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Are bio-based and biodegradable microplastics impacting for blue mussel (*Mytilus edulis*)?

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ABSTRACT

The substitution of petrochemical plastics by bio-based and biodegradable plastics are in need of an evaluation for the potential toxic impacts that they can have on marine wildlife. This study aims to assess the toxicological effects of polylactic acid microparticles at two concentrations, 10 and 100 μg/L, during 8 days on the blue mussel, *Mytilus edulis*. No significant oxidative stress (catalase, glutathione-S-transferase and superoxide dismutase activities), neurotoxicity (acetylcholinesterase), or immunotoxicity (lysosomal membrane stability and acid phosphatase activity) were detectable. The multivariate analysis of metabolomic data allowed us to differentiate the individuals according to the exposure. From the loading plot of OPLS-DA, 48 ions down-regulated in the individuals exposed to microplastics. They were identified based on HRMS data as glycerophospholipids.

1. Introduction

Plastic pollution is weakening the ability of the oceans to support mankind. The impressive qualities of plastic, such as its resistance, strength, durability, and affordability, make it the most demanded product in the world, as it presents incredible social benefits (Gorman, 1993; Laist, 1987). Increase in production has been seen from 1960 to 2016 upscaling from 0.5 tons/year to over 359 million tons in 2018 (Plastic Europe, 2019). Thompson (2006a) stated that 10% of produced plastic ends up in the oceans. The increased use of poorly managed single-use products, unrestricted or unchecked debris disposal, and inefficient recycling practices are the main reasons for plastic accumulation in the oceans. (Barnes et al., 2009). Since these polymers are either lightweight or buoyant, a huge amount of them is easily carried long distances, while the heavyweight polymers, end up in sinking into the sediments, where they settle and persist for centuries (Moore, 2008; Barnes et al., 2009; Jeftic et al., 2009). The statistics on anthropogenic debris and the aforementioned statements make plastic ubiquitous under micro- and macro-sizes as well as different shapes like filaments, granules, fragments, foams, *etc.*, leading to a perilous situation (Derraik, 2002).

Once in the environment, depending on the conditions, plastic wastes can be fractionated due to UV radiation, hydrolysis, and/or biodegradation. The presence and identification of small floating fragments on the ocean surface was first reported in 1972 by Carpenter et al. (Carpenter and Smith, 1972; Carpenter et al., 1972). Accumulation of plastic fragments was also reported decades ago in the bodies of organisms like birds (Harper and Fowler, 1987), and in others like Zooplankton (Desforges et al., 2014), Polychaeta (Mathalon and Hill, 2014), Bivalves (Mathalon and Hill, 2014; Van Cauwenberghe and Janssen, 2014; Van Cauwenberghe et al., 2015), Crustaceans (Murray and Cowie, 2011; Devriese et al., 2015), Fish (Boerger et al., 2010; Lusher et al., 2013), and Mammalia (Rebolledo et al., 2013). The term 'microplastic' have been widely used and corresponds to plastic particles/fragments with a size less than 5 mm (Arthur et al., 2009).

In regards to microplastic studies, the most studied species in field investigations is the mussel (Phuong et al., 2016). The mode of feeding, easy availability, high tolerance threshold to salinity differences, and broad geographical distribution make mussels ideal organisms for biomonitoring studies. They are benthic, extensive filter feeder organisms

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with a selective mechanism of suspension feeding. Laboratory exposures with mussels have also been conducted to investigate the effects of microplastics. (Von Moos et al., 2012; Browne et al., 2008; De Witte et al., 2014; Avio et al., 2015; Li et al., 2015, 2016). Biomarkers are usually used as warning signs of biological effects caused by the presence of contaminants (Wu et al., 2005). Utilizing various health biomarkers, studies have shown a reduction in feeding activity at a concentration of 0.1 g/L of 10 μm PS beads (Wegner et al., 2012). It has also been reported that ingestion of these beads creates physical blockages in the intestinal tract, leading to reductions in feeding stimuli and function as well as a decrease in reproductive capabilities, primarily due to delays in ovulation and the lowering of steroid hormones (Wright et al., 2013; Canesi et al., 2015). Significant histologic abnormalities due to strong inflammatory responses were also observed after exposing mussels to high-density polyethylene (HDPE) at an acute concentration of 2.5 g/L (Von Moos et al., 2012). A dose as high as 32 μg/L of PS was given to assess the toxicological effects with an increased hemocyte mortality and triggered the cellular oxidative balance (Paul-Pont et al., 2016). In most of exposure studies, the microplastics used were not environmentally representative with spherical and monodispersed sizes and very high concentrations (Phuong et al., 2016). Few recent papers started to use fragments made from milled plastic items (Rainieri et al., 2018; Weber et al., 2018; Revel et al., 2019).

Recently as an alternative to petro-sourced and non-biodegradable plastic, compostable plastics and/or biosourced plastics, have been manufactured to provide a sustainable alternative. (Auras et al., 2004; Kaseem et al., 2012; Hamad et al., 2014; Gomes et al., 2019). Among these biopolymers, poly (lactic acid) (PLA) is getting fame and increased attention for its potential to replace petroleum-based plastics, mainly for low value-added applications, including single-use packaging. It also has impressive properties of high strength, aroma barrier, excellent crease, as well as oil and grease resistance (Ishida et al., 2006). With the increasing awareness of plastic pollution, the need for biodegradable plastics, especially the compostable PLA, has increased, and these plastics are produced on a ton scale today. At the same time, prices have dropped from $1000 USD per kg to a few USD$ per kg during the last 20 years, and are now at a price level similar to that of polystyrene (Schneiderman and Hillmyer, 2017). The tendency to be broken down by hydrolysis and bacteria make PLA an ideal product (Nakatsuka, 2011). However, the favorable conditions required for biodegradation, including a temperature range of 55–175 °C, are difficult to achieve in the natural environment (Garlotta, 2001; Haider et al., 2019). The degradation time of PLA varies from six months to two years, depending on environment conditions. The time for degradation is still less than traditional polystyrene or polyethylene plastics, which, according to literature is reported to take anywhere from 500 to 1000 years (Sinclair, 1996).

Nevertheless, the negative impacts of microplastics in natural environments are inevitable. Indeed, without specific kinds of composting garbage disposal units, PLA wastes have to be thrown into the general waste. This is in contradiction to popular convention, as the 'green' label of these objects tends to disillude consumers into believing that they can be thrown anywhere in nature without negative effects (Haider et al., 2019). This practice emphasizes the need for clear answers about the ecotoxicological effects of PLA. Currently, few papers can provide much information about the biodegradation and ecotoxicology of PLA in soil (Palsikowski et al., 2018; Adhikari et al., 2016). As a result, the effects of PLA microplastics on marine biota remains largely unexplored.

The aim of this work was to evaluate the toxicity effects of bio-based and biodegradable Poly(L-lactide) (PLLA) microplastic fragments at 10 and 100 $\mu g.L^{-1}$ on blue mussels (*Mytilus edulis*) exposed in the laboratory for 8 days. Microplastics have been refined by mechanical milling to obtain a spherical-like powder (0.8 μm < Ø < 10 μm). Health consequences were evaluated using various biochemical markers measured in the gills, *i.e.* antioxidant enzyme activities of catalase (CAT), superoxide dismutase (SOD), glutathion S-transferase (GST) as well as neurotoxicity (acetylcholinesterase activity; AChE). In hemolymph, an immunotoxicity assessment was performed, and acid phosphatase activity (AcP) and lysosomal membrane stability (LMS) were analyzed. Finally, a comprehensive metabolomics study was also conducted to examine the variations of lipid metabolites with and without microplastics.

2. Material and methods

2.1. Microplastic preparation and characterization

The commercial grade, bio-based biodegradable plastic used was a Poly(L-lactide) (PLLA-4032D; NatureWorks LLC (USA); film-grade; Mn (PS) = 143,000; D = 2. According to supplier: D-isomer = 1.4%; relative viscosity 3.94; residual monomer = 0.14%). The initial synthesis of the polymer was a polymerization of lactic acid. The density of the polymer was 1.24. Polymer pellets were crushed using an Ultra Centrifulgal Mill ZM 200 (Retsch) with liquid nitrogen to obtain microplastics of PLLA. The powder was then sieved on a stain sieve of 250 μm. The size distribution particles under 250 μm was analyzed with a laser Mastersizer Hydro 3000 (Malvern) granulometer.

Once milled, the particles were used to constitute a stock suspension prepared in deionized water to determine the size distribution. A second stock suspension was prepared in seawater for the exposure experiments.

2.2. Experimental design

Blue mussels (*Mytilus edulis*) were purchased from a supermarket (Super U). They were collected by dredging in the North Sea in March 2018. Organisms were placed in artificial seawater with air diffusion in two laboratory tanks of 70 L capacity, each for acclimation over one week. This step is needed to help the organisms adapt to their new conditions. Commercial sea salt (Tropic Marin®, France) was dissolved in deionized water to create artificial seawater at the desired salinity of 33 Practical Salinity Units (PSU).

After one week of acclimation, 15 individuals were randomly chosen and were placed in nine aquaria filled with 15 L of artificial seawater with air diffusion. Air diffusion was consistently provided throughout the experimental duration to optimizing the distribution and suspension of microplastics. The average size of mussels was 5.2 ± 0.5 cm (mean ± S·D). Three conditions of exposure were tested: two concentrations of microplastics, D1: 10 μg/L and D2: 100 μg/L, and one control condition. For each condition, 3 experimental replicates were performed, with 3 tanks per condition, each including 15 mussels per tank. The exposure was performed in parallel with the three conditions, for a total of nine experimental tanks, for a duration of 8 days without a depuration period. Mortality was monitored daily and dead mussels were removed immediately.

During the acclimation and exposure period, the water was changed every two days. Two hours after every water renewal, the organisms were fed with artificial food (Procoral phyton, Aquarium Bulle). Stock solution was made of algal powder by taking a concentration of 4.25 g/mL in seawater. One mL of stock solution per 10 L of seawater was given.

The conditions of the chamber for acclimation and exposure were kept constant throughout the experiment. Temperature of the room was adjusted at 13–14 °C and white light was provided with a photoperiod of 12 h:12 h.

For the exposure, the spike of microplastics at the desired concentration was done with diluted suspensions made from the stock suspension. The stock suspension in seawater was sonicated and stirred using a container with a magnetic bar prior to use in order to ensure an adequate homogenization. The diluted suspension was stirred for 5 min before giving the doses. All the aquaria were re-dosed 2 h after every water renewal.

After 8 days of exposure, the animals were quickly freeze-died by putting them in liquid nitrogen to avoid the potential stress caused by a

slow death. They were stored in a freezer at −80 °C until it was time for the analysis. Among the 15 individuals per tank, half of the live individuals were used for the biochemical analysis and the other half were dedicated to the metabolomics analysis. This distribution should lead to more than 20 mussels used to carry out the biochemical and metabolomics analysis, respectively, as per exposure conditions.

2.3. Biological dissection and analysis

The total live mussels for the three treatments were dissected in order to collect the hemolymph and gills needed for further experiments. Half of the organisms of each tank were randomly selected from the exposure conditions, and used for individual biomarker study. The frozen mussels after eight days of exposure were left on ice for thawing to avoid enzyme degradation. Hemolymph was sampled with a syringe and a sterile needle by a puncturing the posterior adductor muscle. Gills were then collected by dissecting the mussels. For GST, 1/3rd of the total gills were used while the rest of the gills were used for AChE, SOD and protein analysis.

Both the soft tissue weight and the total weight of the mussels were measured to calculate the Condition Index (CI) as follows: CI = [(whole soft tissues wet weight /total wet weight)*100].

2.4. Biochemical markers

Oxidative stress was evaluated by studying the GST, CAT and SOD activity in the gills. The procedures were adapted from Habig et al. (1974), Claiborne (1985) as well as Beauchamp and Fridovich (1971), respectively. The neurotoxicity was deducted by AChE activity in gills, adapting the protocol from Ellman et al. (1961). Immunotoxicity was estimated by measuring the AcP and LMS in hemolymph. An Acid Phosphatase Kinetic Method Kit (Biolabo Reagents) was used for AcP determination. Lysosomal membrane stability measures involved a neutral red dye retention (NRRT) assay adapted from the description in the manual on the biomarkers recommended for the MED POL biomonitoring program (1999). Finally, the results were expressed with reference to protein concentrations determined according to Bradford (1976), with bovine serum albumin (BSA) as standard.

2.5. Metabolomics analysis

The body of the mussel was individually thawed, weighed, and ground in a pestle and mortar in 10 mL methanol (>99.8% HiPerSolv CHROMANORM®, HPLC grade). The mixture was sonicated for 10 min before and after the addition of 10 mL of dichloromethane (> 99.8% stabilized HiPerSolv CHROMANORM®, HPLC grade) to each sample. Then, 9 mL of ultra-pure water was added and the sample was left for sonication for another 5 min. By adding these three different solvents of varying polarity, the extract was divided into two phases. After 5 min of centrifugation at 1000 G, 18 °C, the bottom phase, corresponding to the dicholoromethanolic phase, was kept in a pre-weighed tube. The upper layer was re-extracted using 5 mL of methanol and dichloromethane and centrifuged. The bottom phase was then taken out and combined with the previous bottom phase. The organic phase was then evaporated using N_2 at 50 °C. After complete evaporation, tubes were weighed again and stored at −20 °C until analysis.

The frozen extract was thawed and diluted in a mix isoporpanol/chloroform solution (9:1, *v*/v; HPLC grade) to reach a final concentration of 10 mg/mL extract. A final dilution by 100 was performed with isopropanol modified with formic acid (0.1%) and ammonium formate (10 mM). A quality control sample (QC) was prepared as a mixture of all prepared samples.

Samples at a concentration of 0.1 mg/ml were injected (10 μL) and analyzed by high-performance liquid chromatography (HPLC) coupled with High Resolution Mass Spectrometry analysis (HRMS) through a Flow Injection Analysis (FIA) strategy.

The tests were performed using an Acquity H-Class® UPLC™ device coupled to a Synapt™ G2 HRMS Q-TOF mass spectrometer equipped with an electrospray ionization (ESI) interface operating in the positive and negative ionization mode (Waters Corporation, Milford, MA, USA). The mobile phase was composed of acetonitrile (ULC-MS grade, Biosolve Valkenswaard, Netherlands) and isopropanol (ULC-MS grade, Biosolve Valkenswaard, Netherlands) in 9:1 (*v*/v) proportion modified by 0.1% formic acid and 10 mM of ammonium formate, with a flow rate of 0.4 mL/min. The full-HRMS mode was applied for lipid detection (mass-to-charge ratio (*m/z*) range 200–1200) at a mass resolution of 25,000 full-widths at half maximum (continuum mode). The ionization settings were as follows: capillary voltage, +2 kV; cone voltage, 30 V; desolvation gas (N2) flow rate, 900 L/h; desolvation gas/source temperatures, 550/120 °C. Leucine enkephalin solution at 2 μg/mL (50% acetonitrile) was infused at a constant flow rate of 10 μL/min in the lockspray channel, allowing for correction of the measured m/z throughout the batch (theoretical m/z 556.2771). Data acquisition was achieved using MassLynx™ software version 4.1 (Waters Corporation). According to metabolomic standards (Want et al., 2010), 10 μL of all samples were injected in random order using a dedicated Excel macro (Bertrand et al., 2013) with QC samples and blank samples every 10 and 20 injections, respectively.

Native MassLynx data (Waters) was converted into nCDF (common data format) data using DataBridge software (Waters). Automatic feature detection was performed between 0.5 and 4.5 min with MZmine2 software (Pluskal et al., 2010) using parameters selected according observed data variations. Peaks with a width of at least 0.05 s and an intensity greater than either 20 counts (NI) or 200 counts (PI) were selected with a 100 ppm m/z tolerance and deconvoluted. Feature alignment and gap filling were achieved with an m/z tolerance of 100 ppm and a retention time (RT) tolerance of 0.1 min. Only peaks eluted between 0 and 0.3 min were kept to generate the FIAHRMS data matrices containing features based on *m/z* and peak area values. This yielded two data matrixes according to both ionization mode containing 589 and 57 features for positive and negative ionization mode respectively. Both data matrices were concatenated for further data analysis.

2.6. Statistical data treatment

To highlight the significant differences of the condition index and biochemical markers (CAT, GST, SOD, AChE, AcP, LMS) between exposure treatments (control, D1 and D2), Kruskal-Wallis tests were applied with $p < 0.05$.

The matrix was used for statistical treatments, as Principal Component Analysis (PCA) and orthogonal projection of latent structure with discriminant analysis (OPLS-DA), with the software SIMCA13® (UMETRICS). Preliminary annotation of the features of interest was achieved using the Lipid Blast Database (Kind et al., 2013).

3. Results

3.1. Microplastic characterization

After grinding, the L-lactic acid polymer (PLLA) was used in the form of powder. The laser diffraction equipped with ultrasound separated the smallest particles which allowed for the analysis of all sizes of microparticles, including nanoparticles. Fig. 1 shows the density distribution of the powder. The size of particles ranged between 0.8 and 10 μm, with a large cluster between 0.8 and 2.5 μm.

3.2. Mortality and biological conditions in tanks

During the experimental exposure, some of the mussels died in each tank. The total number per condition at the end of the exposure was 37, 36 and 30 bivalves for control, D1 and D2 exposure conditions respectively. The mortality rate was higher in the tanks exposed to 100 μg/L of

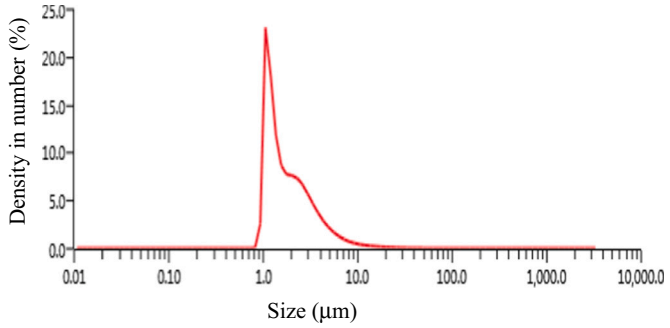

Fig. 1. Size distribution of microplastics used for exposure.

microplastics.

After 8 days of exposure, the condition index of *M. edulis* did not show any significant differences between the three treatments: 34.5 ± 10.9, 33.6 ± 10.8 and 39.8 ± 5.1 for Controls, D1 and D2 respectively.

3.3. Biochemical markers

3.3.1. Oxidative stress

CAT, GST and SOD were the enzymes used to evaluate oxidative stress in organisms. In situations when organisms suffer from oxidative stress, the activity of these three biomarkers differs in exposed organisms compared to negative controls.

Fig. 2 shows the results of the CAT, GST and SOD activities in mussels from the three conditions after 8 days of exposure.

For CAT, the mean obtained for control, D1 and D2 were 0.878 ± 0.707, 2.716 ± 0.968 and 0.884 ± 1.060 µmol/min/mg of protein, respectively. Concerning GST, average values of 0.172 ± 0.070, 0.453 ± 0.211 and 0.123 ± 0.151 nmol/min/mg of protein were observed for the organisms of the control group, D1 and D2 respectively. For both of these enzymes, the activity was found to be higher in the group of organisms exposed to 10 µg/L (D1) compared to control and D2 (100 µg/L), but the differences were not significant. The mean values obtained for SOD activity in gills of mussels in control, D1 and D2 conditions were not significantly different from each other and reached 2.298 ± 0.530, 1.938 ± 0.356 and 1.995 ± 0.120 U/min/mg of protein, respectively.

3.3.2. Neurotoxic effects

In the current study, no neurotoxic effect was observed as indicated by the activity of AChE in gills (Fig. 2). Control results showed a mean activity of 11.7 ± 3.8 nmol/min/mg of protein, which was not significantly different to the contaminated condition activities of 8.0 ± 1.3 and 9.62 ± 1.02 nmol/min/mg for D1 and D2, respectively.

3.3.3. Immunological effects

Hemolymph was extracted to analyze Acid Phosphatase (AcP) and lysosomes membrane stability. The mean AcP values obtained for each condition were not significantly different from each other, reaching 18.59 ± 16.22, 12.34 ± 7.80 and 11.40 ± 6.00 UI/L for the control, D1 and D2 conditions.

Fig. 3 shows the neutral red dye retention (NRRT) in lysosomal membranes as seen visually with the aid of a light microscope for the three treatments and 3 times after coloration, *i.e.* 15 min., 30 min. and 60 min.

NRRT is an assay used as an indicator of the health of the lysosomes. Shape deformity or leakage of dye in cytosol is an indication of wall instability. For the control condition, Fig. 3A, B and C show that lysosomes were round, in perfectly normal shape and with a dye retention of 50% for 60 mins. No sign of shape anomaly was observed in the different pictures of lysosomes from contaminated mussels but leakage of dye for both treatments D1 and D2 was visually observed after comparing Fig. 3D, E and F as well as 3G, 3H and 3I to those of control, particularly

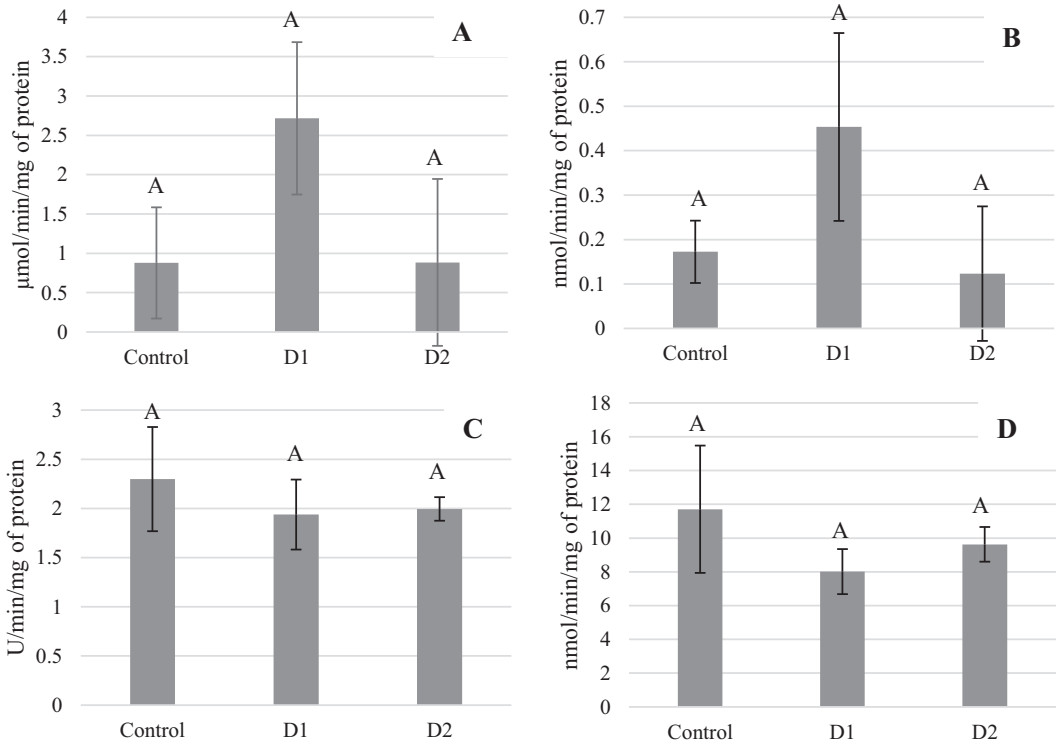

Fig. 2. A: Catalase activity (CAT); B: Glutathione S-transferase activity (GST); C: superoxide dismutase activity (SOD); D: Acetylcholinesterase activity (AChE) measured in gills from mussels exposed to three conditions: control (no contamination); D1 (microplastics at 10 µg/L); D2 (microplastics at 100 µg/L). Values are means with error bars as standard deviation, $n = 19$, 19 and 16 respectively for control, D1 and D2. Different letters correspond to significant differences between treatments (Kruskall Wallis test; $p < 0.05$). U corresponds to an arbitrary unit.

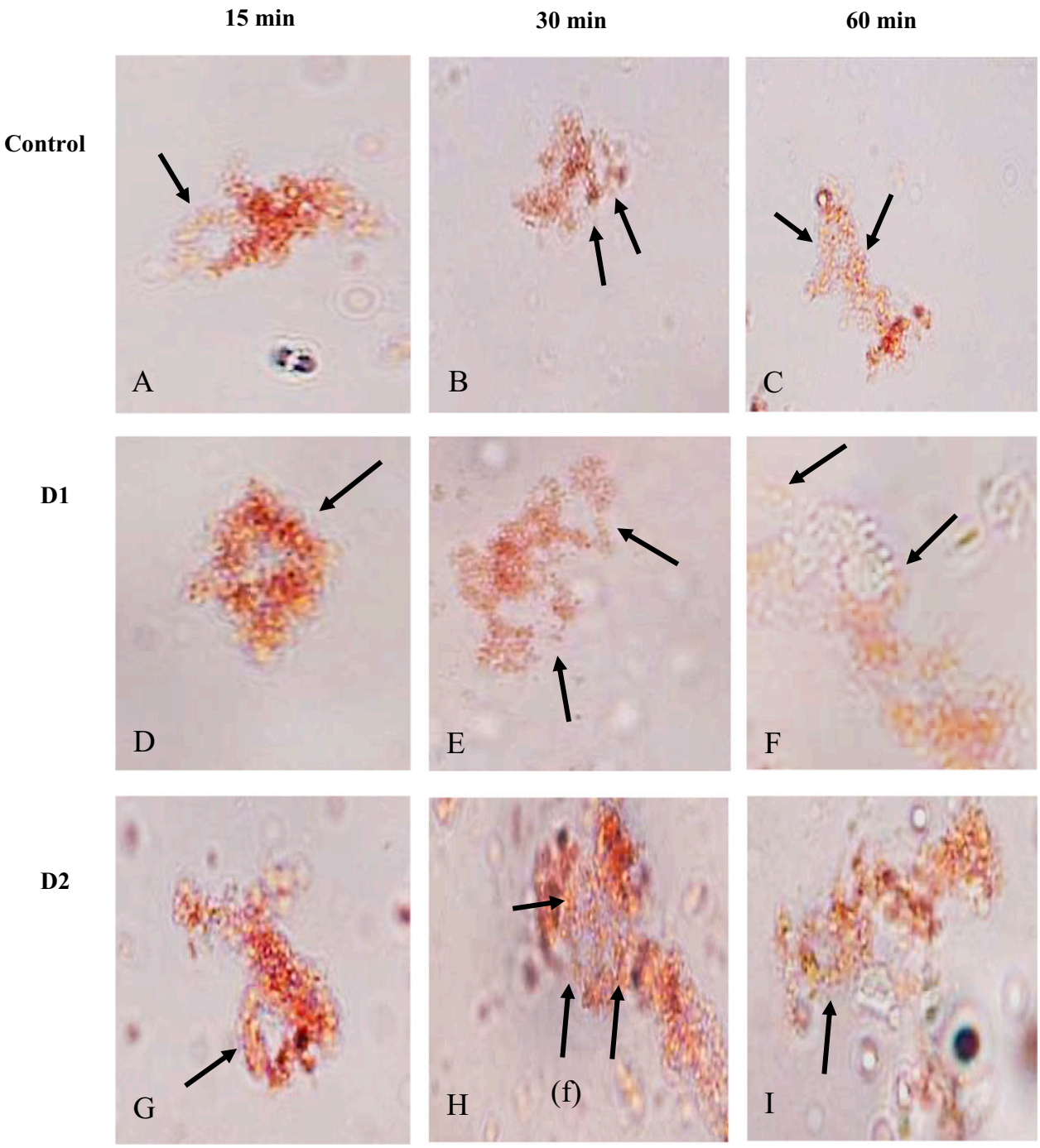

Fig. 3. Lysosomal wall stability shown by dye retention capacity. Arrows in the pictures indicate the position of lysosomes. A, B and C show the dye uptake and retention by lysosomes of organisms of control group after 15, 30 and 60 min of labialization period respectively. D, E and F correspond to contamination of microplastics at D1 (10 μg/L) and G, H and I at D2 (100 μg/L).

after 60 min.

3.4. Metabolomics analysis

To explore lipid modification in relation to exposure, lipids were extracted from individual *M. edulis* biomass. To rapidly evaluate the chemical composition of lipid extract, all extracts were analyzed by FIA-HRMS (Hsu, 2018). All ions detected were extracted from the generated fingerprints using automatic peak picking open source software (Pluskal et al., 2010), yielding the data matrix. The data corresponds to detected features observed in each mussel, provided as detected *m/z* along with their signal peak area. To explore metabolic alteration, a preliminary multivariate unsupervised data analysis was performed using PCA (Fig. 4A). The first two components (PC1 and PC2) explained 18.3% and 9.8% of the total variance of the data matrix. No clear clustering was observed within the first two axes of the PCA, however, an exposure gradient can be observed along the second axis, with unexposed *M. edulis* samples being located on the bottom of the PCA and highly exposed samples (D2, 100 μg/L) residing on the upper side. The low (intermediate) exposure (D1, 10 μg/L) is located between 'no' and 'high' exposure along PC2. In addition, discrimination between D1 and D2 were lower in comparison to control. The PC1 was only related to

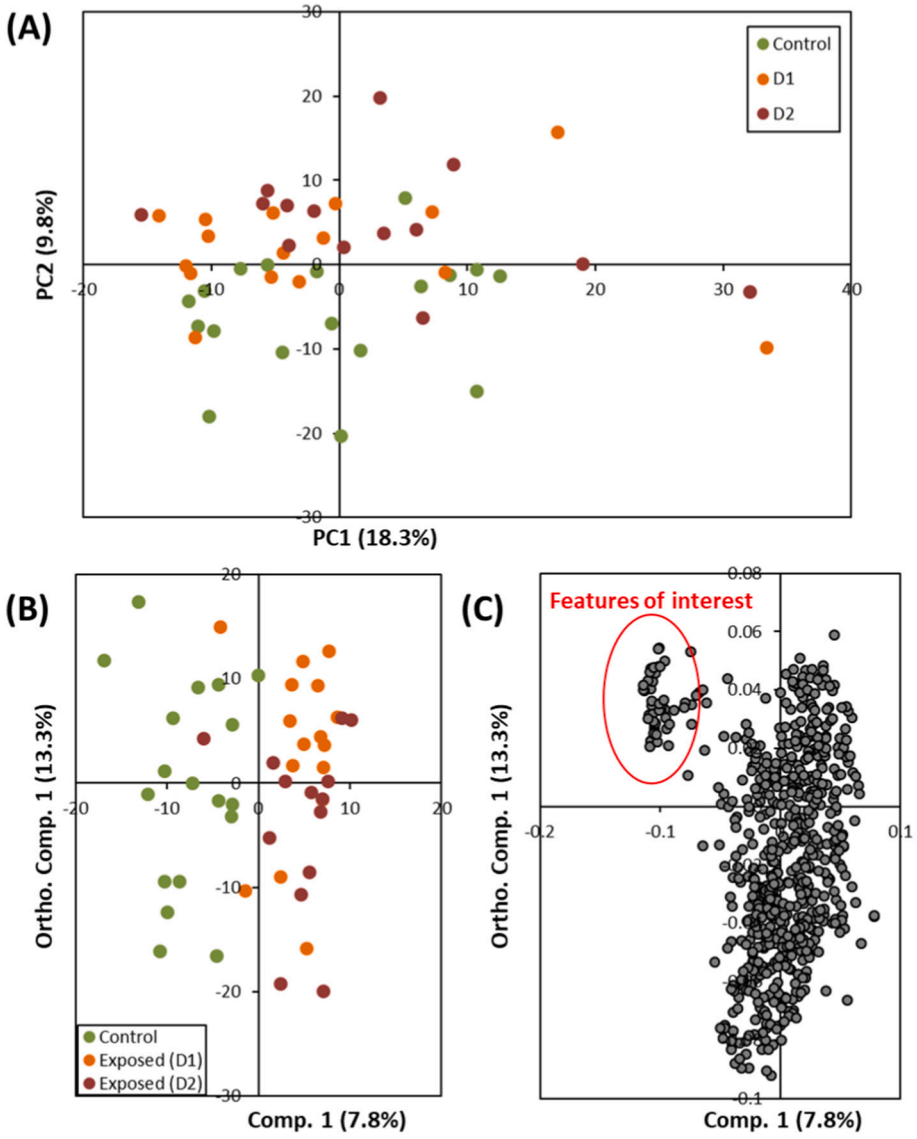

Fig. 4. Multivariate unsupervised and supervised data analysis of FIA-MS data from *M. edulis* extracts showing the impact of microplastic exposure (D1: 10 μg/L and D2: 100 μg/L of microplastics) to its lipidome. A) scores plot of the principal component analysis (PCA, UV scaling (Van den Berg et al., 2006), samples were highlighted according to microplastic exposure. B, C) supervised analysis using orthogonal projection of latent structure with discriminant analysis (OPLS-DA) (UV scaling) highlighting differences between unexposed and exposed (D1 and D2 simultaneously) *M. edulis* samples. The scores plot (B) shows, as expected, the separation between control and exposed samples and the corresponding loading plot (C) highlights the features of interest (in red) based on the OPLS-DA analysis (Table 1).

individual sample variability. The PCA (Fig. 4A) highlights that an exposure effect should be significantly retrieved from the data using supervised multivariate data analysis.

Supervised data analysis was performed using OPLS-DA focusing on searching for the exposure effect, either high (D2, 100 μg/L) or low (D1, 10 μg/L) in comparison to the control (no exposure) (Fig. 4B and C). The OPLS-DA model (R2Y = 0.702) highlights a separation on component 1 (7.8%) (Fig. 4B). The orthogonal axis of the OPLS-DA (Ortho. Comp. 1) was able to distinguish in an unsupervised way between both exposures. The OPLS-DA model was used to highlight features of interest related to the exposure effect. Interestingly, the most significantly altered lipids were down-regulated during exposure (Fig. 4C). This yields to the selection of 48 features (variable importance in the projection: VIP > 2) down regulated in the individuals exposed for 8 days to microplastics whatever the dose (Table 1), all detected in positive ionization mode. Those features were further annotated based on high mass accuracy using the Lipid Blast Database (Kind et al., 2013). Most of these annotated features were putatively identified as glycerophospholipids.

4. Discussion

The current study is aimed at investigating the potential of bio-based and biodegradable plastic to negatively affect the health of the blue mussel, *M. edulis*. Considering the fact that a huge proportion of debris produced globally ends up in the oceans (Thompson, 2006a), it is accurate to say that biodegradable polymers enter the ocean and act in the same way as other kinds of debris. Its tendency to naturally degrade completely reduces the physical conditions necessary for environmental degradation (Garlotta, 2001). Previous studies have already confirmed that non-biodegradable plastics alter gene expression, feeding rates, predation activity, ovulation, development of embryo, biomarker responses, and weight reduction in bivalves (Phuong et al., 2016). The results of the present study validated that biodegradable microplastics also stresses the blue mussel, mainly at the lipidome level.

4.1. Mortality of mussels

The results of mortality showed that during the experimental exposure, some of the mussels died in each tank. The total number per condition at day 8 of exposure was 37, 36, and 30 bivalves for control, D1, and D2 exposure condition, respectively. Even if an acclimation period was performed, the health of the mussels could be negatively affected by their commercialization in the supermarket, which represents a weakness. Nevertheless, the purchase of mussels can present advantages

Table 1
Features of interest highlights by the OPLS-DA analysis (Fig. 4) along with putative annotation using Lipid Blast Database (Kind et al., 2013).

Ionization mode	*m/z*	VIP value	Putative annotations
Positive	980.2780	3.21	
Positive	833.2420	3.17	
Positive	908.2582	3.14	Fatty amids (58:2)
Positive	539.1635	3.09	Sophorolipid lacton form SLP lacton (44:4); Sulfoquinovosyldiacylglycerols SQDG (44:0); Diacylglycerophosphoglycerols PG (50:6); Monoalkylglycerophosphoinositols PI (47:7)
Positive	614.1788	3.07	mono-Rhamnolipid mRL (49:1); Monoacylglycerophosphates PA (52:3)
Positive	613.1817	3.02	
Positive	522.1351	2.96	Monoacylglycerophosphoserines PS (46:3)
Positive	874.2652	2.96	Monoalkylglycerols MG (48:5); Dialkylglycerols DG (48:5)
Positive	836.2351	2.91	
Positive	540.1622	2.86	Monoacylglycerophosphoserines PS(50:5)
Positive	462.1479	2.85	Monoacylglycerophosphocholines PC(41:4); Monoacylglycerophosphoethanolamines PE (44:4)
Positive	446.1211	2.81	
Positive	838.23151	2.78	Fatty nitriles Nitrile (34:5)
Positive	615.1781	2.78	Diacylglycerophosphoglycerols PG(48:0); Monoalkylglycerophosphoinositols PI (45:1); Monoacylglycerophosphates PA (55:5)
Positive	928.2646	2.73	Fatty nitriles (65:3); Hydrocarbons (66:4)
Positive	837.2312	2.73	Fatty amids (30:3)
Positive	764.2159	2.71	
Positive	448.1178	2.69	Diacylglycerophosphocholines PC(40:8); Diacylglycerophosphoethanolamines PE (43:8)
Positive	1002.2838	2.69	Monoalkylglycerophosphocholines PC (15:1); Monoalkylglycerophosphoethanolamines PE(18:1); Ceramide 1-phosphates CerP (23:0)
Positive	762.2163	2.68	Monoacylglycerophosphocholines PC(17:2); Monoacylglycerophosphoethanolamines PE (20:2)
Positive	1150.3187	2.66	Sophorolipid lacton form SLP lacton (13:2)
Positive	929.2670	2.65	
Positive	1501.4001	2.64	Monoacylglycerophosphocholines PC (20:8); Monoacylglycerophosphoethanolamines PE (23:8)
Positive	1075.3032	2.61	Monoacylglycerophosphocholines PC (18:1); Monoacylglycerophosphoethanolamines PE (21:1)
Positive	780.2274	2.58	
Positive	1076.3023	2.55	
Positive	799.2446	2.54	
Positive	629.1961	2.53	Monoacylglycerophosphoserines PS (52:0)
Positive	1001.2860	2.52	Fatty acids FA (9:2); Fatty esters Ester (9:2)
Positive	801.2397	2.51	Monoacylglycerophosphocholines PC (20:4); Monoacylglycerophosphoethanolamines PE (23:4)
Positive	854.2478	2.50	
Positive	689.1978	2.47	Monogalactosyldiacylglycerol MGDG (34:0); Dialkylglycerophosphoglycerols PG (37:3); Monoalkylglycerophosphoglycerols PG (37:3); Diacylglycerophosphates PA (40:1)
Positive	705.2113	2.44	Monoacylglycerols MG (52:6); Alkylacylglycerols DG (52:6); Dialkylmonoacylglycerols TG (52:6)
Positive	1149.3248	2.38	
Positive	1077.2888	2.38	Monoacylglycerophosphocholines PC (18:0); Monoacylglycerophosphoethanolamines PE (21:0); Acylceramides Cer (33:8)
Positive	800.2471	2.38	Sophorolipid lacton form SLP lacton (13:4); Sulfoquinovosyldiacylglycerols SQDG (13:0); Diacylglycerophosphoglycerols PG (19:6); Monoalkylglycerophosphoinositols PI (16:7)
Positive	630.1943	2.35	Dialkylglycerophosphocholines PC (54:5); Monoalkylglycerophosphocholines PC (54:5); Dialkylglycerophosphoethanolamines PE (57:5); Monoalkylglycerophosphoethanolamines PE (57:5); Ceramide 1-phosphates CerP (62:4)
Positive	873.2656	2.26	
Positive	1003.2831	2.23	Dialkylglycerophosphocholines PC (16:1); Monoalkylglycerophosphocholines PC (16:1); Dialkylglycerophosphoethanolamines PE (19:1); Monoalkylglycerophosphoethanolamines PE (19:1); Ceramide 1-phosphates CerP (24:0)
Positive	806.7193	2.20	
Positive	631.1954	2.13	Sophorolipid lacton form SLP lacton (48:6); Sulfoquinovosyldiacylglycerols SQDG (48:2)
Positive	991.0063	2.11	
Positive	685.6968	2.05	Diacylglycerophosphoglycerols PG (19:2); Monoalkylglycerophosphoinositols PI(16:3)
Positive	685.3964	2.03	
Positive	706.2079	2.02	Diacylglycerophosphocholines PC (41:5); Diacylglycerophosphoethanolamines PE (44:5); Dialkylglycerophosphoserines PS (43:6); Monoalkylglycerophosphoserines PS (43:6)
Positive	875.2622	2.01	
Positive	628.3562	2.01	mono-Rhamnolipid mRL (56:7); Sophorolipid lacton form SLP lacton (46:2)

compared to their collection in the field. The organisms are likely more homogenous, allowing for less intraspecies variations, as they are often cultivated in the same conditions, with similar age. Mortality rate was not significantly different between the conditions, but the highest was in the tanks exposed to 100 μg/L of microplastics. The mortality suggested that there was an inability of the organisms to cope with the stress of contamination. The intrinsic property of organisms to acclimate to stressful conditions is without doubt, but the threat of higher concentrations of microplastics cannot be ignored. The highest concentration of microplastics could represent a higher risk of physical blockages in the intestinal tract, as already observed for petroleum-based microplastics (Wright et al., 2013; Canesi et al., 2015). Blockages are physical alterations not dependent on the nature of the plastics themselves and therefore could lead to direct death of organisms.

4.2. Modulation of oxidative stress due to microplastics

Biological systems produce oxygen free radicals in the living cells for better physiological function (Halliwell and Gutteridge, 1999). The upregulation of reactive oxygen species (ROS) and its balance in the body is essential for the health of organisms (Ames et al., 1993; Akaishi et al., 2007: Bouchard et al., 2009). In some cases, during metabolism, 2–3% of free radicals escape the antioxidant mechanism, resulting in oxidative damage to the cell. The imbalance between production and neutralization of ROS creates oxidative stress (Davies, 1995). Aquatic organisms have shown an increased sensitivity towards pollutants by exhibiting oxidative stress (Livingstone, 2001; Valavanidis et al., 2006; Lackner, 1998). CAT, GST, and SOD are involved in the removal of reactive oxygen and hydroxyl radicals from the bodies of marine

organisms. The elevated activity of the SOD enzyme reflects the need for rapid conversion of oxygen anions into less damaging H_2O_2 to prevent oxidative damage. CAT and GST are involved in scavenging the necessary H_2O_2, which can potentially be converted into hydroxyl radicals. GST deals mainly with the peroxide produced metabolically, while CAT is a defense mechanism acts on exogenous sources of peroxide molecules (Regoli and Giuliani, 2014).

The effect of the microplastics studied using biomarkers lead to no significant differences between exposed organisms and controls. Nevertheless, microplastics have slightly, but not significantly, elevated the activity of CAT and GST in mussels after exposure to PLLA microplastics at the 10 μg/L concentration.

In order to compare the effects of bio-based and biodegradable microplastics to petroleum-based microplastics on bivalves, Table 2 lists works of the literature dealing with laboratory exposures. However, the comparison is not easy because the experimental designs are not equivalent between the studies in terms of species, microplastics (type, form, size, concentration), duration, and biomarkers.

Most of the studies highlighted some oxidative stress in the different tissues of the organisms, mostly in the digestive glands and the gills (Trestrail et al., 2020). For a somewhat similar exposure times between 7 and 10 days, Avio et al. (2015) showed limited variations of antioxidant defenses in the digestive tissues of *Mytilus galloprovincialis* exposed to 1.5 g/L of <100 μm powdered PE and PS, virgin or with pyrene sorption. Inhibition was also observed for Se-dependent glutathione peroxidases and CAT. Moreover, a higher genotoxic effect (nuclear alterations and micronuclei damage) in the haemocytes of exposed mussels hypothesized an enhancement of ROS production in response to microplastics, specifically to virgin PS. In the same exposure timeframe, the study of Paul-Pont et al. (2016) showed a reduced activity of CAT, lipid peroxidase (LPO), and enhanced GST and SOD activity in the digestive glands of *M. edulis* and *M. galloprovincialis* after an exposure of marine mussels to PS microbeads of 2 and 6 μm at 32 μg/L. But these upregulations were observed at T14, during a depuration phase, and it was concluded from the study that organisms exposed to PS microbeads showed a great capacity for rapid conversion of reactive oxygen into less harmful H_2O_2. The 6-day exposure of the bivalve *Dreissena polymorpha* exposed to PS microbeads of 1 and 10 μm at 5.10^6 particle/L displayed a modulation of CAT and GST (Magni et al., 2018). Another recent work about petroleum-based plastics, Revel et al., 2019, involving 10 days of exposure showed perturbations of enzymes in gills of blue mussels, *M. edulis* after exposure with a mix of PE and PP fragments at 100 μg/L. The modulation of antioxidant enzyme activities measured with SOD and CAT increased in gills of exposed organisms. The comparison of these 3 studies is impossible, due to massive variation in experimental particle sizes. For example, in Avio et al., 2015, sizes ranged from 100 to 1000 μm, while in Paul-Pont et al., 2016, microplastic sizes were 2 and 6 μm. The concentrations studied were also widely varied, from 32 μg/L (Paul-Pont et al., 2016) to 1.5 g/L (Avio et al., 2015) and even expressed in a other units than m/v, *i.e.* $1.5.10^7$ particles/L, which leads once again to an impossible comparison.

In regards to other studies implementing time exposures, from as low as 3 h (Kinjo et al., 2019), 24 h (Détrée and Gallardo-Escarate, 2017) and 48 h (Webb et al., 2020) to as high as 14 days (Ribeiro et al., 2017), even 26 days (González-Soto et al., 2019) and 28–64 days (Détrée and Gallardo-Escarate, 2017), only one conclude to oxidative stress (Ribeiro et al., 2017) or upregulations of genes relative to it (Détrée and Gallardo-Escarate, 2017). According to the results of the present study, it would seem that bio-based and biodegradable microplastics are not a threat to the integrity of the physiology of the organism by inducing oxidative stress. Nevertheless, it does not mean that PLLA would be ecosafe compared to petroleum-based microplastics since the induction of oxidative stress is not reported in all the studies using petroleum-based microplastics for experiments. A gap still exists in the normalization of the environmental risk assessment of microplastics, even if some papers already discuss it (Phuong et al., 2016; Paul-Pont et al., 2018).

4.3. Induction of neurotoxicity

Literature has reported the presence of AChE enzymes at cholinergic and non-cholinergic neurons as well as in the central ganglia of the nervous system in mollusks (Giller and Schwartz, 1971; Galloway and Handy, 2003). Its role, however, is still unclear in mollusks (Heyer et al., 1973; Dauberschmidt et al., 1996; Rickwood and Galloway, 2004). Some studies have raised the speculations on the involvement of AChE as a function of the growth factor (Soreq and Seidman, 2001). An increased neurite growth was observed in cholinergic and dopaminergic neurons of *Aplysia californica* (Srivatsan, 1999). However, for hazard identification in risk assessment, a void of information exists on the intraspecies variations, which illustrates the inability to scientifically interpret the neurotoxicity data.

The results of the current study showed no neurotoxic effects of PLLA microplastics in mussels. On the contrary, Avio et al. (2015) reported a significant down regulation of AChE activity in the gills of *M. galloprovincialis* of contaminated groups by microplastics (<100 μm), of virgin PE, and PS at 1.5 g/L or pyrene sorbed, after 7 days exposure. To the best of our knowledge, no other work about the neurotoxic effects of microplastics on mussels has been published. Concerning other species, clams (*Scrobicularia plana*) exhibited significant inhibition of AChE after 20 μm PS microplastic exposure at 1 mg/L for 14 days, followed by 7 days of depuration, the phase after exposure corresponding to a phase of maintaining organisms without contamination. (Ribeiro et al., 2017). Oliveira et al. (2013) worked on common goby (*Pomatoschistus microps*). After giving 96 h exposure of 1–5 μm sized PE microspheres (0, 18.4 and 184 μg/L) in the presence and absence of pyrene (20 and 200 μg/L), 22% inhibition of the aforementioned enzyme was observed. Moreover, concentrations as high as 500 μg/L resulted in inhibiting AChE by 42% in common goby after 96 h of exposure (Oliveira et al., 2013).

4.4. Response of lysosome to microplastics

Lysosomes are the structures which are involved in the detoxification of foreign objects or pollutants (Izagirre et al., 2009). They contain more than 50 hydrolytic enzymes, such as acid phosphatase (AcP), and have a semi-permeable membrane (Moore, 1976; Barret and Heath, 1977; Ferreira and Dolder, 2003). Macrostructures are engulfed and broken down in the process of autophagy by these organelles. Additionally, toxic chemicals, upon entering the body, are also detoxified by lysosomes (Moore et al., 1980a, 1980b; Nott et al., 1985; Viarengo et al., 1985; Sarasquete et al., 1992; Cajaraville et al., 2000; Moore et al., 2004; Koehler et al., 2008; Von Moos et al., 2012). Previous studies were conducted to validate the use of AcP as lysosomal biomarkers (Jayakumar et al., 2008). Lysosomal membrane stability (LMS) was also demonstrated as a sensitive biomarker for pollutants (Moore, 1985, 1988; Viarengo et al., 2007). If the concentration of contaminants increases the capacity of the cells to withhold dye, the membrane becomes leaky and unsteady. In severe stress cases, hydrolytic enzymes burst out of the lysosome walls and enter the cytoplasm, with the potential to cause cell death (Koehler et al., 2002). In the case of an impairment in the lysosome membrane, uptake and retention of dye used for the LMS essay will be reduced over time (Lowe et al., 1992).

In the current study, the measure of AcP activity coupled to LMS from the hemolymph of mussels allowed us to monitor the health of lysosomes after microplastic exposures. The use of AcP and LMS as lysosomal biomarkers were validated by the coherent results of both parameters. The two assays showed insignificant health implications of microplastics to lysosomes. No significant difference of AcP activity was observed between the different exposure conditions. Lysosomes from the hemolymph of the exposed mussels showed no real differences in retention of dye in the current study, alluding to no observable impairment of the membrane. The amphiphilic nature of dye meant it became trapped in the cell wall matrix in the process of protonation (Moore et al., 2004). The insignificant differences in exposed and

Table 2
Changes in biomarkers related to laboratory exposures of bivalves to microplastics.

Species	Nature	Form	Particle size	Conc.	Duration	Endpoints	Ref.
Mytilus edulis	HDPE	fluff	>0–80 μm	2.5 g/L	96 days	- accumulation in lysosomal system - histological changes - inflammatory response - lysosomal membrane destabilization	Von Moos et al., 2012
Mytilus galloprovincialis	PS PE (with or without pyrene)		100–1000 μm <100 μm virgin	1.5 g/L	7 days	- DNA strand broke in hemocytes - Nuclear anomalies - AChE decreased in gills - Inhibition of Se-dependent – Decreased GST and CAT activity - Lysosomal integrity affected	Avio et al., 2015
Marine mussels	PS	Microbeads	Mix of 2 and 6 μm	32 μg/L	7 days exposure/ 7 days depuration	- Hemocyte mortality - Increased production of ROS in hemocytes - Decreased granulocyte concentration - Enhanced phagocytosis activity - Reduced activity of CAT and LPO and enhanced GST and SOD activity in DG - Significant effects on mRNA levels	Paul-Pont et al., 2016
Mytilus Galloprovincialis	PE	Microbeads	1–50 μm	$1.5.10^7$ MP/L	24 h	Gene expression in 4 tissues: - up-regulation of genes relative to carbon metabolism, oxidative stress, immune response and apoptosis, in the mantle and digestive gland - down-regulation of genes involve in carbon metabolism was observed in the haemolymph and gills	Détrée and Gallardo-Escarate, 2017
Scrobicularia plana	PS	Microbeads	20 μm	1 mg/L	14 days exposure/ 7 days depuration	- oxidative stress in gills and DG - inhibition of AChE - genotoxicity	Ribeiro et al., 2017
Mytilus Galloprovincialis	HDPE	Microbeads	1–50 μm	$4.6.10^5$ MP/L	28 days exposure/ 18 days depuration/ 64 days exposure	- 1st exposure: production of stress and immune-related proteins and diminution of energy allocated to growth - depuration: activation of apoptotic processes and up-regulation of immunereceptors and stress-related proteins - 2nd exposure: stress-memory upon microplastics based on gene expression data	Détrée and Gallardo-Escarate, 2018
Dreissena polymorpha	PS	Microbeads	1 and 10 μm	Mix 1: 5.10^5 MP/L both 1 and 10 μm Mix 2: 2.10^6 MP/L both 1 and 10 μm	6 days	- Induction of neurotoxicity by increasing dopamine production - Modulation of CAT and GST in the organisms exposed to Mix 1.	Magni et al., 2018
Dreissena polymorpha	PS	Microbeads	1 and 10 μm	Mix 1: 5.10^5 MP/L both 1 and 10 μm Mix 2: 2.10^6 MP/L both 1 and 10 μm	6 days	Protein modulation lacking in the lowest concentration group while 78 proteins differently modulated in the other group.	Magni et al., 2018
Mytilus galloprovincialis	PS	Microbeads	0.5 and 4.5 μm	$7.44.10^5$ MP/mL for 0.5 μm 1000 MP/mL for 4.5 μm	26 days	0.5 μm MPs: more toxicity for DNA and cell composition of digestive tubules. Increased inflammatory response in tissues. 4.5 μm: found in stomach contents and epithelial cells of digestive system.	González-Soto et al., 2019
Mytilus edulis	PE/PP (v:v)	Fragments	0.4–950 μm	0.008, 10 and 100 μg/L	10 days exposure/	- Increases of SOD and CAT in digestive gland (0.008 and 10 μg/L) - Increases of SOD and CAT in gills (100 μg/L) - Increase of acid phosphatase activity in hemolymph (0.008 and 10 μg/L)	Revel et al., 2019
Mytilus galloprovincialis	PS	Microsphere	1, 10 and 90 μm	Aliquots of 15,000 microspheres	3 h	GRT was found to be directly related to the sizes with highest GRT for MPs of 90 μm	Kinjo et al., 2019
Perna canaliculus	PE	Microbeads	38–45 μm	1 g/cc	48 h	Decrease of: - Clearance rate - Oxygen respiration rate - Byssus production	Webb et al., 2020

PS: polystyrene, PE: polyethylene, HDPE: High-density polyethylene; MP: microplastics; AChE: acetylcholinesterase; GST: glutathion S-transferase; SOD: superoxide dismutase activity; DG: digestive gland; CAT: catalase; ROS: reactive oxygen species; LPO: lipid peroxidase; GRT: gut retention time.

unexposed mussels over time indicated no prominent effects on the working of the proton pump. It can be hypothesized from the results of the current study that microplastic exposure did not threaten the cellular physiology enough to initiate indiscriminate autophagy (Versteeg and Giesy, 1985).

As for oxidative stress, even if some publications exist about the response of microplastics, the comparison of the data remains difficult to highlight the implications of the nature-based microplastics. Von Moos et al. (2012) reported lower dye retention in lysosomes of *M. edulis* after 96 h exposure of 2.5 g/L of >0–80 μm HDPE fluff, which represents an acute exposure (short exposure time/high concentration). Avio et al. (2015) showed that lysosomal integrity appeared more sensitive than antioxidant biomarkers in *M. galloprovincialis*, as it decreased after exposure to both virgin and pyrene-sorbed PE and PS microplastics at a high concentration of 1.5 g/L, during 7 days. Recently, Revel et al. (2019) revealed a significant increase of AcP activity in lysosomes, from the hemolymph of mussels exposed to 0.008 and 10 μg/L of a mix of PE and PP particles during 10 days. The similarities of experimental designs between this last study and the present one allows to conclude than PLLA would be safer than petroleum-based microplastics. However, further investigations are needed to confirm this hypothesis. Moreover, study on lysosomes in digestive glands would be helpful in evaluating the impacts of bio-based microplastics on lysosomes. The gills play a role in the uptake of particles *via* microvilli by the process of endocytosis, and with the help of cilia the particles can navigate their way to stomach, intestines, and eventually to the digestive glands (Von Moos et al., 2012). Nevertheless, all these processes are totally dependent on the size of the particles (Paul-Pont et al., 2018).

4.5. Metabolomics implication

Metabolomics analysis is increasingly used to investigate organismal responses at the molecular level in conjunction with the development of the analytical technology and the methods of data treatments (Dumas et al., 2020). Metabolomics aim to profile the end products of the regulatory processes of a cell and hence are closely related to the biological condition of an organism when compared to transcriptomics or proteomics (Miracle and Ankley, 2005). Nuclear Magnetic Resonance (NMR) spectroscopy, or mass spectrometry, are the analytical techniques involved in the metabolomics analysis. The metabolic phenotype of the organisms can be considered as an indicator of tissue differentiation, disease or environmental stresses, such as climate changes or pollution (Bundy et al., 2009; Hines et al., 2010; Flores-Valverde et al., 2010; Van Aggelen et al., 2010; Cubero-Leon et al., 2012). This tool can be used with organisms either from the field (Southam et al., 2014) or employed during laboratory exposures. For example, metabolomics has been previously used to investigate responses in mussels after exposures to nickel and chlorpyrifos (Jones et al., 2008).

Mass spectrometry-based profiling techniques were performed in the present study. The multivariate analysis of the ion intensities observed in each mussel using PCA depicted the discrimination of controls from PLLA microplastic exposed mussels, regardless of the dose of exposure. These results showed that the microplastic exposure lead to an effect on the mussels at the molecular level. The 48 down-regulated features were responsible for the discrimination of mussels in the OPLS-DA. Most of them were identified as glycerophospholipids based on HR-MS accuracy (Table 1). Glycerophospholipids are one of the major components of lipid membranes. They fulfill several roles, such as messengers, in the regulation of the binding of proteins to membranes, and in the participation in signaling such as cell cycle and apoptosis (Hannun and Obeid, 2008).

With coarse-grained molecular simulations, Rossi et al. (2014) showed that polystyrene (PS) nanoplastics were able to permeate easily into lipid membranes. They changed the membrane properties and lateral organization, affecting membrane protein activity and cellular function. Nevertheless, these results involved the use of small nanoplastics, which is not the case of the present study. The results of the simulation of Rossi et al. (2014) were mentioned by Tallec et al. (2018) as an explanation for the malformations of oyster embryos after exposure with PS particles at different sizes (50 nm, 500 nm and 2 μm) and concentrations (0.1, 1, 10, and 25 μg/mL), which let them consider future lipidomic approaches to assess lipid membrane composition. Only the smallest sizes, *i.e.* 50 nm, lead to significant effects on oyster gamete and embryo-larvae life stages. These results highlighted the difference between nano- and micro-plastics whose behavior, bioavailability, and toxicity are known to be very different (Paul-Pont et al., 2018). Such suggested lipid membrane disturbance can presumably originate from interaction between biological membranes and plastic particles due to their physical properties. It is of great importance when particles are smaller than 100 nm, as it changes their surface area and subsequent interaction with biological membranes (Klaine et al., 2012). The permeation of microplastic fragments between 0.8 and 10 μm through the lipid membranes is probably not envisaged, but the possible presence of additives in the polymer could be a way of explaining the effects observed on glycerophospholipids.

Finally, metabolomics and precisely lipidomic analysis, is (i) a complementary tool to biochemical analysis in ecotoxicology studies, (ii) a contribution to the understanding of the microplastic action on cellular mechanisms of and (iii) a promising tool in risk assessment of coastal environments.

4.6. Environmental relevance

In this study, the concentrations of microplastics used in the tanks, *i.e.* 10 and 100 μg/L, were chosen to be quite small compared to other studies (Von Moos et al., 2012; Avio et al., 2015). They are in accordance with others using 32 μg/L (Paul-Pont et al., 2016) and 0.008, 10 and 100 μg/L (Revel et al., 2019), the latter of which are environmentally relevant concentrations. Nevertheless, these concentrations cannot be compared to others expressed in number of particles per volume unit, which is more representative of concentrations measured in the field. Concerning bio-based and biodegradable microplastics, the concentrations are probably not representative of the field, since this type of polymer is quite rarely detected in the works evaluating the environmental contamination (Phuong et al., 2016).

At the highest concentration of 100 μg/L, the results were not significantly different to the controls, even if the mortality was the highest in this group (discussed above). Moreover, the findings of biomarker studies showed that microplastic concentrations of 10 μg/L lead to slight, but non-significant, biochemical effects (CAT and GST) on *M. edulis* after 8 days of exposure. Consequently, the correlation between effects and microplastic concentrations would not be linear. This result could be explained by the feeding behavior of mussels. The filtration activity of mussel could be disturbed in cases of high presence of microplastics, leading to lower impregnation by contaminants, as well as lower impacts. In literature, the filtering activity of *M. edulis* was shown as significantly dependent on different types of suspended particles (Navarrao et al., 1996), likely including particles of plastics as it was shown with nanoparticles of PS at varying concentrations (0.1, 0.2 and 0.3 g/L) used for exposure experiments (Wegner et al., 2012). Valve opening, in millimeters, decreased from 4 mm to 1 mm in the presence of aggregated nanoparticles after 20 min of exposure. The time was necessary for the mussels to determine that nanoparticles of PS were a low nutritional food (Wegner et al., 2012). Finally, the same study concluded that the time decreased feeding and increased production of pseudo-feces, which lowered the energy acquisition and consequently starved the mussels.

5. Conclusions

This experiment illustrated that combinations of biomarkers can be utilized to characterize the dose and effect relationships of bio-based

and biodegradable microplastics on the blue mussel (*M. edulis*). From the results obtained from the PLLA microplastic exposure studies at 10 and 100 μg/L, with size ranging from 0.8 and 10 μm, during 8 days, it is concluded that organisms exhibited no significant signs of both oxidative stress and neurotoxicity. Slight, but not significant, increased activities of CAT and GST biomarkers in the organisms exposed to 10 μg/L were found. It is correlated with an upregulation of antioxidative enzymes, implying an increase in the physiological challenges the organisms faced after microplastic exposure. No significant difference in lysosomal membrane structures was observed as well. It can be hypothesized that microplastics did not threaten the cellular physiology in gills enough to initiate indiscriminate autophagy. The results showed the lipidome of mussels would be impacted by microplastics at both doses. Glycerophospholipids, important structural lipids of biological membranes, seemed to be the most disrupting lipid category in individuals exposed to microplastics. The comparison of the results of the present study to those of the literature does not allow us to confirm that bio-based and compostable particles are safer than petroleum-based ones. Finally, biochemical and metabolomic approaches were complementary tools in the assessment of the environmental risks of microplastics. However, more research is needed to be done to evaluate the prospective toxicity of bio-based plastics by using other potential endpoints.

CRediT authorship contribution statement

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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