each biological parameter analyzed was calculated.

Results

At the beginning of the experiment ($t = 0$), all mussels were placed on the bottom of the aquaria. However, over time some migrated and attached themselves on the aquarium walls, close to the water surface, partially emerged (exposed to air). At the end of the experiment, 16 mussels (20%) were fixed at the surface. From this total, 11 mussels were from control group and 5 were exposed to PVC. All of them did not have apparent changes in their filter-feeding habits or other sign of stress due to their position in aquaria. Therefore, none of these mussels were removed from the data sampling but the fact that some were attached partially emerged was considered during data interpretation. Filter-feeding activities were confirmed in both control and exposed groups through the presence of feces and pseudofeces in the aquaria. Feces of exposed organisms were always highly contaminated with E/M PVC, confirming microplastic ingestion. Pseudofeces production was observed to occur in higher quantities in the aquaria of organisms exposed to PVC. Feces and pseudofeces were differentiated by morphology and position in relation to mussel's valves. Pseudofeces are amorphous and eliminated through the base of the inhalant opening, whereas feces are composed by fecal pellets with line shapes eliminated by the anus, near the exhaling siphon. During the 90 days of experiment, none of the individuals died.

No significant effects in physiological rates, biomarkers, and health condition were evident for mussels chronically exposed to E/M PVC microparticles (all $p > 0.05$, Figs. 1, 2). Also, there were no outliers within data. All effects analyzed had responses that varied widely among individuals, resulting in high values of coefficient of variation (CV) (Fig. 2). The monitored abiotic factors did not vary significantly.

Discussion

After 90 days of exposure to 0.125 g/L of E/M PVC, mussels did not show health impacts. Such lack of physiological changes, however, goes against what current researches have indicated (Von Moos et al. 2012; Besseling et al. 2013; Avio et al. 2015; Sussarellu et al. 2016), making necessary a critical understanding of our experimental features and their possible effects on the results. To illustrate, previous studies have found that feeding activity of copepods *Centropages typicus* and lugworms *Arenicola marina* were negatively affected by microplastics (Cole et al. 2013; Besseling et al. 2013) and that mollusk bivalves expressed oxidative stress, inflammation, and apoptotic responses to microplastics exposure (Von Moos et al. 2012; Avio et al. 2015). One study in particular used the same species (*P. perna*) and

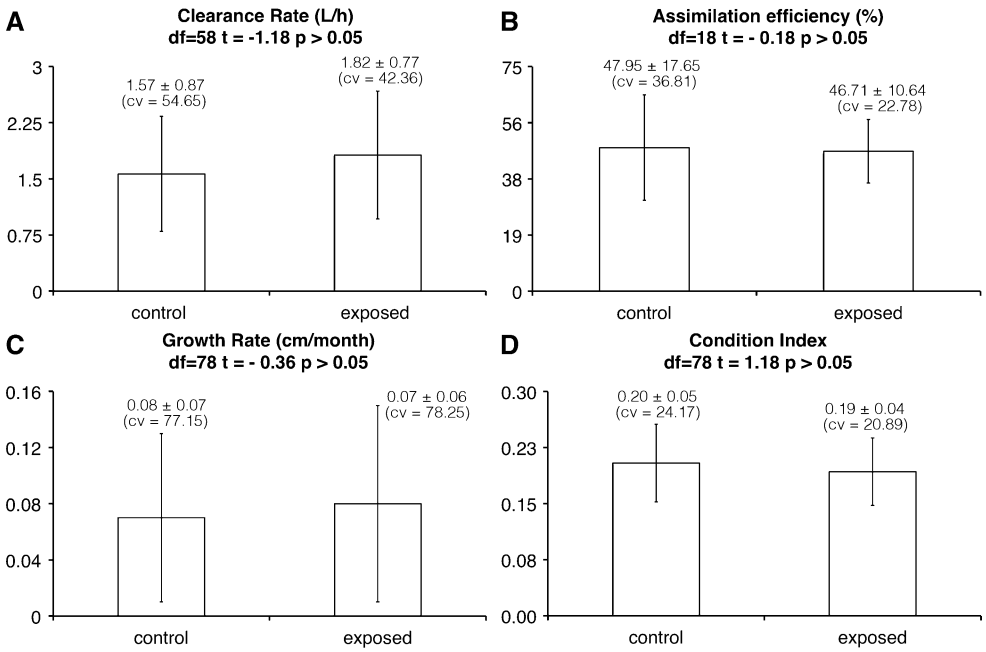

Fig. 1 Physiological rates and condition index results (mean ± standard deviation; *cv* coefficient of variation; and Student's *t* test. *df* degree of freedom, $\alpha < 0.05$) of control and exposed mussels to emulsion/microsuspension PVC microparticles (E/M PVC) under chronic conditions (0.125 g/L for 90 days). **a** Clearance rate (L/h); **b** assimilation efficiency (%); **c** growth rate (g/month); and **d** condition index. For Student's *t* test of growth rate, data were transformed using $\sqrt{(x)}$

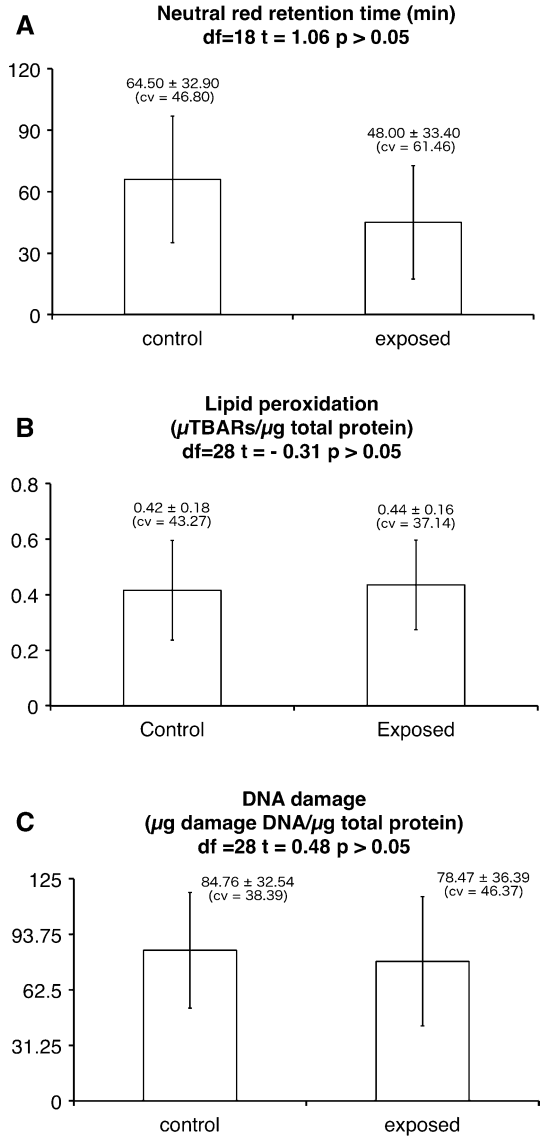

Fig. 2 Biomarkers results (mean ± standard deviation; *cv* coefficient of variation; and Student's *t* test. *df* degree of freedom, $\alpha < 0.05$) of control and exposed mussels to emulsion/microsuspension PVC microparticles (E/M PVC) under chronic conditions (0.125 g/L for 90 days). **a** Lysosomal Integrity measured by neutral red retention time assay (min); **b** lipid peroxidation (μTBARs/μg of total protein); and **c** DNA damage (μg damage DNA/μg of total protein). For Student's *t* test of lysosomal integrity, lipid peroxidation and DNA damage, data were transformed using $\log(x)$, $\log(x + 1)$, and $\sqrt{(x)}$, respectively

the same microplastic model (E/M PVC, i.e., same type of polymer, size and additives) but observed stress signs after microplastic exposure (Santana 2015). Having the same plastic model but opposite responses (effects vs. noneffects) suggests that the present results are not related to PVC type, size, and/or additives composition. So other features of microplastic exposure need to be explored, such as intensity and duration. In this sense, previous studies exposed organisms from hours to a few days (maximum of 28 days; Besseling et al. 2013) and to higher concentrations of microplastics compared with our experimental design. Thus, the lack of effects observed on *P. perna* could be related to the relatively lower concentration of microplastics per aquaria and the long-term exposure evaluated in the study.

The tested concentration of exposure (0.125 g/L of PVC) was sufficiently high to promote pseudofeces by exposed mussels, whereas controls rarely produced it. Mussels' pseudofeces is responsible for rejecting nonnutritive particles before intake and for defending organisms against high quantities of particulate matter suspended in the water (Ward and Shumway 2004). Although microplastics were not quantified in the feces and pseudofeces of exposed mussels, PVC was found within mussels' feces, which is an indication of microplastic intake. Microplastic intake, in turn, suggests that mussels did not identify PVC as nonnutritive. Therefore, pseudofeces should be related to mussels' defense against an overload of particulate matter (Jørgensen 1981) due to the high concentration of plastics in the water, and like that the 0.125 g/L of PVC used in this work should still be considered high for mussels, making this exposure feature a weak reason for the observed results. To better understand dose-responses of microplastic exposure on mussels, studies will need to reduce drastically the concentration of plastics in aquaria, which should be done based on environmental data of different microplastic types to relevantly simulate in situ conditions and their impacts (GESAMP 2016). However, and despite advances in field data, current methods for sampling microplastics in nature are limited, underestimating the environmental abundance of particles of smaller sizes (e.g., < 100 μm—GESAMP 2015). Although these microplastics are the most susceptible to the uptake by marine organisms and the most used in experimental studies of microplastics' biological impacts, experimental works remain restricted to hypothetical concentrations of microplastics in the environment. While reliable field data about microplastics smaller than 100 μm are not available, evaluating the production of contaminated pseudofeces and feces of exposed mussels could be an alternative method to estimate relevant concentrations of microplastics for future laboratory studies.

If not related to concentration of exposure, the nonsignificant effects on *P. perna* could be related to the ability of organisms acclimate to stressors in the long-term, making period of exposure a key factor for our results. To corroborate with this idea, exemplars of *Ostrea edulis* had no effects on filtration activity and growth after being exposed to 0.48–363 μm plastics during 60 days, regardless of concentration and type of microplastic particles (Green 2016). Likewise, the isopod *Ideota emarginata* had no growth changes after 6 weeks of microplastic exposure (Hämer et al. 2014) and the freshwater *Potampoyrgus antipodarum* did not show impacts on morphology after 2, 4, and 8 weeks of

experiment in aquaria overloaded with microplastics (Imhof and Laforsch 2016). Physiological acclimation, tolerance, or compensation can be defined as modifications of organisms' physiological parameters to maintain or increase their net energy gain and health. In terms of rates and efficiencies, mussels' acclimation has been suggested for other stressful scenarios rather than microplastic exposure. Bayne et al. (1987, 1993), for instance, observed blue mussels (*M. edulis*) regulating rates of filtration, digestion absorption and rejection of pseudofeces to maintain a consistent level of nutritive particles uptake after 12 days and 2 weeks of exposure to seston with different levels of inorganic and organic matters. *M. edulis* also was shown to acclimate to high and low temperature under laboratory conditions, compensating the filtration rate after 14 days and having no changes on assimilation efficiency after 28 days of experiment (Widdows and Bayne 1971). In our case, the production of pseudofeces by exposed mussels could have compensated the presence of PVC in the water, helping mussels not to overload themselves with microplastics and to maintain feeding (i.e., CR) without any significant increase. Pseudofeces production has already been suggested as defense mechanism to nanoplastics exposure, with increasing production related to increasing amount of plastic in the water (Wegner et al. 2012). Along with pseudofeces, the slightly and nonsignificant adjust of the CR of exposed mussels (15.9% higher than the control) might have been enough to make mussels efficiently assimilate nutritive particles for survival (Foster-Smith 1975; Navarro et al. 1996) and could explain why absorption efficiency and growth rate were not significantly different for mussels in contact with PVC. Nevertheless, further studies should evaluate what happens at earlier exposure times, between the beginning and the end of long-term experiments, to better infer time-responses to microplastic exposure and confirm acclimation. This is because acclimation to microplastic under long-term exposures is not a rule. After 8 months of exposure to polypropylene fibers, *Nephrops norvegicus* had reduced feeding and metabolic rates and lost body mass (Welden and Cowie 2016), clearly indicating hazardous effects of microplastics uptake.

E/M PVC did not affect mussels' biomarker responses, which indicates another absence of long-term impact, now related to cellular and molecular structures. Compared with previous publications demonstrating alterations in biomarker signs due to microplastic short-term exposures (Browne et al. 2008; Von Moos et al. 2012; Avio et al. 2015; Santana 2015), our results suggest a time-influence on mussels' mechanisms to avoid or compensate stresses caused by microplastics at lower level of biological organization. Time influence towards acclimation also was suggested by Santana (2015), in which mussels exposed to 0.5 and 2.5 g/L of E/M PVC during 7 days had more signs of cellular impacts (lysosomal integrity and the stress protein pP38-MAPK) during the first hours of experiment than at the end. Likewise, Browne et al. (2008) monitored mussels exposed to 0.5 g/L of PS (3 and 9.6 µm of diameter size) during 48 days and did not observe oxidative effects in their hemolymph. Different pollutants, including microplastics, can increase the intracellular generation of ROS (Itziou et al. 2011; Lushchak 2016), but several organisms also can compensate such impact through various mechanisms, such as producing more antioxidant enzymes. We suspect that positive responses against oxidative stress also might happen for microplastic exposure, although it has not been properly studied yet. The poor information relating acclimate responses to the chosen biomarkers and microplastics indicate a demand for future studies. Understanding organisms' mechanisms for microplastics defense under long-term exposures are key information for microplastic risk assessments, especially considering coastal areas susceptible to constant input of plastics.

The variability of responses among organisms is another feature that could potentially explain our results. Remarkable variability can hinder distinctions between control and exposed groups (Underwood and Peterson 1988), influencing the power of Student's *t* tests. This type of error could be avoided by increasing sample sizes (Underwood and Peterson 1988). However, most studies about microplastic impacts on marine biota used smaller than or similar number of organisms to this study (Browne et al. 2008; Von Moos et al. 2012 and Wegner et al. 2012) and some still found significant differences between control and exposed samples. Also, among all investigated parameters, there were no outliers (as indicated by the values of CV), which suggest a natural variation that could not be avoided. Several intrinsic and extrinsic properties can naturally influence biological variability, from genetic aspects to environmental conditions (Underwood and Peterson 1988). Thermal stress (Helmuth and Hofmann 2001), wave beating (Hunt and Scheibling 2001), food availability (Foster-Smith 1975), size (Zilberberg et al. 2011), and reproductive cycle (Bocchetti and Regoli 2006) are examples of factors influencing organism responses. In our assay, some of these natural influences were controlled (e.g., thermal stress, size and reproductive cycle), but others can have acted (e.g., food availability and air exposure due to mussels' depth of fixation). However, such interferences can also occur in natural habitats and cannot be controlled in experimental studies. Thus, considering our sample size and the fact that both control and exposed mussels were submitted to the same conditions and had high variability in their responses, we do not invalidate the results presented here. If the exposure to E/M PVC had significant effects at the concentration and period of exposure tested, differences would be noted despite of other possible natural influences as the ones mentioned. Nevertheless, further studies could be done increasing the sample size to better address this issue.

Some of the observed parameters in this study can be explored in the social context of aquaculture and human food resources. The CI, for instance, is an indicator of mussels' overall health and, regardless of PVC exposure all CI values were considered "good" (i.e., CI between 0.15 and 0.25) by Marques (1988). In addition, E/M PVC did not cause mortality or differences on mussel weight gain over time. These results can indicate that long-term exposure to microplastics does not significantly affect the commercial production of *P. perna*. Mussels are still healthy and gaining weight as the noncontaminated ones, important factors for the fisheries industry. This is particularly relevant if we consider that culture systems can be a chronic source of microplastic, making farmed mussels more contaminated than wild ones (Mathalon and Hill 2014). Indeed, by (i) comparing the significant average growth gain of cultured *P. perna* (0.875 cm/month, according to Ferreira and Magalhães 2010), with the slightly growth gain of both control and exposed mussels on this experiment (0.07 ± 0.06 and 0.08 ± 0.07 cm/month, respectively) and considering that (ii) 20% of mussels spent part or all experiment in the air–water interface of aquaria and that (iii) both groups of mussels did not exceed, on average 50% of absorption efficiency, we suggest that the lack of constant food (which might be influenced by the depth of mussels in the water column; Henriques 2004) may have a greater impact on mussel farming activities than the constant exposure to 0.125 g/L of microplastics. This emphasizes the lack of data about cumulative impacts on organisms of commercial importance that considers microplastics as one of the impact factors. Nevertheless, this was just one scenario of microplastics exposure and other levels of factors (i.e., other periods of exposures, concentrations, and types of polymer) should be investigated to reinforce the hypotheses discussed here. Even though mussels do not display significant effects in response to long-time exposure to E/M PVC, the potential health effects to consumers (i.e., human) still needs to be evaluated. Whereas *P. perna* showed a possible acclimation, other cultured and wild organisms might not have the same ability, highlighting the importance of understand microplastic impacts on multiple coastal and commercial species susceptible to its ingestion.

Conclusions

Assessing the effects of microplastic long-term contamination is of environmental relevance but also of social importance due to the impacts associated with commercial species. After exposure to 0.125 g/L of E/M PVC for 90 days, microplastic were effective to induce tolerance in brown mussel *P. perna*, enabling the species to live regularly under these circumstances. This has important implications for environmental health and human food resources. For the explored scenario, the concentration of microplastics was considerably high compared with current environmental concentrations but not capable of inducing negative effects in filter feeding bivalves. In this case, time, depth, food availability, and potential acclimation seemed to be important features for the physiological responses of the mussels. However, this might not apply to other scenarios of exposure and to the susceptibility of other marine organisms to microplastic intake, especially nonfilter-feeding organisms, which may not have adaptive mechanisms of rejection and/or depuration of particles. Given the importance of this result for risk assessments of microplastics pollution, we recommend that further studies explore similar and different scenarios of chronic exposures using more biological models.

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Compliance with Ethical Standards

Conflict of interest The authors declare no conflict of interest. The founding sponsors had no role in the design of the study, in the collection, analyses, or interpretation of data, in the writing of the manuscript, or in the decision to publish the results.

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