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Effect of size continuum from nanoplastics to microplastics on marine mussel *Mytilus edulis*: comparison *in vitro* / *in vivo* exposure scenarii

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Abstract

For several decades, plastic has been a global threat in terms of pollution. Plastic polymers, when introduce in the aquatic environment, are exposed to fragmentation processes into microplastics (MPs) and nanoplastics (NPs) which could potentially interact with living organisms. The objective of this work was to study the effects of plastic particles representative of those found in the environment, on the marine mussels *Mytilus edulis*, under two exposure scenarii: *in vivo* and *in vitro*. Whole mussels or cultured hemocytes were exposed for 24 hours to NPs and MPs generated from macro-sized plastics collected in the field, but also to reference NPs, at concentrations found in the environment: 0.08, 10 µg and 100 µg.L^{-1}. Results showed that immune response was only activated when mussels were exposed *in vivo*. However, cytotoxicity (hemocyte mortality) and genotoxicity (DNA damage) parameters were induced after both types of exposure, but in a dose-dependent manner after *in vitro* hemocyte exposure to all tested plastic conditions. These results indicate that *in vitro* approaches could be considered as potential predictors of *in vivo* exposures.

Keywords: *Mytilus edulis*, Nanoplastic, Microplastic, genotoxicity, cell viability, immune response

Introduction

Plastic increasing production and use since 1907 (Sorensen and Jovanović, 2021) has led to its continuous accumulation in ecosystems. Resulting from various anthropogenic activities such as industry, agriculture or the building sector, it could be accidentally or voluntarily released into natural environments, and has a significant longevity over time, from a decade to several millennia for the simplest products of human consumption as bottles or bags. In this context, plastic is one of the main environmental pollutant, due to its capacity to be found in all compartments of the environment, e.g., soil, air, water.

Plastics released into aquatic ecosystems under the action of environmental conditions such as waves, ultraviolet radiations, microorganisms etc.... generate particles of tiny sizes called microplastics (MPs) when the size range between 1µm and 5 mm, and nanoplastics (NPs) when the size is lower than 1000 nm (Andrady, 2011; Law & Thompson, 2014; Smith et al., 2018; Waite et al., 2018; Revel et al., 2019). Therefore, due to their small sizes, MPs and NPs have been observed in many aquatic organisms entering their body via food, contact or filtration and different toxicities related to these pollutants have been described at different levels of biological organization and in particular in bivalves that, as filter feeder, are more likely to interact with these polymers. Indeed, most of the studies were conducted with in vivo exposure in mesocosm or microcosm scenario approaches and MP and NP (of different types, sizes, origin) have demonstrated effects on bivalves at cellular as well as individual levels (Li et al., 2020; Pedersen et al., 2020; Nobre et al., 2020).

However, the diversity of MPs and NPs size, shape and chemical composition (additives) raises questions about the possibilities of testing all these particles for future regulatory needs. In this context, the use of miniaturized assays such as cell culture appears as an alternative. The *in vitro* technique of exposure has the notorious advantage of reducing the use of too many whole organisms, and thus of respecting the 3R rule (replace, reduce, refine) (Revel et

al., 2021). It allows to screen many different conditions of exposure (tested chemical, concentration, time of exposure...) and rapidly generate large quantity of data. Short-term *in vitro* experiments were previously conducted to reveal the acute toxicity of MPs and NPs, in a particle size-dependent manner, as shown in *M. galloprovincialis* hemocytes exposed to NPs (50 nm) (Canesi et al., (2016) or in various mussel species exposed to MPs (Paul-Pont et al., 2016). Cell-based assays provide a detailed understanding of the mechanisms involved in the organism's response to xenobiotic exposure, while remaining within a highly monitored framework. The review of Ringwood (2021) is in accordance with the use of high throughput screening evaluation highlighting the importance of developing diagnostic approaches to characterize potential environmental risks associated with emerging contaminants on bivalve species, which are biological sieves based on critical particle capture and processing pathways. For emerging contaminants such as MPs and NPs, this approach could allow a high throughput screening evaluation of different types of particles before verifications are made with *in vivo* experiments. Indeed, to be considered as relevant alternatives, *in vitro* effects must reflect effects observed after *in vivo* exposures.

In the present study, the common blue mussel, *Mytilus edulis*, was used, due its status as a sentinel species in biomonitoring programs (Viarengo & Canesi, 1991; Su et al., 2018), i.e., abundant, well distributed, sedentary, bio-accumulative due to its ability to filter water and as a species of sufficient size for the study and well known in the literature. Only few cell cultures are available for marine aquatic invertebrates and cell culture of hemocytes from *M. edulis* has been previously demonstrated to be suitable for high throughput screening of the toxicity of other emerging contaminants (e.g. nanomaterials) (Barrick et al., 2019). Hemocytes, belonging to the innate immune system of bivalve, are sensitive to non-self-substances and have been shown to react to the uptake of MPs and NPs (Canesi et al., 2016).

The main objective of this study was to compare *in vitro* and *in vivo* scenarii of exposure by evaluating the toxicity effects of environmental MPs and NPs on the marine mussel *M. edulis*. This preliminary study will allow to compare toxicity profiles of MPs and NPs between the two approaches in order to discuss the possible consideration of *in vitro*–based assays for assessment of larger range of different MPs and NPs, in term of size, composition, associated additives for future regulatory needs.

Material and methods

Collection, preparation and characterization of environmental derived MPs and NPs

Plastic wastes were collected by hand with pliers on the right bank of the Garonne River at low tide, near the Langoiran bridge (44°42'14.56"N, 0°24'3.91"W). The most oxidized plastic debris were sampled, rinsed in the Lab with ultra-pure water, dried at 45°C during 48h before preparation of micro and nanoplastic solutions.

Environmental micro and nanoplastics production

Environmental microplastics (ENV MPs) and nanoplastics (ENV NPs) were generated from macro-sized plastic debris according to the protocol described by Blancho et al., (2021). Briefly, NPs and MPs were produced through a process coupling agitation and sonification in aquatic media. Size range was between 1 and 1200 nm for ENV NPs and between 1.2 and 300 µm for ENV MPs. ENV NPs and ENV MPs were characterized in terms of composition, size, shape and surface properties by Pyrolysis (Pyrolyzer PY-3030 Frontier Lab) coupled to gas chromatography-mass spectrometry (Py-GC-MS) (5977B, Agilent Technologies). Plastic analysis showed that ENV NPs and ENV MPs were mainly composed of polyethylene (PE) (95%), that they were anisotropic, polydisperse in term of size and possessed high levels of carboxylic groups onto their surface. Carboxylated polystyrene nanobeads (200 nm) were used as reference material (PS NPs).

Acidic digestion and ICP-MS measurements

In order to optimize the total digestion, 100 mg of microplastics and nanoplastics powder were acid-digested (12 N HNO_3 sub grade) using a multi-step procedure with a microwave oven (MW7000 system from Anton-Paar; increasing ramp of temperature of 6.6°C per minute until reaching 250°C, then 25 min at 250°C under 140 bar of pressure). The solution of three tubes were mixed, evaporated at 90°C and solubilized in 0.37 N HNO_3 prior to ICP-MS measurements. Metal concentrations were measured by Inductively Coupled Plasma-Mass Spectrometry (ICP-MS) from Agilent Technologies (7700x Model, Agilent) (Supplementary Data Table A). The digestion and analyses process were validated using reference materials (ERM-EC 680 and ERM-EC 681) from the Joint Research Centre of the European Commission (JRC, Ispra, Italy).

Animal collection

M. edulis individuals were recovered from a distribution chain, obtaining supplies from a mussel farming site located in Bricqueville-sur-Mer, France (SMBCP, 2012). Mussels were

placed in artificial sea water (30 psu, at 15°C with a 12-hour light/dark cycle under aeration) for a 4-day acclimatization period prior to testing.

Primary cell culture of hemocytes and in vitro exposure

After the acclimatization period, *M.edulis* hemolymph was extracted with a syringe and filtered through a 70 µm filter into a falcon tube at 4°C. Cell viability is tested by the trypan blue exclusion technique and must have a survival percentage greater than 80%. Approximately 500 000 hemocytes were suspended in a L-15 medium (20.2 $g.L^{-1}$ NaCl, 0.54 $g.L^{-1}$ KCl, 0.6 $g.L^{-1}$ $CaCl_2$, 1 $g.L^{-1}$ $MgSO_4$, 3.9 $g.L^{-1}$ $MgCl_2$, 100 $units.mL^{-1}$ penicillin G, 100 $\mu g.mL^{-1}$ streptomycin, 1% gentamycin, 10% glucose and 10% Foetal Bovine Serum (FBS), pH 7.0) for 24h to establish the primary cell culture. After 24h, the medium was removed and replaced with a L-15 cell culture medium supplemented with ENV MPs, ENV NPs or PS NPs in suspension (0.08, 10 and 100 µg.L-1). Cells were then incubated for 24h at 18°C (Barrick et al., 2019).

In vivo mussel exposure

Mussels (15) were placed in 3L aquaria and maintained in the same condition as the acclimatization period (30 psu ; 15 °C). ENV MPs, ENV NPs or PS NPs previously prepared suspensions were spiked into each aquarium for the three same concentrations as the *in vitro* exposure (0.08, 10 and 100 $\mu g.L^{-1}$). After 24h of exposure under oxygenation, *M.edulis* hemolymph was extracted prior to analysis.

Cell viability

Cell viability was determined with the MTT test quantifying proportionally the mitochondrial enzymatic activity of living cells, *via* the reduction of tetrazolium salt (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide) into coloured purple formazan. After *in vitro* exposure and following the protocol of Katsumiti et al., (2014), 50 µL of MTT were added in each well containing 500000 hemocytes for 3h at 18°C. The supernatant was then removed and 200 µL of DMSO was added.

After *in vivo* exposure, hemolymph was extracted and hemocytes were suspended in PBS (1,100 mOsm). Following Katsumiti et al., (2014), 150 µL of MTT were added to 50 µL of *M.edulis* hemocytes. Then, the supernatant was removed and 200 µL of DMSO was added. The absorbance value of each sample was then measured at 570 nm in a spectrophotometer microplate reader (TECAN® Sunrise). Optical density was then normalized in percentage to quantify the cell viability.

$$Cell\ viability\ (\%) = \frac{OD\ sample}{OD\ blank} \times 100$$

Comet assay

The comet assay is a method for measuring the amount of DNA damage present in cells. This test was carried out as previously described (Akcha et al., 2000; Barranger et al., 2014). After *in vitro* exposure, cells were washed 2 times with PBS (1,100 mOsm) and 50 µL of trypsin were added in each well to dissociate the cells for 5 minutes at room temperature. Then, 200 µL of cell culture medium containing 10% FBS was added to stop trypsin activity and cells were collected by centrifugation at 600g for 5 min at 4°C. Finally, the obtained pellet was washed with PBS (1,100 mOsm) 3 times and cells were conserved in 200 µL of PBS (1,100 mOsm). Regarding *in vivo* exposure, after testing cell viability, *M.edulis* hemocytes were suspended into a low melting point (LMP) 0.5% agarose gel and placed on microscope slides, previously covered with LMP agarose 0.8%. After a few minutes between 4 and 8 ° C, the slides were removed and a new layer of LMP agarose (0.5%) is affixed. Slides were then immersed in a lysis solution (NaCl 2.5M (Sigma® S9888); EDTA 0.1M (Sigma® E5134); Tris 0.001M (Trizma-base Sigma® T6791) and Triton (Sigma® TritonX 100)) over the night in the dark, at 8°C. The slides were rinsed 3 times with PBS 1X, before 15-minutes DNA denaturation (NaOH 0.3M (Sigma® 221465); EDTA 0.001M (Sigma® E5134)) following a 10-minute electrophoresis at 300mA. Consecutively, 3 washes with PBS are necessary, before fixing the slides with 70% ethanol for 10 minutes. DNA is revealed by adding 30 µL of ethidium bromide and observed using an optical fluorescence microscope (Olympus BX60, x 40) equipped with a CDD camera (Lucas-S, Andor Technology) and a Komet 6 image analysis system (Kinetic Imaging Ltd). DNA damage were expressed in percentage of the DNA present in the Comet tail (% Tail DNA) for each nucleus.

Acid phosphatase activity

Hemocytes are circulating immune cells engaged in several cellular reactions and the synthesis of immunoactive molecules. Acid phosphatase is one of the acid hydrolases that highlights the specific activity of proteins and lysosomes (Bergmeyer et al., 1974) and is known to be a biomarker for assessing immunity in contaminated environments (Wootton et al., 2003). *M. edulis* hemocytes collected from *in vivo* and *in vitro* exposure were conserved at -80°C allowing cell membrane rupture (Polge, 1957), and also, a quantification of proteins (Du Plessis et al., 2007). The amount of total protein was quantified using the Bradford method (Bradford, 1976). Phosphatase acid (AcP) activity was determined

spectrophotometrically at 340 nm (ε = 18.3 $mM^{-1}.cm^{-1}$; TECAN® Sunrise; Acid phosphatase Assay kit Sigma® CS0740), measuring the appearance of p-nitrophenol (pNP) by the hydrolysis of p-nitrophenylphosphate (4-NPP) and expressed in $nmoles.min^{-1}.mg^{-1}$ of protein.

Statistical analysis

Because of the non-normality of the results, the statistical test used was oriented towards a non-parametric test. A Dunn (1964) test, comparative by pairs, was performed for each dataset of each test (MTT assay, Comet assay and Acid Phosphatase), with a significance threshold set at p-value<0.05. The statistical analysis using the Principal Components Analysis (PCA) was carried out with XLSTAT 2019 software (version 21.4.63762). The PCAs were performed on a matrix containing all the known and previously obtained parameters for all the ENV NPs, ENV MPs, PS NPs at the three concentrations.

Results

Hemocyte viability

In vitro

Global cellular activity showed a concentration-dependent decline regardless the type of plastics with only significant differences when mussels were exposed to all the plastics to the highest concentration (100 µg.L^{-1}) (Figure 1.A). Regarding differences between exposure concentrations, 100 µg.L^{-1} induced a significant decrease in cell viability compared to 0,08 µg.L^{-1} for both ENV NPs and ENV MPs.

In vivo

Cellular viability related to the MTT test showed a significant decrease when mussels were exposed to 0.08 and 10 µg.L^{-1} of ENV MPs, 0.08 µg.L^{-1} of ENV NPs and to 10 µg.L^{-1} of PS NPs compared to the control group (Figure 1.B). Hemocyte cell viability was significantly decreased after exposure to 10 µg.L^{-1} of PS NPs compared to 0.08 µg.L^{-1} while a significant increase was observed when mussels were exposed to 10 µg.L^{-1} of ENV NPs compared to 0.08 µg.L^{-1}. Regarding the comparison between environmental and reference NPs, a significant decrease was depicted after exposure to 10 µg.L^{-1} of PS NPs compared to the same concentration of ENV NPs. Cellular viability was significantly lower after exposure to 10 µg.L^{-1} of ENV MPs compared to the same concentration of ENV NPs.

Genotoxicity

In vitro

All the plastic tested conditions induced significant induction of DNA damage in mussel hemocytes as compared to control exposure and the effects were observed in a concentration-dependant manner (Figure 2.A). DNA damages were significantly more abundant after exposure to 100 µg.L^{-1} of both environmental and reference NPs, compared to the lowest ones (0.08 and 10 µg.L^{-1}). Genotoxicity was significantly higher when mussels hemocytes were exposed to 10 µg.L^{-1} of ENV MPs compared to 0.08 µg.L^{-1} and also after exposure to 100 µg.L^{-1} ENV MPs in comparison to 0.08 and 10 µg.L^{-1}. Concerning effect of plastic types, a significant higher level of DNA damages was observed after exposure to 100 µg.L^{-1} of ENV NPs compared to the same concentration of PS NPs. The size of plastic induced significantly more DNA strand breaks when organisms were exposed to 10 µg.L^{-1} of ENV MPs compared to the same concentration of ENV NPs.

In vivo

DNA damages were significantly higher in hemocytes for all the conditions of exposure except for 0.08 µg.L^{-1} of ENV NPs in comparison to control mussels (Figure 2.B). Comparing DNA damages in hemocytes of mussels exposed to different concentration showed a significant increase in genotoxicity when mussels were exposed to 100 µg.L^{-1} of PS NPs compared to 0.08 and 10 µg.L^{-1}. Exposure to 10 µg.L^{-1} of ENV NPs induced significantly more DNA damages than the exposure to 0.08 µg.L^{-1} as the exposure to 100 µg.L^{-1} of ENV NPs compared to 0.08 and 10 µg.L^{-1}. The exposure to 100 µg.L^{-1} of ENV MPs induced a significant higher genotoxicity than the exposure to 0.08 and 10 µg.L^{-1} whereas 10 µg.L^{-1} of ENV MPs decrease significantly DNA breaks compared to 0.08 and 100 µg.L^{-1}. Regarding plastic types, DNA damages were significantly induced after exposure to 0.08 µg.L^{-1} of PS NPs compared to the same concentration of ENV NPs whereas the opposite was observed with significantly less DNA damages after exposure to 100 µg.L^{-1} of PS NPs compared to the same concentration of ENV NPs. Results showed a significant higher levels of DNA damages after exposure to 0.08 µg.L^{-1} of ENV MPs compared to the same concentration of ENV NPs whereas the opposite was observed after exposure to 10 µg.L^{-1} of ENV MPs compared to the same concentration of ENV NPs.

Immunity

In vitro

No significant differences were depicted for acid phosphatase (AcP) activity after *in vitro* exposure to all tested plastic whatever the tested concentration (Figure 3.A).

In vivo

Acid phosphatase activity was significantly higher in hemocyte of mussels exposed to 0.08 µg.L^{-1} of the three plastics and also to 10 µg.L^{-1} of PS NPs than in hemocyte from the non-exposed mussels (Figure 3.B). Regarding concentration-effects, all the lowest concentrations of plastic particles induced an increase in AcP activity compared to the highest ones. In this sense, the exposure to 100 µg.L^{-1} of PS NPs induced a significant decrease of AcP activity compared to 0.08 and 10 µg.L^{-1}. AcP activity was significantly reduced when mussels were exposed to 100 µg.L^{-1} and 10 µg.L^{-1} of ENV NPs and ENV MPs respectively, compared to 0.08 µg.L^{-1}.

Comparison between size and type of plastics

Additionally, PCA were performed using all the data issued from our study to analyse the ecotoxicological effects on cell viability, genotoxicity and immunity after *in vitro* and *in vivo* exposure to the three types of plastics. The resulting PCAs were presented in figure 4 and 5.

For *in vitro* exposure (Figure 4), the PCA first axis was represented mainly by the parameters describing concentrations of exposure which were closely correlated with data from genotoxicity (comet). The parameter describing cell viability (MTT) of *M. edulis* hemocytes was anticorrelated with the concentrations and genotoxicity. Indeed, DNA damages and cell mortality were more important when exposure concentrations were increasing. The resulting biplot of type and size of plastics allowed to group the MPs and NPs in three main clusters. The first cluster was formed by the two environmental types of plastics (ENV NPs and ENV MPs) at the lowest concentrations (0.08 and 10 µg.L^{-1}) while another cluster was composed of PS NPs also at the lowest concentrations (0.08 and 10 µg.L^{-1}). The third group comprised both NPs and MPs from the environment and tested at the highest concentration (100 µg.L^{-1}).

In the PCA characterizing the *in vivo* exposure (Figure 5), the first axis was also represented by the parameters that described concentrations that appeared closely correlated to genotoxicity (comet). However, immunity (AcP activity) was negatively correlated with the concentrations and comet assay that confirm that immunity and DNA damage are not directly linked but probably more ROS production and DNA damage. The biplot allowed to group MPs and NPs in two clusters. The main group was composed of both ENV NPs and ENV MPs and also PS NPs at the same concentrations (0.08 and 10 µg.L^{-1}) while the second cluster was formed by the three plastic types at 100 µg.L^{-1}.
In vitro exposure allowed to discriminating effects depending on the concentration and type of plastics whereas *in vivo* exposure showed impacts depending on the concentration tested.

Comparison between in vivo and in vitro exposure
Data analysis using PCA comparing results between *in vitro* and *in vivo* exposure were presented in figure 6. The first axis was driven by parameters describing genotoxicity (comet) and concentrations while the second axis was explained by immunity (AcP) and cell viability (MTT). The resulting biplot allowed to form groups of MPs and NPs according to *in vitro* and *in vivo* exposure scenarii. Results showed the discrimination of 4 clusters, anti-correlated two by two. The first cluster (on the left) was composed of all the plastics types (PS NPs, ENV NPs and ENV MPs) and the lowest concentrations (0.08 and 10 µg.L^{-1}) after *in vitro* exposures while the anti-correlated cluster (on the right) was composed of the same plastics

after *in vivo* exposure to 100 µg.L^{-1}. In this sense, the *in vivo* exposure to 0.08 and 10 µg.L^{-1} of PS NPs, ENV NPs and ENV MPs were grouped above the second axis and anti-correlated to the cluster composed of the same plastics after *in vitro* exposure to 100 µg.L^{-1}.

Discussion

In a context of global plastic pollution, aquatic environments and their populations are the most impacted by polymers, of different sizes and concentrations. In marine environment, plastics are subjected to weathering processes due to environmental stresses such as sunlight, heat, moisture, pollutants, mechanical stresses and biological growth (Pickett, 2020). Their weathering can be a serious threat to environmental sustainability and human health. Beyond polymer degradation, environmental micro and nanoplastics can adsorb and release some chemical additives, potentially acting as carrier of toxic chemical pollutant (Rai et al., 2022). Because of this cocktail of stressors, the detailed evaluation of ecotoxic effects on aquatic organisms is considered as a real challenge. In this context, the main objective of this study was to compare two exposure scenarii *in vivo* and *in vitro* using a continuum of size of environmental plastics, from NPs to MPs, on the marine mussel *M. edulis*. For this comparison, MPs and NPs were tested at concentrations representative of those found in the marine environment, in the coastal regions or gyres, 0.08, 10 and 100 $\mu g.L^{-1}$ (Goldstein et al., 2013; Ivar Do Sul et al., 2014).

Hemocytes has been chosen as a good biological model as it allows *in vivo* and *in vitro* comparison. Hemocytes are commonly used in *in vitro* studies due to their role in the innate immunity of the organisms. Hemocytes also present similar structure and function to mammalian immune cells, which could be interesting for regulatory studies (Barrick et al., 2018).

Among marine organisms, bivalves have an immune system known to be able to assess the toxicity of plastics particles (Sendra et al., 2020). NPs and MPs, after their filtering and ingestion by aquatic organism as filter-feeder such as mussels, can accumulate in gills and gut and then, depending on their size, be transferred in hemolymph (Sendra et al., 2020). Upon contact with cells, they could be internalized via endocytic and non-endocytic pathways, eventually accumulating into lysosomes (Gaspar et al., 2018). This internalization of MPs and NPs may cause cytotoxicity, oxidative stress, changes in enzyme activities, lysosomal membrane destabilization, immunotoxicity, genotoxicity and transcriptomic/proteomic changes (Canesi et al., 2015; Paul-Pont et al., 2016; Sussarellu et al., 2016; Gaspar et al., 2018; Sendra, Carrasco-Braganza, et al., 2020; Sendra, Saco, et al., 2020; Sendra et al., 2021). Gonzalez-Soto et al. (2019) showed that the bioaccumulation of MPs in *Mytilus galloprovincialis* digestive gland, gills and gonads led to histopathological lesions. Li et al., (2020, 2021) also observed these effects on *Corbicula fluminea* visceral mass, gills and mantle exposed to NPs (80 nm) and intestines damages after exposure to both NPs (80nm)

and MPs (2µm). Other studies present the physiological responses triggered by plastic exposure. For example, Pedersen et al., (2020) highlighted a significant reduction of the clearance rate of *Dreissena bugensis*, associated with impairments on the filtration rate, reproduction and oxygen consumption and an increase in mortality. At the cellular level, some studies have shown that exposures to MPs and NPs can lead to genotoxicity in mussels and in oysters, respectively (Brandts et al., 2018; Nobre et al., 2020). In addition, according to Sussarellu et al., (2016) and Jiang et al., (2022), MPs and NPs cause reproductive toxicity in marine organisms, after exposures of oysters to MPs for 2 months, or exposures of *Ruditapes philippinarum* to PS MPs for 15 and 30 days.

Comparison of cellular responses induced by MP and NP between in vitro and in vivo exposures

In our study, all tested plastics (ENV NPs, ENV MPs and PS NPs) caused impairment of hemocyte cell viability and DNA damages after both *in vitro* and *in vivo* exposure. However, the immune response was induced only in the hemocytes of mussels exposed *in vivo* to the lowest concentrations (0.08 and 10 µg.L^{-1}) of both MPs and NPs but not after exposure of the hemocyte *in vitro*. This could be explained by the fact that the interaction of MP and NP with hemocytes are different *in vitro* as compared to *in vivo* since *in vivo*, MP and NP could interact with biomolecules forming a bio-corona at their surface that interferes with the toxicity of the polymer itself. MP/NP-protein corona might also be formed in biological fluids of marine invertebrates that may affect MP/NP–cell interactions and that could explain the induction of immune response only after *in vivo* mussel exposure.

Cell viability

Concerning cell viability, this might indicate involvement of endocytic activity as well as direct diffusion of MPs and NPs through cell membrane as demonstrated by Katsumiti et al. (2021). Moreover, cells exposed *in vitro* showed a dose dependant decrease in cell viability. This dose dependant response was also depicted on *Crassostrea gigas* hemocytes after exposure for 24 h to 0.05 µm NPs (10^2, 10^3, 10^5, 10^6, 10^8, 10^9 and 10^{12} part.mL^{-1}), 0.5 µm MPs (10^2, 10^3, 10^5, 10^6, 10^8 and 10^9 part.mL^{-1}) and 4.5 µm MPs (10^2, 10^3, 10^5, 10^6 and 10^8 part.mL^{-1}) (Katsumiti et al., 2021). In accordance to our results, some studies demonstrated that MPs and NPs have a dose dependent effect on hemocytes resulting in a decline in cell viability in the blood clam *Tegillarca granosa* (Sun et al., 2021), in the zebra mussel *Dreissena polymorpha* (Magni et al., 2018) and in *Mytilus galloprovincialis* (González-Fernández et al., 2018).

Particle properties such as surface charge, shape and size as well as the cell type are main determinants of uptake mechanisms and intracellular fate of NPs and MPs. Size of plastic has been demonstrated to differentially interact with plasma membrane. Katsumity et al. (2021) showed in mussels that NPs (0.05 µm or less) can be internalized by ATP-independent passive transport or endocytic mechanisms whereas small MPs (0.5 µm or less) could be internalized through clathrin or caveolae-mediated endocytic pathways or via macropinocytosis, and larger MPs (approximately 1 µm or larger) are taken up by phagocytosis.

Genotoxicity

Concerning genotoxicity, an important cellular stress caused by MPs and NPs could induce DNA damages resulting in a reduced hemocytes viability. Main functions of the cell are mostly related with the DNA integrity (Han et al., 2019). In this sense, aggravated DNA damages could be correlated with decrease in cell viability. In our study, results showed that all type of plastics and concentrations tested induced DNA damage in mussel hemocytes exposed both *in vitro* and *in vivo*. Concerning *in vitro* exposure, Katsumiti et al., (2021) also tested the genotoxic impacts of 0.05 µm NPs, 0.5 µm MPs, 4.5 µm MPs at different concentrations on *Crassostrea gigas* hemocytes. In accordance with our results, the exposure time of 24 h produced significantly more DNA strand breaks at all the tested concentrations compared to controls (Katsumiti et al., 2021), that may be partly due to ROS overproduction. Others studies have also reported genotoxic effects after *in vivo* bivalve exposure to MPs (Ribeiro et al., 2017; Brandts et al., 2018; González-Soto et al., 2019; Revel et al., 2019). A significant increase of DNA damage was demonstrated in hemocyte when mussels *Mytilus. spp* were exposed to 10 $\mu g.L^{-1}$ or higher concentrations of PE-PP MPs after 10-day (Revel et al., 2019) or in hemocytes of *M. galloprovincialis* exposed to PS-NPs for 96 h (Brandts et al., 2018). Contrary to our results, PS NPs (50 nm), PS MPs (20 µm) and polyamide microfibers (10x30 µm) did not cause any genotoxic damages on *M. edulis* hemocytes following 24 h or 7-days of exposure to 500 $ng.mL^{-1}$ (Cole et al., 2020).

Interestingly, *in vitro* approach showed differences of DNA damage between cells exposed to ENV NPs and MPs as compared to REF NPs. In addition, at 10 µg/L a difference of genotoxicity was demonstrated between ENV NPs and ENV MPs. Smaller particles are known to be more reactive due to their higher surface to volume ratio as compared to larger particles (van der Merwe and Pickrell, 2018) in murine immune cells, PP MPs 20 µm induced

more toxicity compared to larger particles (25 – 200 µm) (Hwang et al., 2019). DNA integrity is compromised after acute exposure and may be due to the incapacity of the mussel's antioxidant system to reduce an overproduction of ROS generated by the exposure to MPs and NPs. ROS have an effect on DNA bases by oxidizing the molecules inducing oxidative damages that can finally lead to cell death as observed in the present study.

Immune response

As regards to immunity, phagocytosis is the primary cellular defense reaction in invertebrate hemocytes and is first induced by interaction of particles with cell plasma membrane. Our results showed significant increases of acid phosphatase (AcP) activity when mussel hemocytes were exposed *in vivo* to the lowest concentrations (0.08 and 10 $\mu g.L^{-1}$) of both MPs and NPs. AcP is an enzyme implicated in immune response induction and its increase correspond to the destabilization of hemocyte's lysosomes after exposure to contaminant (Ray et al 2020). The study of Revel et al., (2019) also reported an enhancement of AcP activity in mussels *Mytilus spp* exposed to the lowest concentrations (0.008 and 10 $\mu g.L^{-1}$) of PE-PP MPs, as well as Auguste et al., (2020) after exposure to 10 $\mu g.L^{-1}$ of PS-NH_2 for 96h. The study of Capolupo et al., (2021) showed that size could play a role in modulating immunological and detoxication pathway with an inhibition of hemocyte phagocytosis after exposure to 1.5, 15, and 150 ng/L of PS-MP (3-µm) and PS-NP (50-nm) for 21 days on *Mytilus galloprovincialis* as well as the study of Wang et al., (2021b) on *Mytilus coruscus* exposed to PS-NPs (70 nm) and PS-MPs (10 µm). The enhancement observed in our study could be related to the increase in endolysosomal activity when hemocytes were exposed to MPs and NPs. An enhancement of AcP activity could be a consequence of the activation of cellular defences as AcP is secreted as a humoral defence factor in mussel hemocytes (Cajaraville et al., 1995) and could indicate that immune parameters such as AcP may act as compensatory mechanisms to maintain immune homeostasis after an exposure to polystyrene particles (Auguste et al., 2020a). Katsumiti et al. (2021) demonstrated an increase in AcP activity in the same cells also exposed for 24 h to 0.05 µm at 10^{12} part./mL, to 0.5 µm at 10^{9} part./mL and to 4.5 µm MPs at 10^{8} part./mL which are much higher concentrations as compared to the one tested. In the present study, no significant induction of AcP activity was measured after *in vitro* mussel exposure. This could be explained by the fact that immune response together with ROS production could have been earlier induced and lead to DNA damage and cell death after 24h hemocyte exposure.

Interest to use in vitro and in vivo approaches

Our results showed that concerning viability and genotoxicity measured parameters following mussel exposure to the different types of plastics, *in vitro* and *in vivo* approaches gave similar general results as regards to their toxicity. This indicates that *in vitro* approaches could be considered as potential predictors of *in vivo* exposures. Cell culture as tools for assessing and quantifying ecotoxicological impacts of MPs and NPs has been described as a good and ethical alternative to the use of individuals (Revel et al., 2021). Indeed, the huge variety of plastics (size, shape, composition, additives...) makes difficult their toxicity testing for future regulatory perspectives. As a global concern, there is an urgent need to generate data on the toxicity mechanisms implicated by MPs and NPs, and in this context, the use of *in vitro* approaches appears relevant to rapidly screen a large range of chemicals (Barrick et al., 2017; Revel et al., 2021). In addition, others studies showed that cell cultures could be an alternative method to animal experiments (Castaño et al., 1996; Knauer et al., 2007; Barrick et al., 2019).

Another asset of using cell culture that it allows to test many endpoints including cytotoxicity, genotoxicity, cell viability and enzymes activities under different conditions and it could be useful to understand the cellular mechanisms associated to an exposure to MPs and NPs. Although primary cell cultures of mussels are relatively well known, they remain little exploited because of their limited longevity. Cell culture, under these conditions, only allows to envisage acute exposure for the moment. More research is needed to develop environmental cell culture models in order to be able to generate large set of data as regards to the toxicity of MPs and NPs. *In vitro* testing strategies can provide a means in which contaminants can be quickly screened, allowing for the identification of chemicals that require more indepth analysis through *in vivo* testing (Barrick et al., 2018).

Future recommendations for MP and NP studies

In the present study, results showed that globally, PS NPs did not present the same toxicity as compared to ENV NPs and ENV MPs towards *M. edulis* hemocytes, both followed *in vitro* and *in vivo* exposure, which could be due to the high abundance of metals in the NPs and MPs associated to macroplastics coming from the field. Metals are present in MPs and NPs as additives incorporated during their production or after being adsorbed on the surface of plastic particles (Godoy et al., 2019; Huang et al., 2021; Liu et al., 2021). The use of MPs and NPs generated from a common set of macroplastics collected from the field allow to understanding the effects of size only on toxicity mechanisms of those plastics having the same

characteristics (composition, adsorbed contaminants). Results showed that ENV NPs and ENV MPs induced significative differences between parameters tested (cell viability, DNA damage, AcP activity) that highlighted the effect of size of plastics on their toxicity profiles. Another study showed a size-dependent accumulation in the digestive tract and allows to highlight the dynamic ingestion of nano/microplastics in mussels which are one of the main marine organisms for accumulating microplastics (Wang et al., 2021a). Since MPs and NPs aggregation depends upon their chemical composition and characteristics, it is necessary to investigate the effects of NPs and MPs from an environmental source that can modulate their toxicity (Arini et al., 2022).

Conclusion

The main aim of this preliminary study was to compare *in vitro* and *in vivo* exposure approach targeting mussel hemocyte's cells response to NPs and MPs contamination. Similar genotoxic and cytotoxic effects were observed for both *in vitro* and *in vivo* exposures to MPs and NPs indicating that *in vitro* experiments could potentially be alternatives to predict *in vivo* effects. This could be of great interest in order to generate rapidly ecotoxicity data helping to define regulation needs. Further studies have to be performed to compare both approaches on hemocytes responses to environmental MPs and NPs exposure, by testing complementary biomarkers such as enzymes of oxidative system also coupled with transcriptomic studies. The aim is to be able to use in vitro approaches to compensate for the heaviness and the costs of setting up *in vivo* approaches. Some *in vivo* experiments should also be conducted in addition of *in vitro* ones to better determine mechanisms and signalling pathways of these emerging contaminants on mussels.

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Compliance with Ethical Standards

Ethical Approval: Not applicable. This article does not contain any studies with human participants. The animal used in this article is an aquatic invertebrate.

Consent to Participate: All authors agreed with the content of this article.

Consent to Publish: All authors gave explicit consent to submit this article.

Authors Contributions: Micro and nanoplastics were generated and analysed by Julien Gigault. Chemical analysis of the micro and nanoplastic were conducted by Charlotte Catrouillet. Pauline Mahé participated in clam sampling, exposure and dissection. Analysis were performed by Pauline Mahé and Coraline Roman. The first draft of the manuscript was written by Amélie Châtel, Isabelle Métais, Coraline Roman, Oihana Latchere and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

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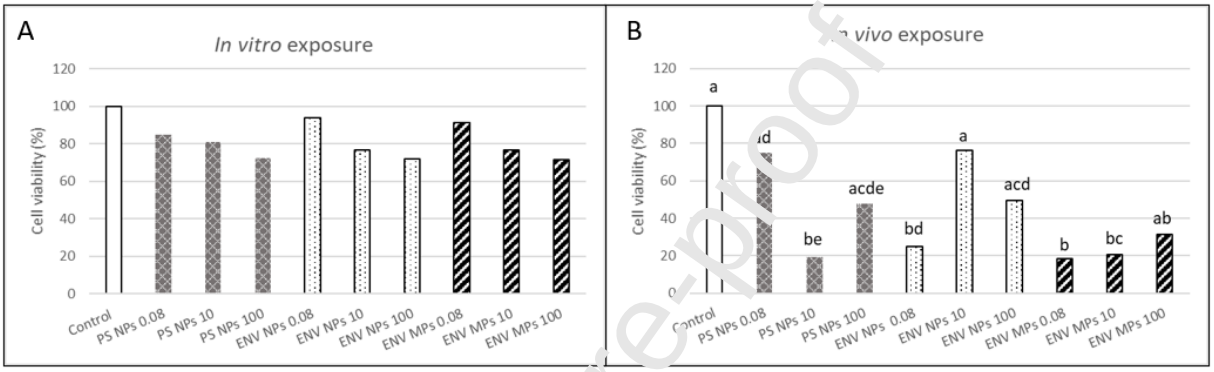


Figure 1: Cell viability (%) of hemocyte samples from M. edulis, exposed in vitro (A) and in vivo (B) for 24 hours to ENV NPs, ENV MPs and PS NPs to 0.08, 10 and 100 µg.L^{-1}. a, b, c, d showed significant differences between conditions; p<0.05)

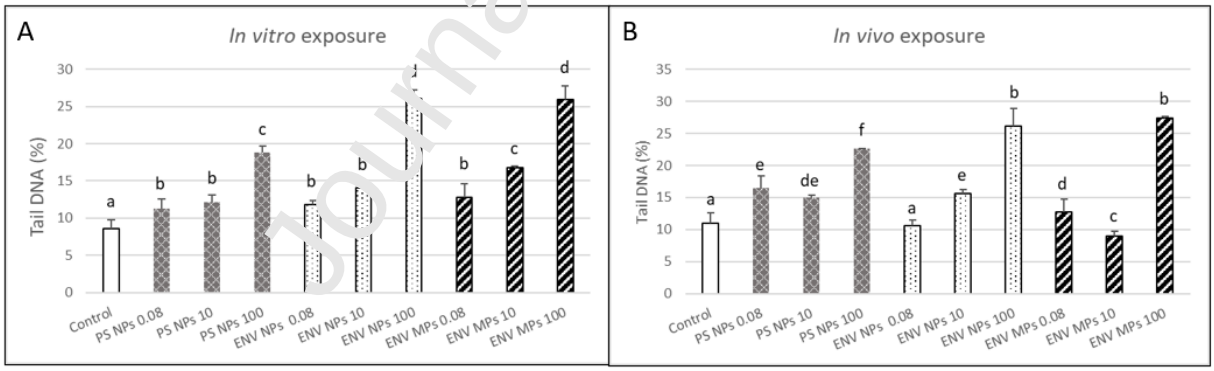


Figure 2: DNA integrity (%) of hemocyte samples from M. edulis, exposed in vitro (A) and in vivo (B) for 24 hours to ENV NPs, ENV MPs and PS NPs to 0.08, 10 and 100 µg.L^{-1}. a, b, c, d, e, f showed significant differences between conditions; p<0.05)

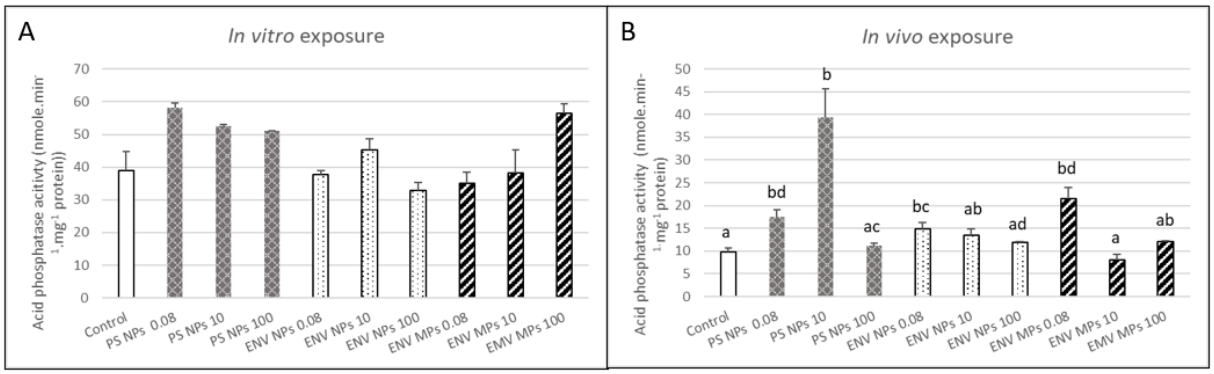


Figure 3: Specific activity of AcP in hemocyte samples from M. edulis, exposed in vitro (A) and in vivo (B) for 24 hours to ENV NPs, ENV MPs and PS NPs to 0.08, 10 and 100 µg.L^{-1}. a, b, c, d showed significant differences between conditions; p<0.05)

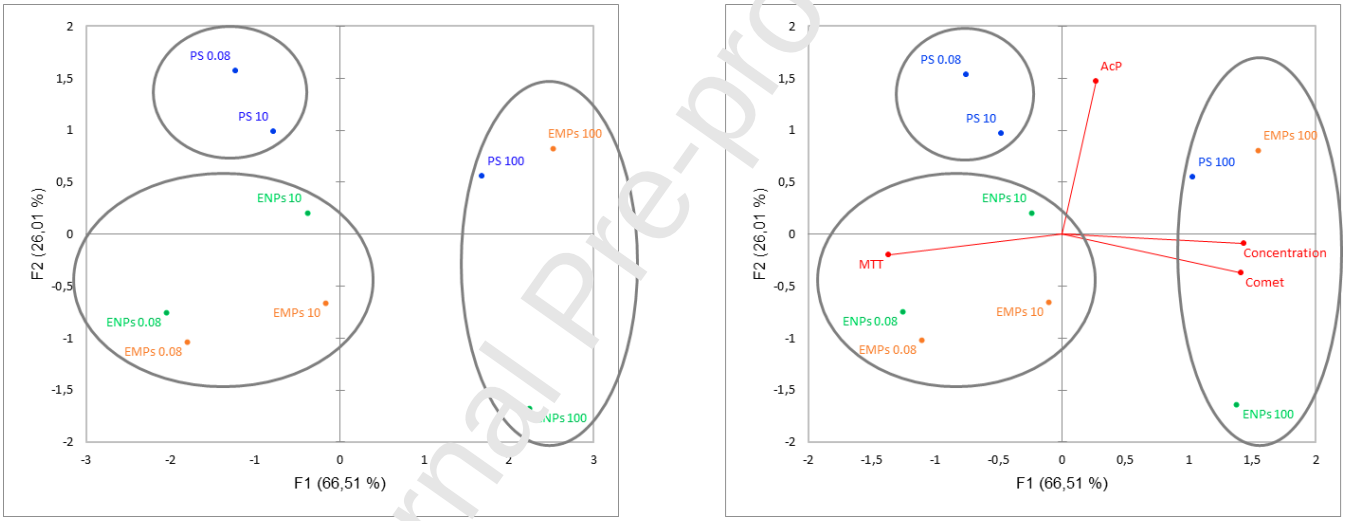


Figure 4: PCA analysis of the ecotoxicological effects on cell viability (MTT), genotoxcity (Comet) and immunity (AcP) after in vitro exposure for 24 hours to ENV NPs, ENV MPs and PS NPs to 0.08, 10 and 100 µg.L^{-1}. PS: PS NPs ; ENPs: ENV NPs ; EMPs: ENV MPs.

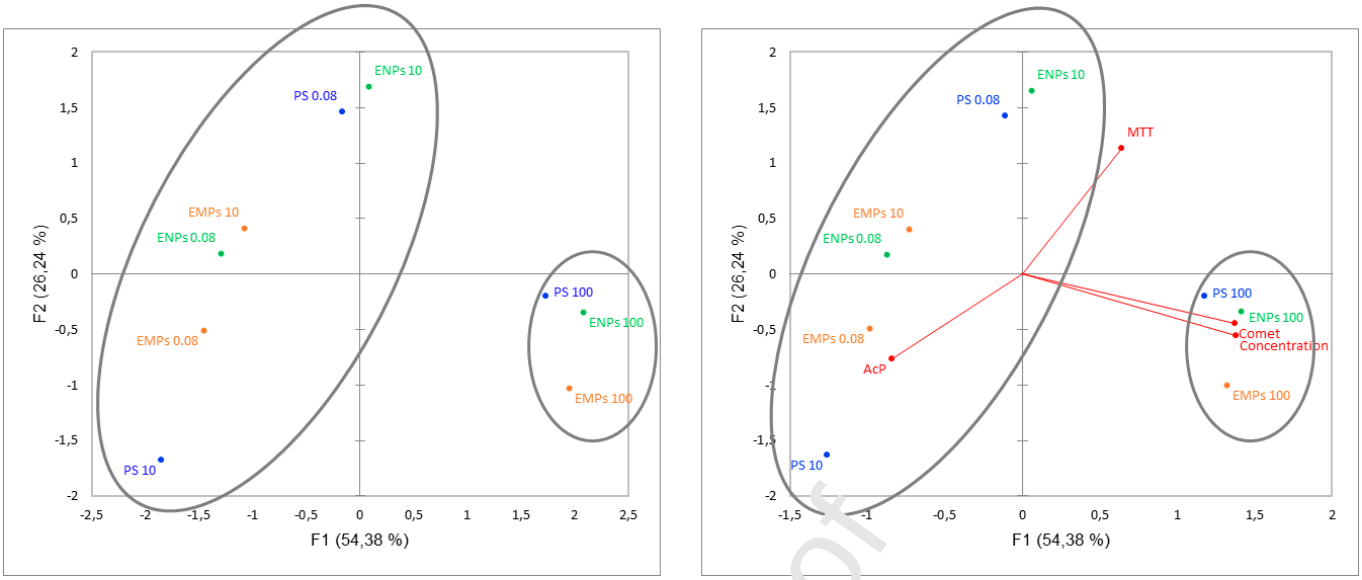


Figure 5: PCA analysis of the ecotoxicological effects on cell viability (MTT), genotoxicity (Comet) and immunity (AcP) after in vivo exposure for 24 hours to ENV NPs, ENV MPs and PS NPs to 0.08, 10 and 100 µg.L^{-1}. PS: PS NPs ; ENPs: ENV NPs ; EMPs: ENV MPs.

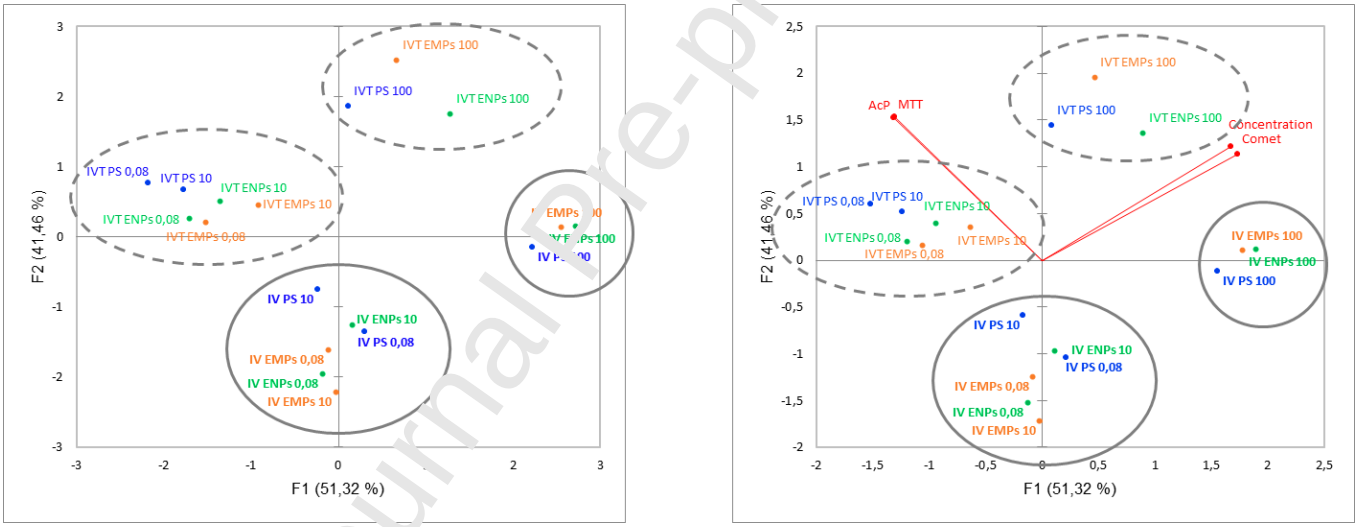
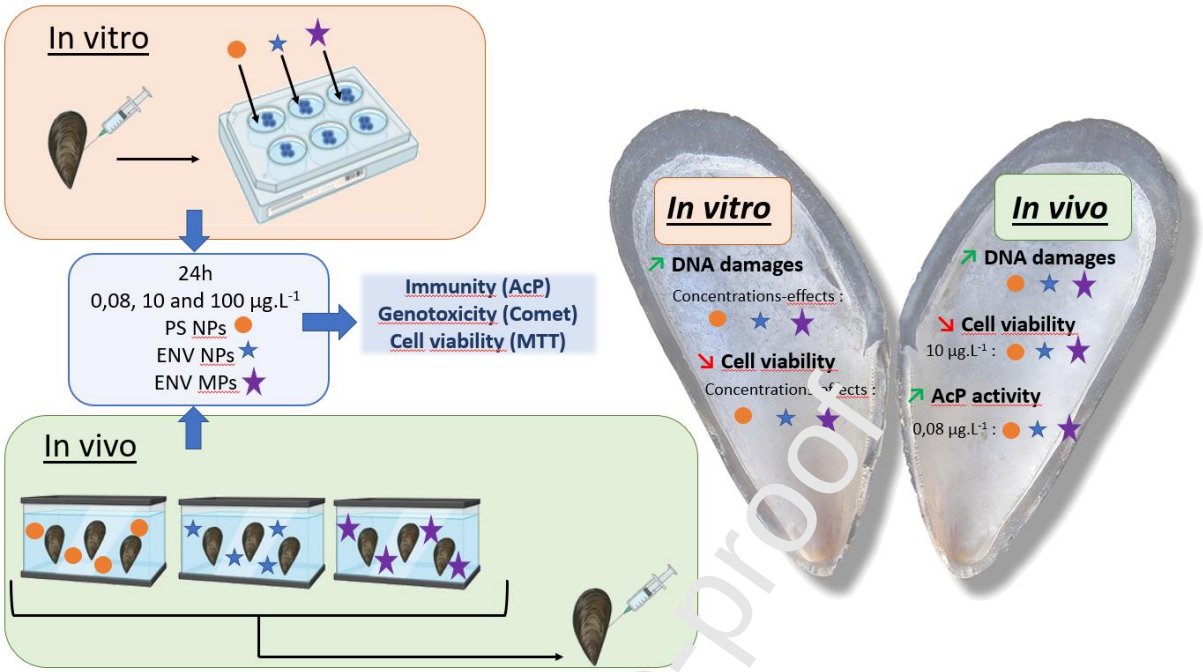


Figure 6: PCA analysis of the comparison of ecotoxicological effects on cell viability (MTT), genotoxcity (Comet) and immunity (AcP) after in vitro and vivo exposure for 24 hours to ENV NPs, ENV MPs and PS NPs to 0.08, 10 and 100 µg.L^{-1}. PS: PS NPs ; ENPs: ENV NPs ; EMPs: ENV MPs ; IV: in vivo ; IVT: in vitro.

Graphical abstract



Highlights

- Different in toxicity in mussels exposed to environmental MP and NP vs reference NP
- Dose dependant genotoxicity and cytotoxicity in mussels exposed *in vitro*
- Induction of immune response only after *in vivo* exposure
- *in vitro* and *in vivo* mussel exposure show similar genotoxicity and cytotoxicity