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Lipidomic analysis of mussel hemocytes exposed to polystyrene nanoplastics

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ABSTRACT

Plastics production and usage has exponentially increased in the last decades around the world. Due to the insufficient waste management, a significant amount of plastic ends up in the environment, where they tend to fragment into micro- and nano-plastics (NPs), and accumulate in aquatic organisms with still unknown effects. Although studies have indicated that lipid metabolism is a main target of NPs, this mechanism has not been extensively explored. In this study, we evaluated changes in the lipidome of mussel hemocytes after exposure to polystyrene (PS) NPs of 50 and 500 nm, at two different concentrations (10^6 and 10^9 particles/mL) for 24 h. The lipidome of hemocytes, analyzed by FIA-ESI (±) Orbitrap, was characterized by a relatively high abundance of cholesteryl esters (CEs) and phosphatidylcholine-plasmalogens (PC–Os/PC-Ps), involved in cell's defense against oxidative stress and membrane reorganization. In hemocytes exposed to PS NPs, a number of highly unsaturated membrane lipids were down-regulated, indicating a reorganization of the cell membranes after exposure to the particles and an oxidation of lipids with a high number of double bonds. This reduction was more evident after exposure to 50 nm NPs –both concentrations- and 500 nm NPs –high concentration–. The analysis of culture medium suggested increased release of vesicles enriched in triglycerides (TGs). The relevance of these responses to NP exposure on the immune function of hemocytes remains to be investigated.

1. Introduction

Nowadays, plastics are used all over the world and their production has exponentially increased in the last decades. Due to the insufficient waste management, a significant amount of plastics ends up in the environment, threatening human and environmental health (Kögel et al., 2020; Sendra et al., 2021). It is estimated that 4.8 to 12.7 million tons of plastic were released into the ocean in 2010, and this amount could reach 250 million tons by 2025 (Jambeck et al., 2015). Once in the environment, plastics tend to fragment into micro-plastics (MPs) (1 μm–5 mm) and NPs (1–1000 nm) under the action of biotic and abiotic factors, including solar radiation, temperature, waves and wind (Thompson, 2015; Song et al., 2017; Jang et al., 2018). Apart from the formation of MPs and NPs by plastic fragmentation (secondary origin), they can also be intentionally manufactured (primary origin) for different applications (cosmetics, nanomedicine) (Blair et al., 2017). These particles can easily find their way into the environment, via wastewater or through incidents during the manufacturing, processing, transport and recycling of products (Boucher and Friot, 2017). Calculations based on plastic production suggest that the concentration of MPs (<5 mm) range between 4.7×10^{-4} and 3.5×10^{3} particles/L, and it is expected that concentrations of NPs will be 10^{14} times higher than those being reported for MPs (Besseling et al., 2019).

Once in the aquatic environment, MPs and NPs are taken up by aquatic organisms, including crustaceans, filter feeding species and fish (Carbery et al., 2018; Eltemsah and Bøhn, 2019). They accumulate in the gut and gills, enter the circulatory system and reach other organs (Abbasi et al., 2018). Due to their filter feeding habits and their sessile lifestyle, mussels are key targets to plastic particles (Ward et al., 2019; Sendra et al., 2021), and thus, suitable sentinel organisms for assessing

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the potential effects of MPs and NPs in the marine environment (Wegner et al., 2012; Brandts et al., 2018; Bråte et al., 2018; Li et al., 2019). Additionally, mussels are also important particle vectors, as they are a major food source for predators and humans (Li et al., 2019). Recent investigations have demonstrated that MPs and NPs reduce the filtering activity and induce changes in feeding behavior in mussels (Wegner et al., 2012), alter shell formation during embryo development (Balbi et al., 2017), induce lipid peroxidation and oxidative damage (Brandts et al., 2018), neurotoxicity (Avio et al., 2015), immune responses (Pittura et al., 2018; Green et al., 2019), genotoxicity (González-Soto et al., 2019), and alter gene expression (Avio et al., 2015; Paul-Pont et al., 2016; Détrée and Gallardo-Escárate, 2017; Capolupo et al., 2018). Moreover, the translocation of different sizes of polystyrene NPs in mussel's hemolymph has been reported, being faster for the smallest particles (50 nm) than for the largest ones (1 μm) (Sendra et al., 2020a).

Hemocytes are the first line of defense of bivalves against pathogens or foreign particles. Mussel hemocytes, especially the granulocytes, are professional phagocytes showing a well-developed endo-lysosomal system (Cajaraville and Pal, 1995). Polystyrene (PS) NPs and MPs are internalized by mussel hemocytes primarily into lysosomes via endocytic and non-endocytic pathways (Sendra et al., 2020b; Katsumiti et al., 2021). Phagocytosis was the main uptake mechanism in mussel hemocytes for 1 μm PS particles, while the caveolae and clathrin routes were shown to play important roles in the uptake of 50 and 100 nm PS NPs (Sendra et al., 2020a). Hemocytes are often used to investigate the impact of environmental pollution on the immune system of mussels (Cajaraville et al., 1996; Burgos-Aceves and Faggio, 2017), and primary hemocyte cultures have been proposed as a robust in vitro system for screening the effects and mechanisms of action of nanoparticles in bivalves (Canesi et al., 2012; Katsumiti and Cajaraville, 2019). Changes in hemocyte abundance and subtypes, alterations in cell viability, plasma membrane integrity, lysosomal and phagocytic activity, the production of reactive oxygen species (ROS) and gentotoxicity are among the toxic effect of NPs in mussel hemocytes (Pittura et al., 2018; Sendra et al., 2020a, 2020b; Shi et al., 2020; Cole et al., 2020; Katsumiti et al., 2021).

Recently, -omic approaches have been applied to characterize the complex interactions between hemocytes and pollutants (Nguyen and Alfaro, 2020; Balbi et al., 2021). The biomolecular information obtained –e.g. structure, function, and expression dynamics of genes, proteins and metabolites–, provides a better understanding of the underlying mechanisms of toxicity, and can often help to better interpret the physiological responses (Koelmel et al., 2020). Lipids are ubiquitous and diverse biomolecules with key biological roles as structural components of cell membranes, energy storage and cell signaling. Lipid metabolism appears as one of the main targets of MPs and NPs in several aquatic organisms (Barría et al., 2020; Kögel et al., 2020; Kim et al., 2020). Thus, the possibility that NPs may affect the lipidome of bivalve hemocytes, and consequently modulate immune functions, is an intriguing mechanism that deserves to be explored (Balbi et al., 2021). In this context, this study aims to improve our understanding of the ability of polystyrene NPs to modulate the lipidome of mussel's hemocytes and, possibly, the immune response. We applied a lipidomic-based approach to identify changes in the hemocytes exposed to two concentrations (10^6 and 10^9 particles/mL) of PS NPs (50 and 500 nm) for 24 h. Additionally, the culture medium was analyzed to assess whether exposure to NPs could trigger the release of specific lipids through for instance exocytosis related processes.

2. Materials and methods

2.1. Nano-plastic characterization

The plastic particles selected for this work were unlabeled PS particles of 50 nm (Polysciences Inc., cat. # 08691) and 500 nm (Polysciences Inc., cat. # 07307). Hydrodynamic size and surface charge of 50 nm and 500 nm NPs (10^6 and 10^9 particles/mL) suspended in culture medium (Basal Medium Eagle (BME) supplemented with 0.001% gentamicin) were assessed by dynamic light scattering (DLS; Zetasizer Nano ZS, Malvern, UK). Particle aggregation was greater in 50 nm NP suspensions (7700 nm) than in suspensions of 500 nm (2328 nm) as described in Katsumiti et al. (2021). Both particle sizes showed negatively charged surface in BME, with Z-potential values of approximately −23 mV (Katsumiti et al., 2021).

2.2. Hemocyte culture and in vitro exposures

Hemocyte culture was carried out as described before (Katsumiti et al., 2021). Mussels (*Mytilus galloprovincialis*) of 3.5–4.5 cm shell length were collected in February 2021 from a relatively clean site in Plentzia, Bay of Biscay (43°24′41.9″N 2°57′01.1″W) (Bellas et al., 2013) (water temperature approximately 15 °C) and acclimatized for 2 days in aerated tanks with seawater at 18 °C, daily food supply (Marin Coraliquid Sera GmbH, Heinsberg, Germany) and 12/12 h photoperiod in the aquaria facility of the Cell Biology in Environmental Toxicology research group (CBET, UPV/EHU). After acclimatization, hemolymph from 20 animals was withdrawn from mussel's posterior adductor muscle under aseptic conditions in a vertical laminar airflow cabinet (Cultair BC100, Cultek S.L., Madrid, Spain). The hemolymph was pooled and diluted 9:1 in anti-aggregation solution (171 mM NaCl; 0.2 M Tris; 0.15% v/v HCl 1 N; 24 mM EDTA) to obtain 1×10^6 cells/mL (>95% viable according to trypan blue exclusion assay). Then, 200 μL of cell suspensions were seeded into each well of 96 well microplates, centrifuged (Beckman Coulter, Palo Alto, USA) at 270 *g* for 10 min at 4 °C in order to favour cells to attach and kept in culture media (BME, 1040 mOsm/kg, pH 7.4, supplemented with 0.001% gentamicine) for 24 h at 18 °C in a Sanyo incubator (Osaka, Japan) to establish the primary cell cultures before performing the exposures.

Once established the cell culture, hemocytes were exposed to 10^6 and 10^9 particles/mL of 50 and 500 nm PS NPs for 24 h. These exposure concentrations were found to induce cellular responses in mussel hemocytes (Katsumiti et al., 2021). Exposures were carried out in sextuplicate and repeated in three independent experiments (18 wells per treatment). Unexposed cells were used as controls. After exposure, cells and culture medium were collected in separated tubes and frozen at −80 °C for lipid analysis.

2.3. Lipid extraction

For lipid analysis, samples from 2 wells (replicates of the same treatment) were combined to increase the amount of sample. A total of 9 samples per treatment were analyzed. Hemocytes (4×10^5 cells in 400 μL; 10^6 cells/mL) and culture medium (400 μL) were extracted with a mixture of methanol:chloroform (1:2 v/v) containing 0.01% butylated hydroxytoluene (Folch et al., 1957). Briefly, samples were diluted 20x with the solvent mixture and vortexed. Hemocyte samples were placed in an ultrasound bath for 3 min to break cell membranes. Samples were incubated at room temperature under gentle agitation (110 rpm) for 30 min; thereafter, a solution of 0.88% KCl (100 μL and 600 μL for hemocyte and culture medium samples, respectively) was added to induce phase separation. The organic phase was collected and the extraction was repeated twice. The organic phases were evaporated under gentle nitrogen flow, and the extracts were stored at −20 °C under an argon atmosphere until analysis.

2.4. Analysis of lipids

FIA – (+/− H-ESI) Orbitrap – Exactive was applied for analysis of lipid extracts as previously described by Marqueño et al. (2019). Briefly, 5 μL of the reconstituted extract (methanol) were injected at a rate of 50 μL/min using methanol:dichloromethane (80:20) as mobile phase. The acquisition mass range was set to m/z 200–2000 and the total analysis time was 2 min. Ultrahigh resolving power defined as R: 100,000 (m/z

200, FWHM) was applied. The mass peaks considered were single positively charged sodium molecular ions [M^+Na^+] for triglycerides [TGs], diglycerides [DGs], cholesterol esters [CEs], phosphatidylcholines [PCs], lyso-PCs, PC-plasmanyl [PC-Os]/PC-plasmalogens [PC-Ps], phosphatidylglycerols [PGs], and ceramides [Cer]. Single negative charged [$M\text{-}H^+$] were considered for phosphatidylethanolamines [PEs], PE-plasmanyl [PE-Os]/PE-plasmalogens [PE-Ps], lyso-PEs, phosphatidylserines [PSs] and phosphatidylinositols [PIs]). Identification of the lipids was based on the accurate mass measurement (error <5 ppm), isotopic distribution, charge, adduct formation, and elements in the molecular formula. Mass spectra were processed using Xcalibur 2.1 (Thermo Fisher Scientific, Bremen, Germany). Quantification was performed with an internal standard consisting of a mixture of representative lipids for each lipid subclass. The deuterated lipids included in the internal standard are detailed in Table S1 (Supplementary information), together with the relative standard deviation (RSD) intra-experiment calculated for each lipid. Lipid species were annotated as < lipid class > − < total fatty acyl chain)>:<total number of double bonds>.

2.5. Statistical analysis

Lipid profiles were analyzed using the online software MetaboAnalyst 5.0 (Pang et al., 2021). Beforehand, missing values were estimated using K-Nearest Neighbors (feature-wise) and data was auto-scaled. Subsequently, Partial Least Squares-Discriminant Analysis (PLS-DA) was used to compare control and exposed samples, and to identify the lipids responsible for the discrimination based on the variable importance in the projection (VIP) parameter. The relevance and predictability of the PLS-DA model was assessed using the R^2 and Q^2 values, respectively, to examine the quality of the model. Volcano plots, representing a combination of fold change (FC) and Student's *t*-test (FC > 1.5; $p < 0.05$) were applied to visualize the magnitude and the significance of the changes detected in the lipidome of control and exposed samples.

3. Results

3.1. Lipid profile of hemocytes and culture medium

The flow injection analysis of lipid extracts of control hemocytes allowed the identification of 132 lipids across 12 lipid classes. The most abundant lipid subclasses were PCs (~36% of total analyzed lipids), followed by plasmanyl/plasmenyl analogues (PC–Os/PC-Ps), which represented up to ~25% of the detected lipids, and CEs (~17%). TGs and PEs represented ~8% and ~5% of the analyzed lipids, respectively. The other lipid subclasses were present in smaller amounts (<3%) (Fig. 1). Regarding fatty acids, stearic acid (18:0) was the major constituent (40%) followed by palmitic acid (16:0; 34%) and oleic acid (18:1; 3%) (Table S2, Supplementary information). Polyunsaturated fatty acids (PUFAs) represented about 12% of total free fatty acids, being 22:6 (linoleic acid) the most abundant (3.0%).

Interestingly, the analysis of lipids in the culture medium (50 lipid species identified across 8 lipid classes) offered a completely different profile. TGs was the most abundant lipid subclass (~51%), followed by PCs (~29%), while other lipid subclasses were present in smaller amounts (<7%) (Fig. 2). Regarding fatty acids, stearic acid (18:0) was the major constituent (44%) followed by palmitic acid (16:0; 39%) and oleic acid (18:1; 3.7%). Polyunsaturated fatty acids (PUFAs) represented 3.9% of total free fatty acids (Table S2, Supplementary information).

3.2. The effects of nanoplastic exposure on the lipidome of hemocytes and culture medium

Fig. 3 depicts the concentration of the different lipid families in control and exposed hemocytes. A tendency towards decreased levels of membrane lipids and TGs is observed in exposed hemocytes, although only the decrease of PC-Os/PC-Ps after exposure to NPs -high concentration-was statistically significant. The multivariate PLS-DA analysis shows low discrimination between the lipidome of control and exposed hemocytes (Fig. S1, supplementary information). Even though, up to 47 lipids (VIP ≥1) were identified as responsible for the discrimination between control and exposed hemocytes, these lipids were mainly membrane lipids (PCs, PC-Os/PC-Ps, PIs) and some TGs, and almost all of them were down-regulated, particularly after exposure to 500 nm NPs -high concentration- and 50 nm NPs –low and high concentrations- (Fig. 4). Furthermore, volcano plots show the specific lipids that were significantly downregulated in hemocytes after exposure. Namely, a 2-fold decrease of TGs (56:7, 58:7, 58:10), PCs (40:6, 32:0), PCO 32:0, PCO 34:1/PCP 34:0 and PI 38:6 was observed after exposure to 10^9 part/mL of 500 nm NPs. Similarly, TGs (50:4, 58:7, 58:10), PC 36:6, PCO 38:5/PCP 38:4 and PIs (38:6, 40:7) decreased (2-fold) after exposure to 10^9 part/mL of 50 nm NPs, while DG 32:1 increased. For the low concentration conditions, no significant changes were observed after exposure to 500 nm NPs, while 24 h exposure to 50 nm NPs lead to a significant decrease of TG 52:4, PCs (32:0, 36:4, 36:6, 40:4), PE 40:5 and PI 40:7 (Table 1).

Regarding culture medium, no significant differences were observed for the concentration of the different lipid families in control and exposed samples (Fig. S2, supplementary information). This is in agreement with the multivariate PLS-DA analysis, which shows low

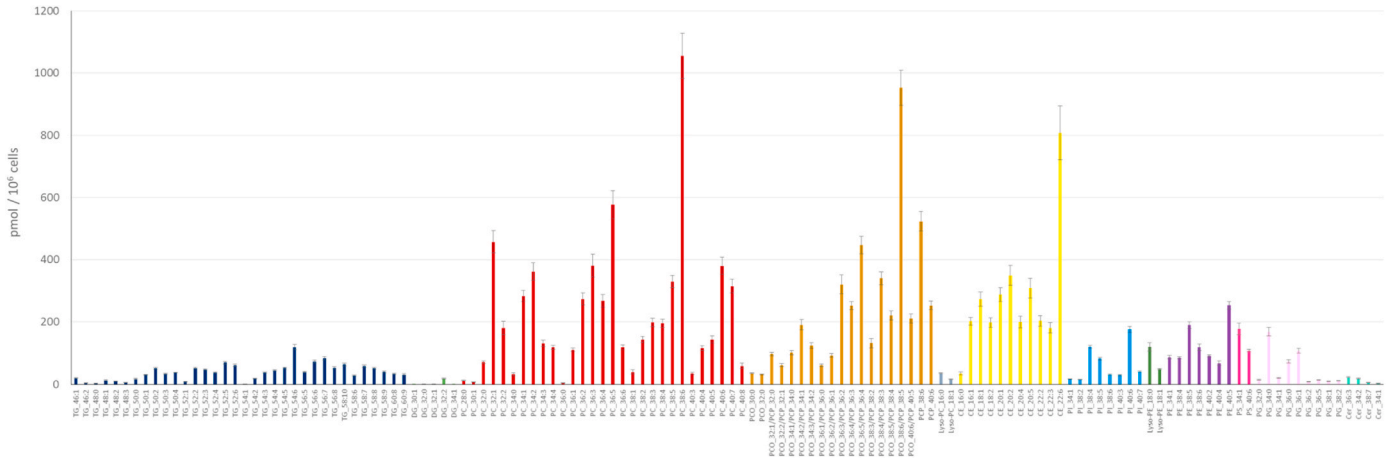

Fig. 1. Lipid profile of control hemocytes indicating the different lipid species quantified (pmol/10^6 cells). Values are mean ± SD (n = 7). Lipid species are annotated as lipid class followed by the number of carbons and the number of double bonds.

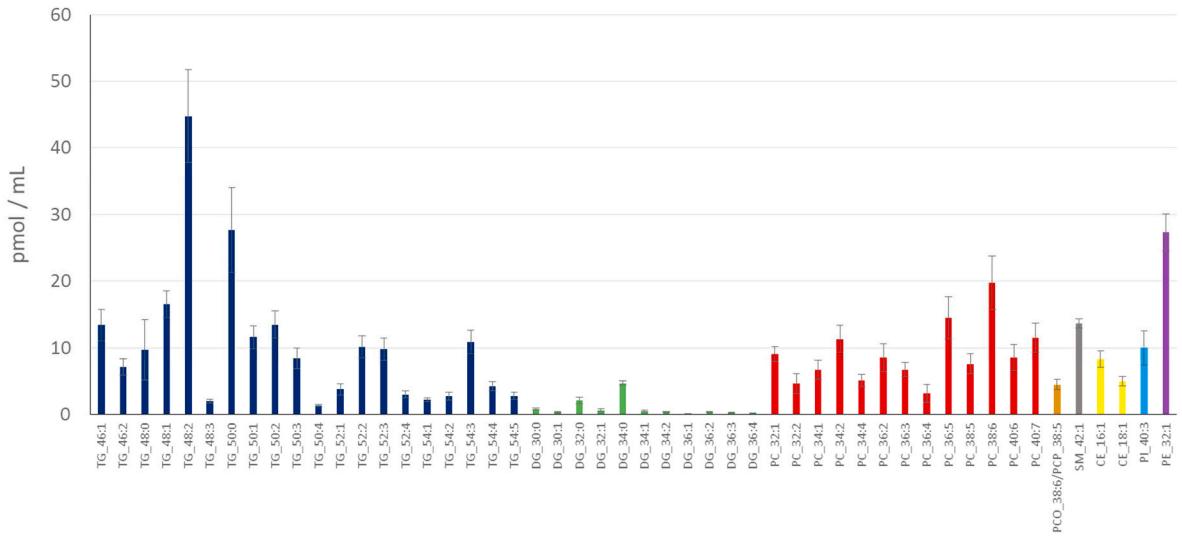

Fig. 2. Lipid profile of culture medium of control hemocytes indicating the different lipid species quantified (pmol). Values are mean ± SD (n = 8). Lipid species are annotated as lipid class followed by the number of carbons and the number of double bonds.

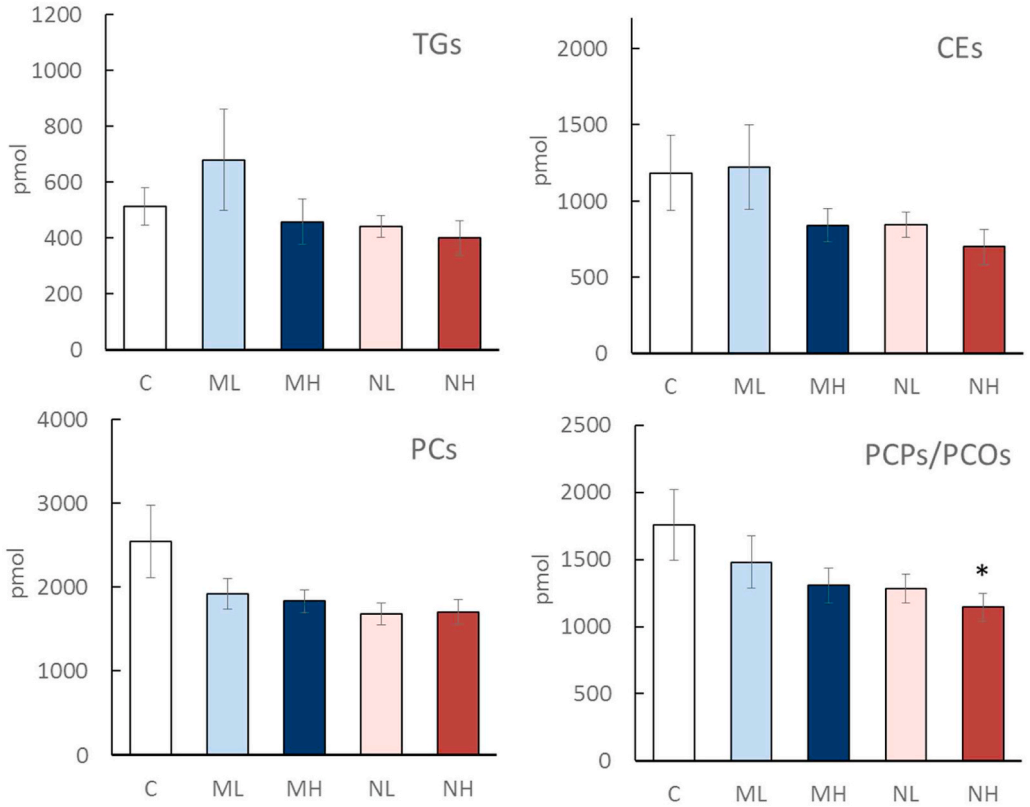

Fig. 3. Concentration of the different lipid families in control hemocytes (C), and hemocytes exposed to low (L) and high (H) concentrations of 500 nm (M) and 50 nm (N) NP for 24 h. Values are mean ± SD (n = 7). *Statistically significant differences respect to control.

discrimination between the lipids detected in the culture medium of control and exposed hemocytes (Fig. S3, supplementary information). Univariate analysis (volcano plots) confirmed a trend towards an increase in lipids for all exposure conditions. PC 34:1 and TG 48:0 increased (2–3 fold) after exposure to 500 nm NPs (10^9 part/mL). DG 34:0 decreased (2 fold) and CE 18:1, PC 32:1 and TGs (48:0, 52:4) increased (2–3 fold) after exposure to 50 nm NPs (10^9 part/mL). For the low concentration conditions, no significant changes were observed after exposure to 500 nm particles, while 24 h exposure to 50 nm NPs lead to a significant increase of PC 34:1 (2 – fold) (Fig. S4, supplementary information).

4. Discussion

Both sizes of NPs were internalized by hemocytes as evidenced by the transmission electron microscopy (TEM) images described in a previous work (Katsumiti et al., 2021). After 24 h of exposure, the small NPs were mainly found inside membrane-bound vesicles, especially in tertiary

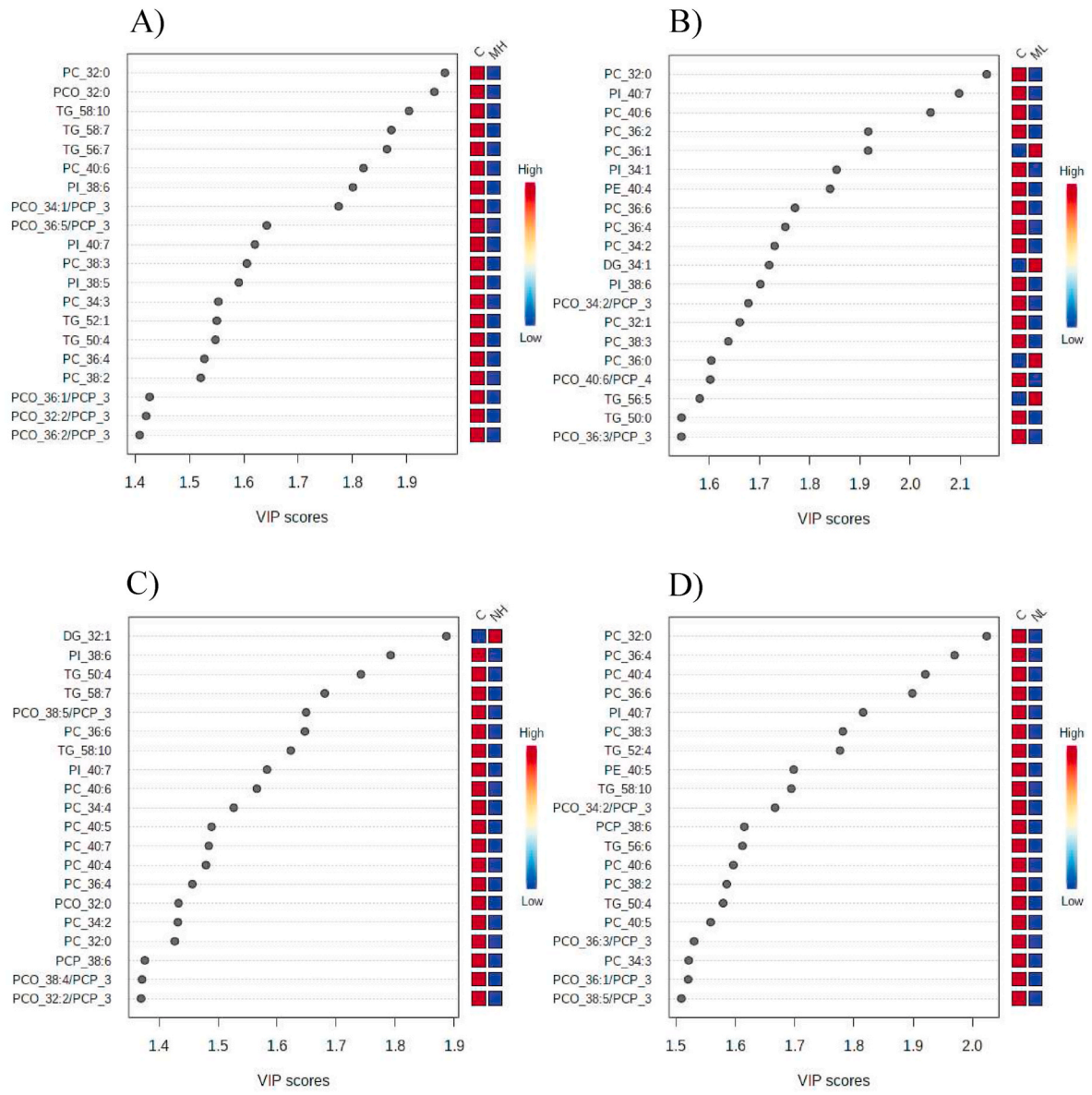

Fig. 4. PLS-DA VIP score lists of the lipids responsible for the discrimination between control and exposed hemocytes. For clarity, only the first 20 lipids have been listed. Heatmaps show the up-regulation (red) and down-regulation (blue) of these specific lipids in control and exposed hemocytes. A) 500 nm; 10^9 part/mL; B) 500 nm; 10^6 part/mL; C) 50 nm; 10^9 part/mL; D) 50 nm; 10^6 part/mL. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

lysosomes or residual bodies containing lipofuscins, while 500 nm NPs were found extracellularly surrounded by cytoplasmic projections and intracellularly within membrane-bound vesicles, suggesting internalization via macropinocytosis or other endocytic pathways. The small NPs were also found free in the cytosol, indicating possible diffusion across the plasma membrane (Katsumiti et al., 2021), as described in primary mammalian cell cultures (Fiorentino et al., 2015). Interestingly, a decrease in hemocyte viability was only detected after exposure to 500 nm NPs (10^9 part./mL) (50 μg/mL), and the two concentrations tested reduced plasma membrane integrity (Katsumiti et al., 2021). Increased lysosomal acid phosphatase activity as well as increased lipofuscin contents indicative of enhanced lysosomal and autophagic activity were observed in hemocytes exposed to 10^9 part/mL of 500 nm NPs, whereas phagocytic activity towards zymosan particles increased after 24 h exposure to both 50 and 500 nm NPs at the two concentrations tested (Katsumiti et al., 2021). All these effects involve substantial rearrangements in hemocyte's membranes and we hypothesized that they could be accompanied by changes in the lipidome.

The analysis of the lipid extracts by FIA-Orbitrap is a powerful tool that allowed a first characterization of the lipid profile of mussel hemocytes in only 2 min acquisition time. As a drawback, due to the absence of column separation, highly complex mass spectra are obtained, and only the most abundant and easily ionized lipids are detected. This precludes the detailed description of the complete lipidome of the hemocytes and a full description of the overall changes in the lipidome potentially induced by PS NPs. Despite this, and the relatively low number of hemocytes analyzed per sample (4×10^5 cells), it was possible to detect that mussel hemocytes were enriched in glycerophospholipids (PCs and PC-Os/PC-Ps) and cholesteryl esters (CEs). Glycerophospholipids, such as PCs and PEs, are the main lipids that make up biological membranes, while PC-Ps, which were very abundant in mussel hemocytes (~25% of the detected lipids) are unique membrane glycerophospholipids that contain a fatty alcohol with a vinyl ether linkage at the sn-1 position and are enriched in polyunsaturated fatty acids at the sn-2 position of the glycerol backbone (Lessig and Fuchs, 2009; Braverman and Moser, 2012). Our analytical approach did

Table 1
Specific lipids that were significantly up- or down-regulated in hemocytes after exposure to NPs (50 and 500 nm) after volcano plot analysis, representing a combination of fold change (FC) and Student's t-test (FC > 1.5; p < 0.05). All lipids were down-regulated except otherwise indicated.

Lipid	500 nm (10^6 particles/mL) fold change	Lipid	500 nm (10^9 particles/mL) fold change
PC36:0	2.26 (up)	DG34:0	3.19 (up)
		PC32:0	1.85
		PC40:6	2.04
		PCO32:0	2.22
		PCO34:1/ PCP34:0	2.50
		PI38:6	1.69
		TG56:7	2.22
		TG58:7	1.82
		TG58:10	1.92
Lipid	**50 nm (10^6 particles/mL)**	**Lipid**	**50 nm (10^9 particles/mL)**
PC32:0	1.92	PC36:6	1.89
PC36:4	2.38	PCO38:5/ PCP38:4	1.82
PC36:6	2.17	PE_32:1	2.27
PC38:3	1.75	PE-P38:6	1.59
PC40:4	2.00	PI38:6	2.33
PE32:1	1.64	PI40:5	1.54
PE40:5	1.67	PI40:7	1.79
PI40:5	1.79	TG50:4	2.08
PI40:7	1.64	TG58:7	2.08
TG50:4	1.69	TG58:10	2.22
TG52:4	1.82		
TG58:10	1.72		

not allow discriminating PC-Ps from the isobaric alkyl ether lipids (PC–Os), therefore, both ether-lipids were quantified together (PC-Ps/PC-Os). Nonetheless, the observed enrichment of mussel hemocytes in plasmalogens is in agreement with previous studies that showed that marine bivalves, and particularly hemocytes, possess a significant amount of plasmalogens (Le Grand et al., 2011).

Regarding their function, plasmalogens are easily oxidized because the hydrogen atoms adjacent to the vinyl ether bond have low dissociation energy. Therefore, they can act as scavengers; protecting unsaturated membrane lipids from oxidative damage (Braverman and Moser, 2012). Moreover, the oxidative products of plasmalogens can no longer propagate lipid peroxidation, which enhances their role in cellular protection (Braverman and Moser, 2012). Thus, the high abundance of PC-Os/PC-Ps detected in mussel hemocytes may serve to protect cell components against oxidative stress, taking into consideration that production of ROS during phagocytosis is one of the main protective mechanisms displayed by hemocytes in the presence of pathogens and foreign particles (Canesi et al., 2012). The high abundance of PC-Os/PC-Ps may also confer increased membrane fluidity, and consequently facilitate processes of membrane fusion and deformation (e.g. phagocytosis), which are characteristics of hemocyte behavior (Cajaraville and Pal, 1995; Le Grand et al., 2011; Canesi et al., 2012). In addition, the role of plasmalogens in endocytosis, cell signaling and the modulation of inflammatory and immunological responses has also been described (Braverman and Moser, 2012; Laudicella et al., 2020).

Interestingly, when hemocytes were exposed to PS nanoparticles, a number of membrane lipids (mainly PCs, PC-Os/PC-Ps, PIs) were down-regulated, suggesting a reorganization of the cell membranes after exposure to the nanoparticles, and possibly an oxidation of the lipid molecules with a high number of double bonds, the most susceptible to oxidation (Borst et al., 2000). This reduction was more evident after exposure to 500 nm NPs –high concentration– and 50 nm NPs –both concentrations– (Table 1). Hemocytes are involved in internal defense through the elimination of internalized particles and increased production of ROS as a defense mechanism. Therefore, it is likely that the internalized PS particles will modulate the oxidative system of hemocytes, leading to increased generation of ROS and lipid oxidation. Indeed, Canesi et al. (2015) reported increased ROS production in hemocytes of *M. galloprovincialis* exposed for 30 min to 50 nm amino modified PS particles. Although Katsumiti et al. (2021) did not observe enhanced ROS production after exposure of mussel hemocytes to PS NPs for 24 h, the authors found increased levels of highly oxidized lipofuscins in the TEM analysis, which are indicative of oxidative damage. The significant decrease of PC-Ps/PC-Os, and particularly PC-P38:4/PC-O38:5, detected after exposure to 50 nm NPs (10^9 particles/mL) may therefore be indicative of increased levels of oxidative stress, which led to a significant oxidation of PC-Ps and their consequent decrease (Fig. 3). Moreover, a reorganization of the cell membranes to deal with particle exposure can also occur. Kim et al. (2020) reported significant changes in the glycerophospholipids of *Caenorhabditis elegans* exposed to nano-PS particles (100 nm), together with the altered expression of some autophagy-related genes. Rossi et al. (2014) showed that PS nanoparticles permeate easily into lipid membranes, and once dissolved in the membrane core PS chains alter membrane structure and significantly reduce molecular diffusion. Accordingly, our results show a significant depletion of unsaturated phospholipids (PC 36:4, 36:6, 38:3, 40:4, 40:6; and PIs 38:6, 40:5, 40:7), which may lead to a reduction of membrane fluidity.

In contrast to the changes of membrane lipids, the levels of CEs were not modulated by exposure to PS NPs. Several studies have shown that in addition to immunological processes, bivalve hemocytes may play a role in other physiological functions, including nutrient transport (Donaghy et al., 2009). The relatively high levels of CEs detected in the present study (~17%) further supports the involvement of hemocytes in lipoprotein transport and storage, although the precise mechanisms are still poorly understood.

The levels of TGs (~8%) in hemocytes were lower than those of CEs (~17%), and several TGs (TG50:4, TG52:4, TG56:7, TG58:7, TG58:10) showed a significant depletion (up to 2-fold) after exposure to NPs (high concentration). TGs can be hydrolyzed to obtain energy, but they can be also a source of fatty acids to repair unsaturated phospholipids that have been oxidized (Chan and Wang, 2018). In the present study, the observed depletion of unsaturated phospholipids (PC 36:4, 36:6, 38:3, 40:4, 40:6; and PIs 38:6, 40:5, 40:7), possibly by oxidation, could have induced a stimulation of the TGs hydrolysis process and consequently, a depletion of TGs as described by Chan and Wang (2018).

The culture medium had a lipid profile composed mainly of PCs and TGs of 46–50 carbon atoms and a low number of double bonds (0–2). This lipid profile can be attributed to the presence of vesicles released by hemocytes, either residual bodies loaded with NPs (Katsumiti et al., 2021) and/or extracellular vesicles involved in intercellular communication. So far, there is little information on the lipid content of extracellular vesicles, which are generally enriched in cholesterol and sphingolipids and carry mostly saturated fatty acids (Sagini et al., 2018). Regarding the impact of plastic particles, no marked changes were observed apart from the specific increase of some PCs and TGs in culture medium after exposure to particles, with the exception of 500 nm NPs (low concentration), which is perhaps indicative of increased vesicle release.

In conclusion, this work evidenced the presence in mussel hemocytes of a large amount of PC-Ps/PC-Os, which are involved in protection against oxidative damage and possibly membrane reorganization for endocytosis processes and in immunological responses; and a relatively large amount of CEs, which evidences the involvement of hemocytes in nutrient transport. When hemocytes were exposed to PS particles, a generalized decrease in PCs, PIs, PC-Ps/PC-Os and TGs was observed, possibly as a consequence of the induction of oxidative stress. This decrease was more evident as the number of particles increased and the particle size decreased. It remains to be explored how these subtle

changes in the lipidome of mussel's hemocytes induced by NP exposure can be translated into potential impacts on the immune function and host/pathogen interactions of these key marine organisms.

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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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.envres.2022.113763.

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