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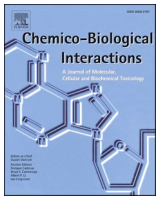

Research paper

Toxicological effects and potential reproductive risk of microplastic-induced molecular changes in protamine-like proteins and their DNA binding

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ABSTRACT

Today, plastic pollution is a widespread problem in all ecosystems and has a particularly severe impact on marine ecosystems and external fertilisers such as the mussel *Mytilus galloprovincialis*. The present study aims to assess the toxicological reproductive health effects in this organism following exposure to two concentrations of polystyrene microplastics (PS-MPs) (0.5 and 1 μg/L), representative of conditions in the Mediterranean Sea. After exposure, the electrophoretic pattern of protamine-like (PL) proteins, the major basic protein component of *Mytilus galloprovincialis* sperm chromatin, was analysed. Compared to the unexposed condition, differences were observed by SDS-PAGE and an increased ability of PL to bind and protect DNA from oxidative damage was then measured, particularly for PL from mussels exposed to 1 μg/L PS-MPs. At this dose of PS-MPs, a reduced release of all PLs from the sperm nuclei was also observed, whereas the digestion by micrococcal nuclease did not show any significant differences between the exposed and the unexposed conditions. Finally, the possibility of poly (ADP)-ribosylation of the PLs was investigated. PL-II showed an increase in poly(ADP)-ribosylation after PS-MPs exposure, which may account for the difference in the ability of the PLs to bind DNA. In conclusion, while all the results might suggest a molecular mechanism of gametic plasticity occurring upon exposure of mussels to PS-MPs 1 μg/L, they also indicate that this dose of exposure could be extremely detrimental to the reproductive health of *Mytilus galloprovincialis* because it could prevent the release of basic nuclear proteins from the sperm DNA at fertilisation.

1. Introduction

A realistic picture of our world shows that it is heavily polluted: coastal regions and oceans are polluted by agricultural fertilisers, manure run-off, sewage and industrial effluent [1–8]. One of the main contributors to water pollution is undoubtedly the excessive use of plastic that characterises today's world. According to the United Nations Environment Programme (UNEP), about 80 per cent of the plastic found in the seas is the result of poor or insufficient waste management on land, due in particular to a limited capacity to reuse and/or recycle plastics. Currently, the annual global production of plastics is about 300 million tonnes, equivalent to about 8 % of the world's oil production, an increase of more than 20 times in the last 60 years [9]. In Europe, between 0.7 and 1.8 million tonnes of microplastics are estimated to be unintentionally released in the EU in 2019 [10]. In fact, plastic pollution has been on the rise over the last twenty years, with impacts on terrestrial and aquatic ecosystems and on air quality. v. Plastics in the size range of 100 nm-5 mm have been termed microplastics (MPs), whereas those in the 1–100 nm range have been defined as nano plastics (NPs) [11] They have been widely found in oceans, urban beaches, sediments, sewage and rivers [12]. These small-diameter plastics can pass through the food chain from lower to higher nutrient levels, and ultimately represent a threat to human health [13,14]. In fact, MPs and NPs have been found in human blood, lungs, faeces, semen, follicular fluid and

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placenta in recent studies [15–17]. The main problem is that plastics take centuries to decompose because few natural microorganisms recognise them. However, sunlight, wind and waves continue to break plastics down into smaller and smaller particles. This means that as plastics age and degrade, these compounds can leach into seawater and be ingested by marine organisms [18]. Microplastics can also act as vectors of other pollutants (organic and inorganic), as well as pathogens of various kinds [19]. For these reasons, as microplastics increase in aquatic ecosystems around the world, numerous scientific studies have been devoted to investigating the toxicological threats to the health of the marine flora and fauna involved, as well as to humans [20,21]. Recent studies have shown that the predominant type of microplastics is often polystyrene (PS; known as PS-MPs). Polystyrene is a thermoplastic polymer with a linear structure that is widely used and belongs to the family of synthetic resins [22]. Polystyrene (PS) is used in many applications because of its mechanical and electrical properties. In the food industry, for example, it is used to make plastic cutlery and plates, egg cartons and yoghurt pots. In research and analysis laboratories, polystyrene petri dishes, test tubes and microplates are widely utilized. Finally, it has applications in the manufacturing industry where a low cost, rigid plastic is required, such as CD and DVD cases, number plate holders and plastic models [22]. To date, the presence of microplastics has been observed in a wide range of seafood products of commercial value for human consumption, including mussels. Mussels have been used as indicators of contamination of marine environments in biomonitoring programmes. This role is due to several important characteristics such as their wide geographical distribution, easy accessibility and high tolerance to a considerable range of salinities [23,24]. The assessment of the presence of microplastics in mussels has been proposed as a parameter of marine and ecosystem health [25–28]. Among different bivalves, specifically, in many scientific studies, those of the genus *Mytilus* have been proposed for biomonitoring microplastic pollution in the ocean, as the microplastics they accumulate reflect those found in sedimentary environmental samples [29]. Specifically, globally, it is the Mediterranean Sea that has the highest concentrations of microplastics (between 0.7 and 1.8 million), due to the constant flow of surface water together with increased anthropogenic pressure along the coastline. Mussels in addition to being excellent bio-indicators, of all the seafood products, are regarded as an extremely valuable source of good nutrients for the human diet as they are high in quality protein, low in fat and high in n-3 fatty acids and other nutrients (including iron, vitamins and some others) [30]. For all these reasons, it is crucial to determine the effects of microplastics on the reproductive health of *Mytilus galloprovincialis* (*M. galloprovincialis*), as microplastics have become an integral part of the bivalve diet and reproductive health is fundamental to the species' survival. Spermatozoa are highly complex cells with the task of transmitting paternal DNA to the oocyte to form new life. In recent years, these cells have also been identified as early sentinels of environmental and organismal health [31]. There is a lack of literature on the effects of microplastics on the reproductive system of *M. galloprovincialis*, in particular on the molecular effects of microplastics on sperm chromatin components and on the oxidative damage and potential epigenetic modifications induced. To fill this gap, we exposed the mussels to two doses (0.5 μg/L and 1 μg/L) of polystyrene microplastics (PS-MPs) for 48 h at 18 °C and then collected male gonads and spermatozoa. The protamine-like (PLs) protein, PL-II (20 %), PL-III (50 %) and PL-IV (6 %) [32], the main Sperm Nuclear Basic Proteins (SNBPs) of *M. galloprovincialis* sperm chromatin, were analysed by electrophoresis and their DNA binding ability was determined. In addition, micrococcal nuclease (MNase) digestion and sperm nuclei release were assessed. Finally, oxidative DNA damage levels were measured and the possibility of poly(ADP)-ribosylation (PAR) of PLs was investigated. The results were analysed to propose a molecular mechanism for the toxicological and molecular effects of microplastics.

2. Materials and Methods

2.1. Ethics statement

The research described in this paper was carried out on the marine invertebrate *M. galloprovincialis*. (Lamarck, 1819). This species is not safeguarded by any environmental agency in Italy. The current study was performed in strict compliance with European (Directive 2010/63) and Italian (Legislative Decree No. 116/1992) legislation on the care and use of animals for scientific purposes.

2.2. Mussel samplings, treatments, and spermatozoa collection

In this study adult specimens of *M. galloprovincialis* were used. The mussels of mixed sex, of average size shell length 4.93 ± 0.17 cm, collected in February 2024 were supplied by Eurofish Napoli S.R.L. Baia, in Naples. 15 mussels of unknown sex were singularly exposed at 18 ± 1 °C for 48h to 0.5 μg/L and 1 μg/L of PS-MPs 5 μm (microParticles GmbH, Volmerstr, Germany), in laboratory plastic tanks (36 × 22 × 22 cm) containing 6 L of 33 ‰ artificial sea water (ASW). The composition of artificial sea water was NaCl 29.2 g, KCl 0.60 g, $MgCl_2$ 1.2 g, sodium bicarbonate ($NaHCO_3$) 0.20 g and $CaCl_2$ 1.08 g, for 1 L. Gametes were sampled from the gonads and the determination of the sexes was carried out by microscopic observation. Male gametes were strained through gauze and then washed by centrifugation at 200×*g* for 3 min at 4 °C. Finally, the spermatozoa were harvested in pellets by centrifuging the former supernatant at 9000×*g* for 10 min at 4 °C.

2.3. Extraction of PLs from M. galloprovincialis

The extraction of PLs from male gonads of unexposed and treated mussels was performed using perchloric acid (PCA) with a final concentration of 5 %, as described by Lettieri et al., 2021 [33]. For this study, two polled gonads were homogenised in the distilled water. Afterwards, PCA was added. Acid extraction was performed under stirring overnight, as described by Lettieri et al., 2022 [34]. Subsequently, the suspension was centrifuged for 30 min at 4 °C at 13000×*g*, and the obtained supernatants containing PLs were dialysed with distilled water to completely eliminate the PCA, and then the proteins were quantified.

2.4. Electrophoretic analysis of PLs

In order to assess the pattern of PLs, two types of electrophoretic analysis were performed: urea-acetic acid polyacrylamide electrophoresis (AU-PAGE), conducted according to the procedure described in Fioretti et al., 2012 [35] and SDS-PAGE according to Piscopo et al., 2020 [36]. SDS-PAGE was perfomed with Novex™ WedgeWell™ 4–20 % Tris-Glycine gel by Invitrogen (ThermoFisher Scientific, Waltham, Massachusetts, USA). The picture of the gels was captured with a Gel-Doc system using ImageLab 6.0.1 software (build 34) (BioRad, Hercules, CA, USA).

2.5. Analysis of the effect of M. galloprovincialis PLs on DNA electrophoretic mobility

DNA binding ability of PLs from mussels exposed to different experiment conditions was evaluated by Electrophoretic Mobility Shift Assay (EMSA) as described in Piscopo, 2019 [24]. For these assays, in all simples were utilized a fixed amount of plasmid DNA (pGEM3) (150 ng) and an increasing amount of PLs, in a PLs protein/DNA wt/wt ratios in the range 0.1–1.8. Protein and DNA were left to interact for 5 min at 16 °C, successively Tris/Borate/EDTA buffer (TBE) was added to all samples to a final 1X concentration before the run, and then analysed on 1 % agarose gel in TBE 1X final concentration. SafeWiev™ classic was used to visualise the DNA. The same conditions were used for EMSA with spermatozoa genomic DNA, but the range of PLs protein/DNA wt/wt

ratios was 0.1–2.0.

2.6. DNA protection analysis

The capacity of PLs to protect DNA from oxidative damage in the presence of 10 μM hydrogen peroxide (H_2O_2) and 5 μM copper chloride ($CuCl_2$) (to induce the Fenton reaction and generate DNA breakage) was estimated using plasmid DNA (pGEM3) and PLs extracted from mussels exposed to the different concentrations of microplastics according to the methods described in Lettieri et al., 2021 [37]. Samples were treated using the EMSA protocol described in the paragraph 2.5, with the only difference being that in this case 150 ng of plasmid DNA (pGEM3) and proteins/DNA wt/wt ratios in a range from 0.4 to 0.8 were used. H_2O_2 and $CuCl_2$ were applied (in order to obtain more than 50 % of the plasmid in the relaxed form) after 5 min of interaction at room temperature between DNA and PLs, and samples were subsequently incubated for 30 min at 37 °C. The samples were then run on 1 % agarose gels at 100V for 30 min in TEB 1X and stained with SafeWiev™ classic. Gel images were acquired from GelDoc Biorad (Hercules, CA, USA).

2.7. Salt-induced release of M. galloprovincialis sperm nuclear basic protein

The sperm nuclei preparation procedure was followed according to Marinaro et al., 2024 [38]. In briefly, the pellets of spermatozoa were resuspended in 1 mL solution A: NaCl 0.15 M, ethylenediaminetetraacetic acid (EDTA) pH 8 25 mM, phenylmethylsulfonyl fluoride (PMSF) 1 M. In briefly, after centrifugation 4 °C at 1900×*g*, the pellets obtained were resuspended in 1 mL of solution B: sucrose 0.25 M, $MgCl_2$ 5 mM, tris(hydroxymethyl)aminomethane-hydrogen chloride (Tris-HCl) pH 8 10 mM, PMSF 1 mM, Triton X-100 0.38 %. The samples were centrifuged 4 °C at 1900×*g*. Finally, the pellets obtained were resuspended in 1 mL of solution C: sucrose 0.25 M, $MgCl_2$ 5 mM, Tris-HCl pH 8 10 mM, PMSF 1 mM. The samples were centrifuged 4 °C at 1900×*g*. The obtained sperm nuclei were stored at 4 °C. The protocol described by De Guglielmo et al. (2019) [39] was then used to achieve the release of SNBPs from the sperm nuclei. For this aim, the increasing concentrations of NaCl were used as follows: 0.65 M, 0.8 M, 1 M, 2 M, 3 M and 4 M. Sperm nuclei were resuspended progressively in 1 mL of each NaCl solution, previously indicated and incubated at 4 C for 30 min under stirring and subsequent centrifugated at 13000×*g* for 30 min. 0.2 M hydrogen chloride (HCl) (final concentration) was applied to the resulting supernatants to obtain the extraction of all SNBPs. The samples were then incubated and kept under stirring for 16h at 4 °C and then centrifuged for 30 min at 13,000×*g*. The supernatants resulting were extensively dialysed with distilled water. In accordance with Fioretti et al. (2012) [35], 4 μg of protein from each sample were analysed by AU-PAGE to estimate the amount of proteins released from sperm DNA as a result of increasing NaCl concentration. The protein bands obtained in AU-PAGE were subsequently quantified using ImageJ 1.54d (https://imagej.net/).

2.8. MNase digestion

For the MNase digestion, sperm nuclei were prepared as described in the above paragraph 2.7 and were resuspended in 0.15 M NaCl, 10 mM Tris - HCl, pH 8.0; 0.5 M $CaCl_2$. Micrococcal nuclease (MNase) digestion was carried out according to Carbone et al., 2023 [40]. In brief, the reaction was carried out with 20 enzyme units (Sigma-Aldrich, Merk Life Science S.r.l., Milan, Italy) at 37 °C using a DNA solution with concentration of 1 mg/mL (A260 = 20). The digestion was arrested at several times (5, 15, 30 and 60 min) with the addition of 2 mM EDTA at pH 8.0 and placing the samples on ice. The obtained digestion products were centrifuged at 1900×*g* for 10 min at 4 °C, and the pellet was resuspended with a solution containing 1 M NaCl and 0.5 % SDS and left for 30 min at 25 °C. Subsequently, a standard phenol/chloroform/isoamyl alcohol protocol was applied to extract DNA. The DNA was then analysed on 0.9 % agarose gels in 1X TBE at 60 V for 1 h.

2.9. Gonadal homogenates for the determination of ɣH2AX levels

The male gonads were homogenised with radioimmunoprecipitation assay (RIPA) buffer: NaCl 150 mM; Tris-HCl pH 7.5 50 mM; Triton X-100 1 %; EDTA 5 mM; sodium dodecyl sulfate (SDS) 0.1 %, proteins inhibitors' tablet cOmplete™ Mini (Merck KGaA, Darmstadt, Germany). Homogenization was carried out at 4 °C with Teflon pestle using 500 μL of RIPA buffer for each male gonad. Following a centrifuge at 6000×*g* for 5 min and supernatant was take. The proteins were quantified with Bradford assay and used for SDS-PAGE and immunoblotting.

2.10. SDS-PAGE and immunoblotting for ɣH2AX analysis

The polyacrylamide gel used for SDS-PAGE was 12.9 % (acrylamide/bis-acrylamide 37.5:1, v/v 30 %) for running gel and 5 % (acrylamide/bis-acrylamide 37.5:1, v/v 30 %) for stacking gels, with tris-glycine 1X. The transfer was carried out at 10 V for 40 min using a buffer consisting of tris-glycine 1X and sodium dodecyl sulfate (SDS) 0.025 %, on nitrocellulose filter (0.22 μm) using mini blot system Invitrogen (Waltham, MA, USA). The transfer was checked with Ponceau S solution 0.1 % (w/v) in 5 % acid acetic (Sigma-Aldrich, St. Louis, MO, USA). Afterwards, the filter was washed for 10min with tris-buffered saline (TBS) 1X supplemented with 0.1 % Tween 20 (TBST 1X) for three time. Subsequently, the filter was blocking with gelatine 3 % in TBST 1X, followed by three washing of filter with TBST 1X. The filter was incubated with primary antibody ɣH2AX (Santa Cruz, 938CT5.1.1, sc-517336) in TBST 1X in gelatine 0.3 % overnight at 4 °C in gentle agitation. The next day was removed the primary antibody and the filter was washed with TBST 1X and incubated with peroxidase (HRP)-conjugated goat anti-mouse IgG (Bioss, BS-0296G-HRP). The filter was visualized with enhanced chemiluminescence (ECL) by ChemiDoc Imaging Systems (Bio-rad). Different times were tested to achieve a correctly exposed filter.

2.11. AU-PAGE and western blotting of PAR and H1 histone

For immunoblotting of PAR, total extracted of protein (20 μg) from *M. galloprovincialis* gonad homogenates were subjected to 12 % SDS-PAGE) in the presence of 0.1 % SDS according to Carbone et al. (2023) [40]. One part of gel was stained with 0.1 % Coomassie Blue R in 10 % acetic acid and 30 % methanol, while the other part was transferred onto a polyvinylidene fluoride (PVDF) filter (0.45 μm; IPVH00010, Merck Millipore, Milano, Italy) at 200 mA in buffer containing 0.025 M Tris, 0.192 M glycine and 0.025 % SDS for 2 h at 4 °C. For immunoblotting, PVDF was treated for 2 h at room temperature with a 3 % gelatine blocking solution in Tris-buffered saline (50 mM Tris-HCl pH 8.0 and 150 mM NaCl, with 0.05 % Tween-20 (TBST). Subsequently, the PVDF filter was incubated for 2 h at room temperature with polyclonal anti-poly (ADP-ribose) polymer antibody (ab14460, Abcam, 2.5 μg/mL) and horseradish peroxidase (HRP)-conjugated anti-chicken secondary antibody (GTX27118, GeneTex, Inc., 1:2000). Regarding immunoblotting of H1 histone, AU-PAGE electrophoresis was performed with PLs protein and H1 histone. The proteins extracted as described in previously paragraph, were electrophoresed at 100 V for 1 h on acetic acid urea polyacrylamide gel (AU-PAGE) with a final concentration of acrylamide of 11.2 % (37.5:1 acrylamide/bis-acrylamide, v/v 30 %) [40]. Subsequently, the gel was cut into two parts, the first was stained in Coomassie Blue R and the second was transferred to a nitrocellulose membrane for 2 h at 4 °C at 100 V in 0.7 % acetic acid. The membrane was blocked with no-fat milk 5 % [41] and then immunoblotting was conducted using anti-H1 monoclonal antibodies (ABclonal A4342). The membrane was acquired by ChemiDoc system (Bio-Rad) Image Lab 5.2.1 Software.

2.12. Statistical analyses

All the data were analysed with one-way ANOVA followed Tukey's test. Data was considered significant with a p-value ≤ 0.05. The data were analysed with GraphPad Prism (ver. 9.5.1.733).

3. Results

3.1. γH2AX expression

The level of γH2AX, a marker of double-strand damage, was measured. Homogenates for each exposure condition of male gonad were used to measure the expression level of γH2AX by immunoblotting. The analyses showed a similar expression of γH2AX in unexposed and exposed to 0.5 μg/L PS-MPs. Instead, the 1 μg/L PS-MPs exposure showed an increase of γH2AX, indicative of double strand break (DBS) of DNA (Fig. 1)

3.2. Electrophoretic analysis of PLs

In order to assess possible alterations in the electrophoretic pattern of PLs extracted from mussel spermatozoa exposed to 0.5 μg/L and 1 μg/L PS-MPs compared to PLs extracted from spermatozoa of unexposed mussels, 11.2 % acetic acid-urea polyacrylamide gel electrophoresis (AU-PAGE) was conducted. To carry out this electrophoretic analysis, 4 μg of PLs were loaded into each lane. In Fig. 2a is shown the electrophoretic pattern of PLs in AU-PAGE. Specifically, lane 1 was loaded with PLs extracted from unexposed mussels, while lanes 2, 3, were loaded with PLs extracted from mussels exposed to 0.5 μg/L PS-MPs and 1 μg/L PS-MPs, respectively. From this type of electrophoretic analysis, PL-II, PL-III and PL-IV of mussels not exposed and exposed to polystyrene microplastics can be observed from top to bottom. This type of analysis showed no significant differences in the electrophoretic pattern between proteins extracted from unexposed mussels and those extracted from mussels exposed to PS-MPs.

SDS-PAGE analysis was also performed on the same samples using a Novex™ WedgeWell™ 4–20 % Tris-Glycine gel by Invitrogen (Thermo Fisher Scientific, Waltham, Massachusetts, USA) (Fig. 2b). For this analysis, lane 2 contained PLs extracted from unexposed mussels, while lanes 3 to 4 contained PLs extracted from mussels exposed to 0.5 μg/L and 1 μg/L PS-MPs, respectively. Lane 1 was loaded with the 250 molecular mass marker. In this case, the three proteins are visible from top to bottom PL-II, PL-III and PL-IV. PL-II and PL-III, which have very close molecular weights (13.8 and 11.8 molecular mass respectively), have a very narrow electrophoretic mobility. SDS-PAGE analysis revealed comigration of PL-III with PL-II in samples of mussel exposed to 0.5 μg/L PS-MPs (lane 3). Furthermore, the presence of an additional band with reduced electrophoretic mobility respect to PL-II in samples from mussels exposed to 0.5 μg/L polystyrene microplastics (lane 3) was observed. Also, at 1 μg/L polystyrene (lane 4) an additional band was detected but, in this condition, the band had an even lower electrophoretic mobility than that obtained in lane 3. This band may correspond to PLs aggregates or post-translational modification PLs or H1 histones, since a lower electrophoretic mobility in the gel indicates a higher molecular weight and/or lower basicity.

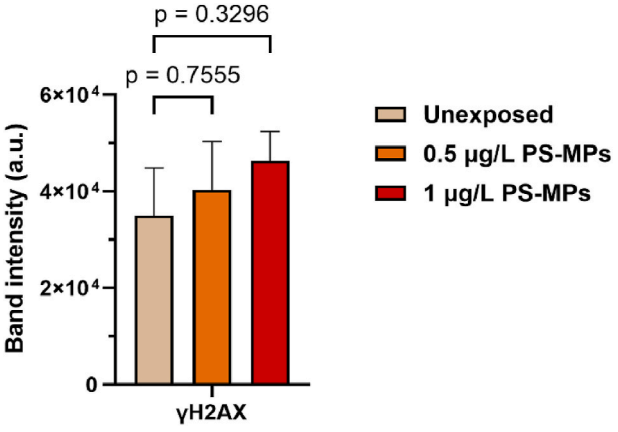

Fig. 1. Western blotting performed on gonadal homogenates from unexposed (Unx) and PS-MPs exposed *M. galloprovincialis*. Histograms show a densitometric analysis of γH2AX bands ± S.D. Differences were not found to be significant. The "p" on top of bar represents the p-value of one-way ANOVA. n = 3.

3.3. Analysis of PLs-DNA interaction by EMSA

Assays were performed to determine whether there were changes in the binding of PLs to DNA, considering the differences observed by SDS-PAGE between PLs extracted from unexposed mussels and from mussels exposed to the two different doses of PS-MPs. The EMSA (Electrophoresis Mobility Shift Assay) is an assay to assess the binding of proteins to DNA. These assays were conducted using the plasmid DNA pGEM3 and protein/DNA weight/weight (w/w) ratios ranging from 0.1 to 1.8. The amount of DNA in each sample is fixed (150 ng), while the amount of protein varies, increasing as the PLs/DNA ratio increases. The analysis is aimed at determining when the DNA becomes saturated. By "saturation" we mean the condition in which the DNA is complexed with the proteins in the form of a single band near the well, so that neither the supercoiled DNA nor the relaxed DNA band is observed. The image in panel a of Fig. 3, which shows the assay performed with PLs extracted from unexposed mussels, reveals that DNA saturation was reached at a w/w protein/DNA ratio of approximately 1.2. In contrast, the assays shown in panels b and c showed that less protein was required to achieve DNA saturation in the case of mussel PLs exposed to 0.5 μg/L or 1 μg/L PS-MPs, which was achieved at a w/w protein/DNA ratio of 1 and 0.6 for 0.5 μg/L and 1 μg/L PS-MPs exposure, respectively. Last but not least, at the exposure condition of 1 μg/L PS-MPs, a sort of binding instability between PLs and DNA was also detected in panel c, (lane 5 and 6). In fact, the intensity of the band corresponding to the supercoiled DNA is higher at the ratio 0.8 respect to 0.6. The reappearance of a fraction of supercoiled plasmid DNA as the amount of PLs increases in the PLs/DNA ratio from 0.6 to 0.8 is an indication of instability of PLs/DNA binding. Presumably, the binding of PL to DNA at the 0.8 ratio is different respect to the same ratio in the unexposed condition, resulting in a loss of a fraction of DNA-bound proteins.

EMSA assays were also performed using genomic DNA extracted from spermatozoa of unexposed mussels. The results obtained, shown in Fig. 4, confirm the higher DNA-binding capacity of PLs from exposed mussels, especially under the exposure condition 1 μg/L PS-MPs, as the DNA saturation is achieved at 0.1 PLs/DNA ratio respect to 0.4 PLs/DNA ratio in the unexposed condition.

3.4. Determination of the ability of PLs to protect DNA from oxidative damage

The ability of PLs extracted from mussels unexposed and exposed to polystyrene microplastics to protect DNA from oxidative damage was assessed by means of DNA protection assays. The experiments were conducted using the plasmid DNA pGEM3. In particular, DNA damage was assessed under pro-oxidative conditions, i.e. in the presence of appropriate concentrations of H_2O_2 and $CuCl_2$, as specified in Materials and Methods, so that the Fenton reaction could take place. As a result of this reaction, radical species are formed that induce DNA damage, which can be seen by an increase in the fraction of relaxed DNA at the expense of supercoiled DNA. In fact, in the lanes numbered 3 of the gels shown in Fig. 5, it can be seen that oxidative DNA damage has occurred in the samples to which plasmid DNA was added with H_2O_2 and $CuCl_2$. The band corresponding to the relaxed DNA is more intense than the band of relaxed DNA in the samples containing only DNA (lane 1) and DNA with

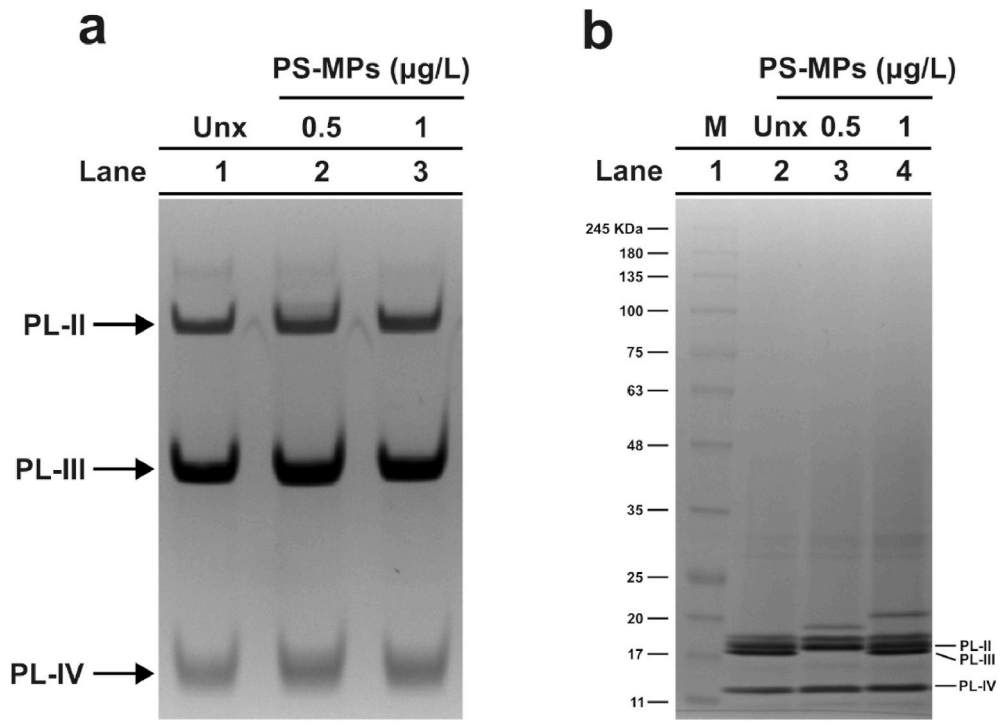

Fig. 2. (a) AU-PAGE of *M. galloprovincialis* PLs extracted from unexposed mussels (lane 1) and exposed for 48h to 0.5 µg/L PS-MPs, and 1 µg/L PS-MPs, respectively, (lanes 2–3); (b) SDS-PAGE of PLs extracted from mussels. 250 molecular mass marker (lane 1); PLs extracted from unexposed (Unx) mussels (lane 2); PLs extracted from mussels exposed for 48h to 0.5 µg/L PS-MPs and 1 µg/L PS-MPs, respectively, (lanes 3–4).

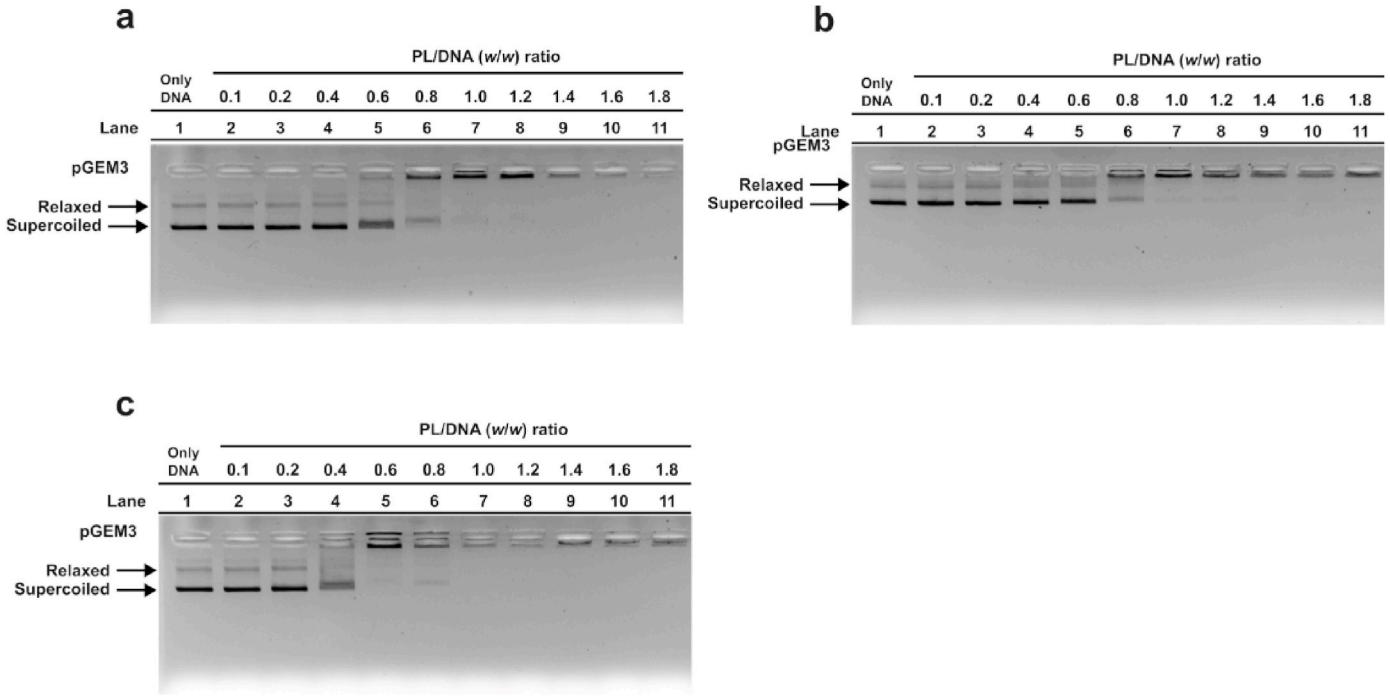

Fig. 3. EMSA conducted with plasmid DNA and PLs extracted from: unexposed mussels (a); mussels exposed to 0.5 µg/L PS-MPs (b); 1 µg/L PS-MPs (c).

H_2O_2 (lane 2). The effects on DNA after the addition of PLs in this condition were assessed from both exposed and unexposed mussels at w/w protein/DNA ratios of 0.2, 0.4, 0.6 and 0.8. The results show that the addition of PLs to the mixture of plasmid DNA, H_2O_2 and $CuCl_2$ reduces the extent of damage in all conditions, as the relaxed DNA band has a lower intensity than that of lane 3 in the presence of PLs as the protein/DNA ratio increases. This indicates that the PLs aggregate the DNA. However, this capacity is more pronounced under exposure conditions, particularly after exposure to 1 µg/L PS-MPs, where very large PLs-DNA aggregates form that cannot migrate in the gel.

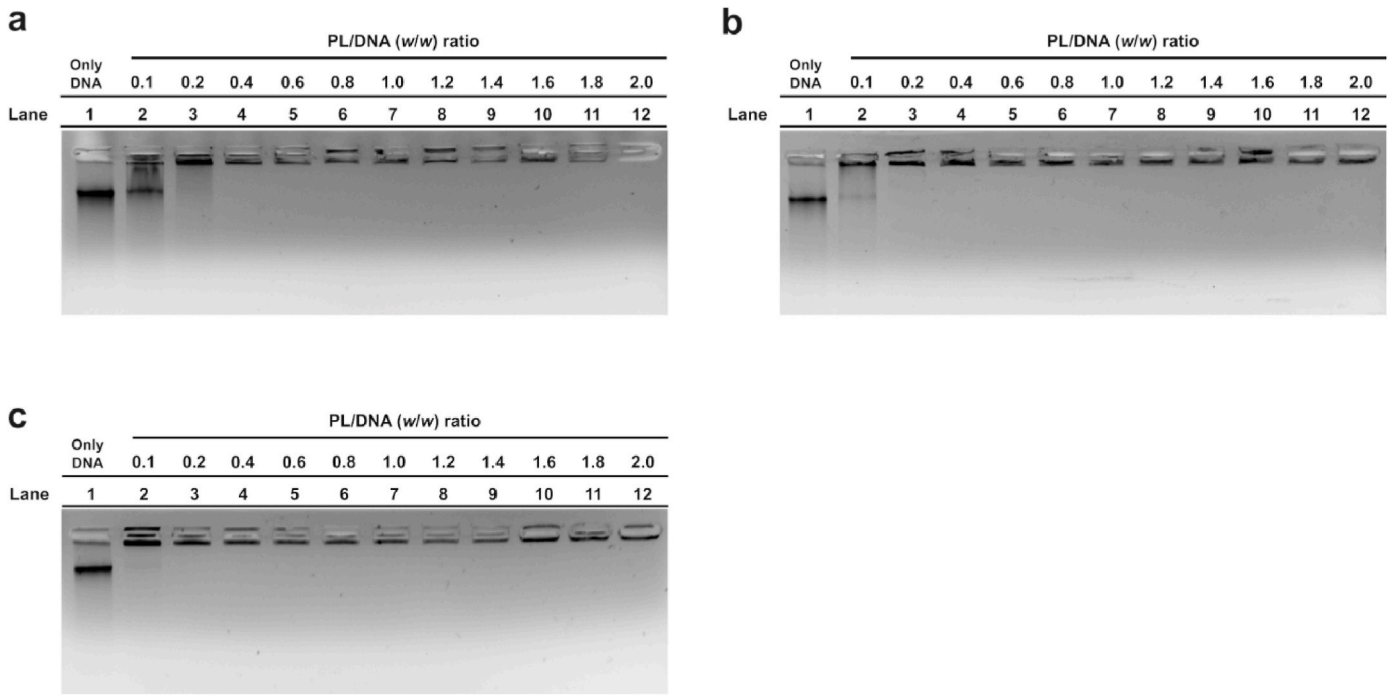

Fig. 4. EMSA conducted with *M. galloprovincialis* sperm DNA and PLs extracted from: unexposed mussels (a); mussels exposed to 0.5 μg/L PS-MPs (b); 1 μg/L PS-MPs (c).

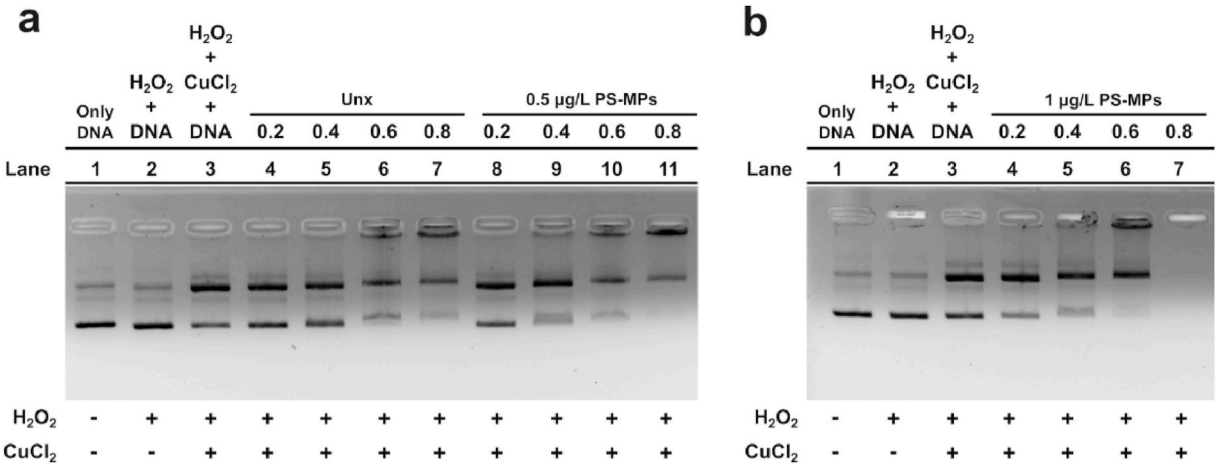

Fig. 5. DNA protection assays with (a) PLs extracted from unexposed mussels and exposed to 0.5 μg/L PS-MPs; (b) PLs extracted from mussels exposed to 1 μg/L PS-MPs.

3.5. Chromatin digestion of M. galloprovincialis spermatozoa with micrococcal nuclease

Digestion with Micrococcal Nuclease was carried out on sperm chromatin from unexposed mussels and exposed to different concentrations of polystyrene microplastics in order to highlight any alterations in the accessibility of this enzyme to sperm chromatin following exposure of the mussels to PS-MPs. DNA obtained at the different digestion times (5, 15, 30 and 60 min) with MNase was analysed on agarose gels (Fig. 6). Panel (a) shows the typical electrophoretic chromatin pattern of spermatozoa from unexposed mussels, where it can be observed that the DNA is progressively degraded with increasing digestion time under the experimental conditions used. On the other hand, under all exposure conditions (0.5 μg/L PS-MPs, 1 μg/L PS-MPs), no significant changes are observed compared to the unexposed condition apart that MNase is slightly less accessible to sperm chromatin.

3.6. Salt solution release of PLs from spermatozoa

Given the differences in the ability of PLs to bind and protect DNA observed after PS-MPs exposure, especially at 1 μg/L PS, the changes in the binding of PLs-DNA were studied. Using increasing concentrations of NaCl, we tested the release of PLs from sperm nuclei (Fig. 7) in exposed and unexposed mussels. This technique evaluates the release of PLs from sperm DNA, which occurs because NaCl acts as a competitor in the electrostatic interactions established between PLs and sperm DNA. Passages are performed on the sperm nuclei with increasing concentrations of NaCl shown on the X-axis of the graphs, while the amount of each PLs released at different NaCl concentrations can be appreciated on the Y-axis. The smaller the amount of PLs released, the greater the binding force that holds the PLs to the DNA. This analysis showed that all PLs (Fig. 7a, b and c) were released at much lower levels after exposure of mussels to 1 μg/L PS-MPs than in the unexposed condition.

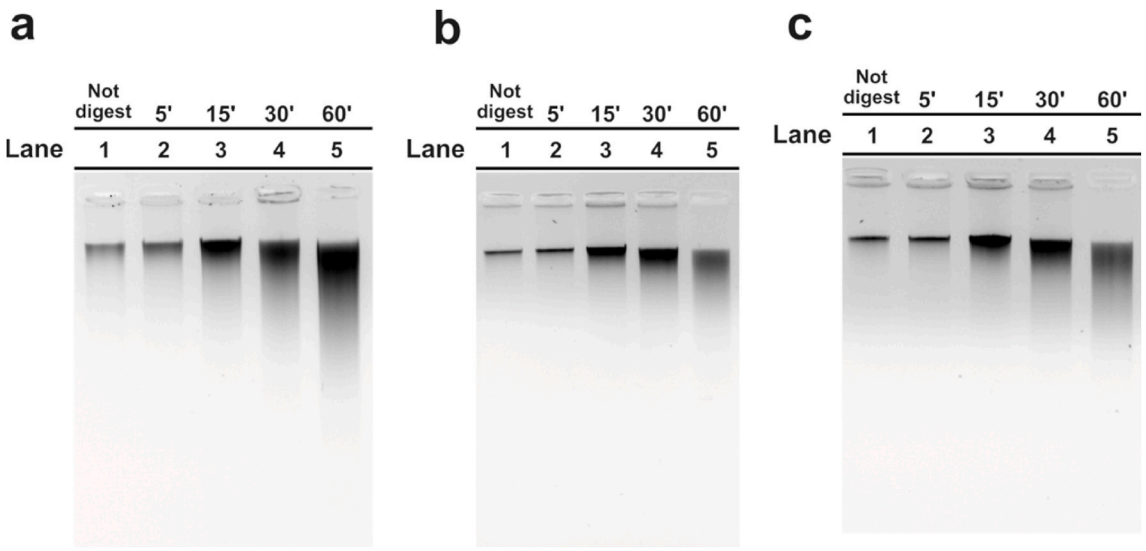

Fig. 6. Analysis of digestion kinetics products with MNase of the sperm chromatin of *M. galloprovincialis*. DNA analysis by 0.9 % agarose gel electrophoresis: unexposed (a) and exposed mussels to 0.5 μg/L PS-MPs (b); 1 μg/L PS-MPs (c).

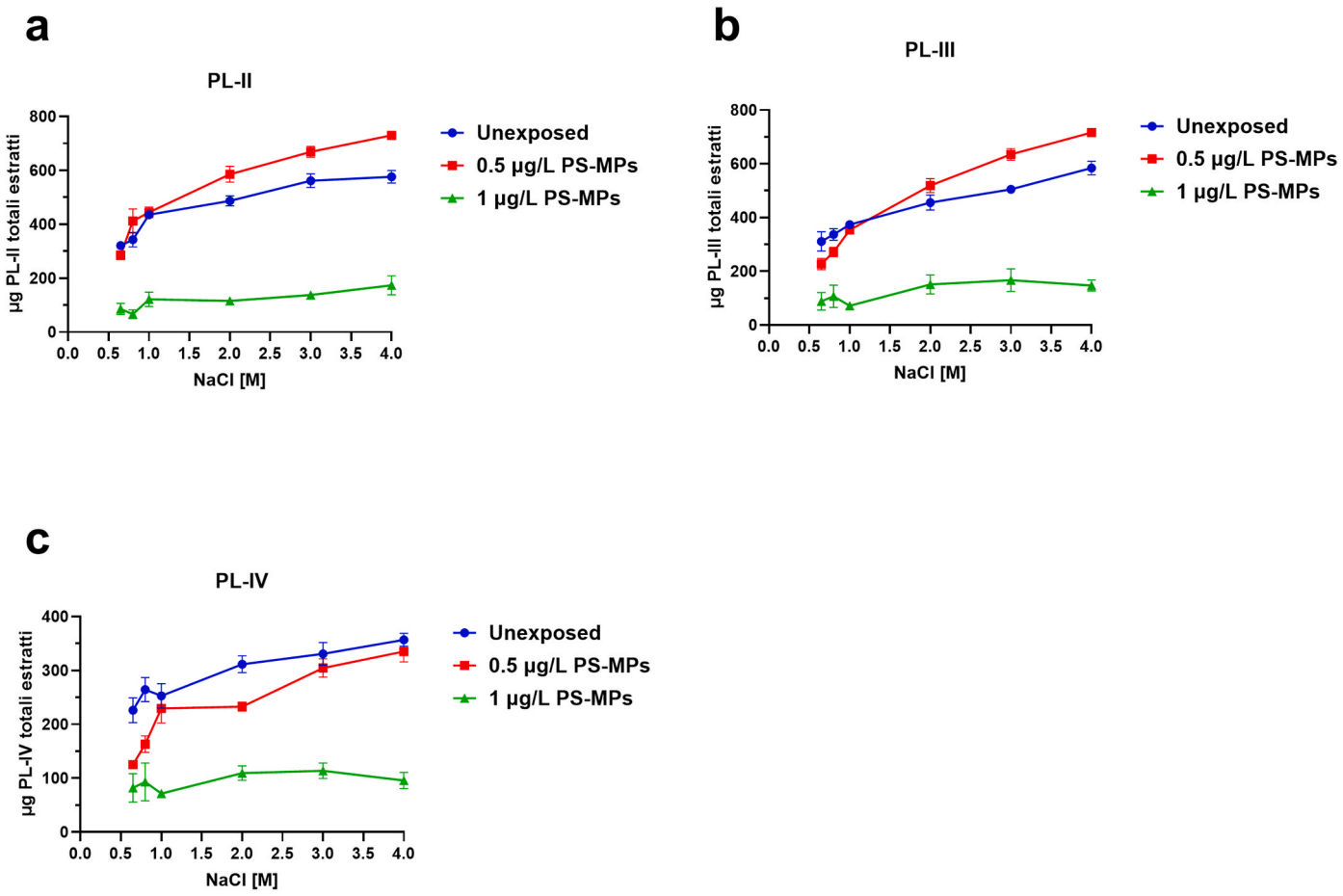

Fig. 7. PLs release from sperm nuclei at different molar NaCl concentrations in unexposed mussels (blue line, circle) and those exposed to 0.5 μg/L PS-MPs, (red line, square) and 1 μg/L PS-MPs (green line, triangle). (a) release of PL-II, (b) release of PL-III and (c) release of PL-IV.

In contrast, when the mussels were exposed to 0.5 μg/L PS-MPs, a slightly higher release of PL-II and PL-III and a slightly lower release of PL-IV were observed, respectively, compared to the unexposed condition. Therefore, the most surprising result is precisely that obtained at 1 μg/L exposure to PS-MPs, because a different, seemingly stronger, bond between PLs and DNA could have negative consequences on fertilisation, as will be better explained in the discussion.

3.7. Poly(ADP)-ribosylation of SNBP

Electrophoretic analysis by AU-PAGE of gonad extracts from *M. galloprovincialis* exposed to different doses of PS-MPs (0.5 μg/L and 1 μg/L) and unexposed (control) showed no significant qualitative and quantitative differences in protein patterns (Fig. 8a). Immunoblotting with anti-PAR identified two immunopositive signals with different electrophoretic migration, corresponding to PL-II and Histone H1 (Fig. 8b). Both signals were also positive to anti-H1 (Fig. 8c).

4. Discussion

The health of the environment is significantly affected by a wide range of contaminants, including heavy metals, pesticides, pharmaceuticals, chemicals and plastic products, which are the result of human activities [21,42–44]. Although they are recognised as being the cause of environmental imbalances in all of the natural habitats, the primary preoccupation with these environmental pollutants is the serious threat they pose to human and animal health. Plastic articles are now an essential part of everyday life. Nonetheless, they have been identified as major environmental pollutants that can enter the food chain and enter the human body [45] and impair the functioning of organs, including those of the reproductive system [46] Plastics undergo chemical-physical transformations that result in their degradation into microplastics (MP). Polystyrene (PS) is one of the most common plastic materials, used in a wide range of commercial products because of its versatility and inertness. PS-MPs have been shown to impair the ability of various aquatic and terrestrial organisms to reproduce, according to published reports [23,33,34,37,38,40]. In addition, data from the literature have demonstrated the capacity of PS-MPs to cause changes in the growth and morphology of larvae and embryos of the *M. galloprovincialis* [47], but there is no study on the molecular effects of PS-MPs in *M. galloprovincialis* spermatozoa. The majority of marine invertebrates, including *M. galloprovincialis*, release their gametes into the sea to form a new individual. As a result, the quality of the spermatozoa is highly dependent on the characteristics of the environment into which the sperm are released. Given the paucity of literature data, the objective of this study was to investigate the genotoxicity of polystyrene microplastics and their molecular mechanisms on *M. galloprovincialis* spermatozoa. The study evaluated oxidative damage at the gonadal level and the response at the molecular level of spermatozoa chromatin components. Experiments were performed with a single exposure time (48 h) and two concentration of PS-MPs (0.5 and 1 μg/L), selected based on the relevant literature on exposure to similar substances, due to the unknown toxicity of PS-MPs to *M. galloprovincialis* spermatozoa. The results reached are fully consistent with the data published in the literature, which classify polystyrene microplastics as harmful and toxic substances, capable of damaging several organisms. The analysis of oxidative damages indicates an increase of the level of ɣH2AX, particularly after exposure of mussels to PS–MPs 1 μg/L, indicative of double strand DNA breakage. Based on these observations, the response of the components of sperm chromatin of *M. galloprovincialis* was evaluated. First, we observed a difference in the electrophoretic pattern of PLs by SDS-PAGE. In particular, aggregates of PLs or H1 histone with different molecular weight or basicity were observed after the two exposure doses to PS-MPs. Furthermore, PL-III comigrated with PL-II after exposure to PS-MPs 0.5 μg/L. These electrophoretic differences in the state of PLs affected the ability of PLs to bind DNA. In fact, in both condition of exposure, but particularly after that of PS-MPs 1 μg/L an increase in ability to bind DNA was registered. However, it should be noted that the PLs-DNA aggregates obtained with PLs from mussels exposed to PS-MPs were of a different nature compared to the unexposed condition, as evidenced by the DNA intensity in the proximity of the well when DNA saturation was reached. DNA intensity was lower in the two-dose microplastic exposures than in the no exposure condition. This suggests that some of the aggregates formed between the PLs and DNA after exposure to microplastics were too big to enter the mesh of the agarose gel and remained in the well when loading the sample. An increased ability of PLs to bind DNA after mussel exposure to microplastics was also confirmed in EMSA assays performed with mussel sperm genomic DNA. Once again, PLs from mussels exposed to 1 μg/L PS-MPs showed the highest DNA binding capacity. DNA protection experiments carried out under pro-oxidative conditions also showed that the DNA aggregation capacity of PLs from mussels exposed to PS-MPs was increased. In fact, it was observed that the DNA was protected at lower values of the PLs/DNA ratio compared to the no exposure condition, and even at the 1 μg/L condition with a PLs/DNA ratio of 0.8, the DNA was no longer visible in the gel. This was probably due to the fact that the DNA had aggregated to such a high weight that it was no longer able to enter the gel. As a result of the electrophoretic changes in PLs and their increased ability to bind and protect DNA after exposure to PS-MPs, especially after the exposure to 1 μg/L, there was almost equivalent accessibility of

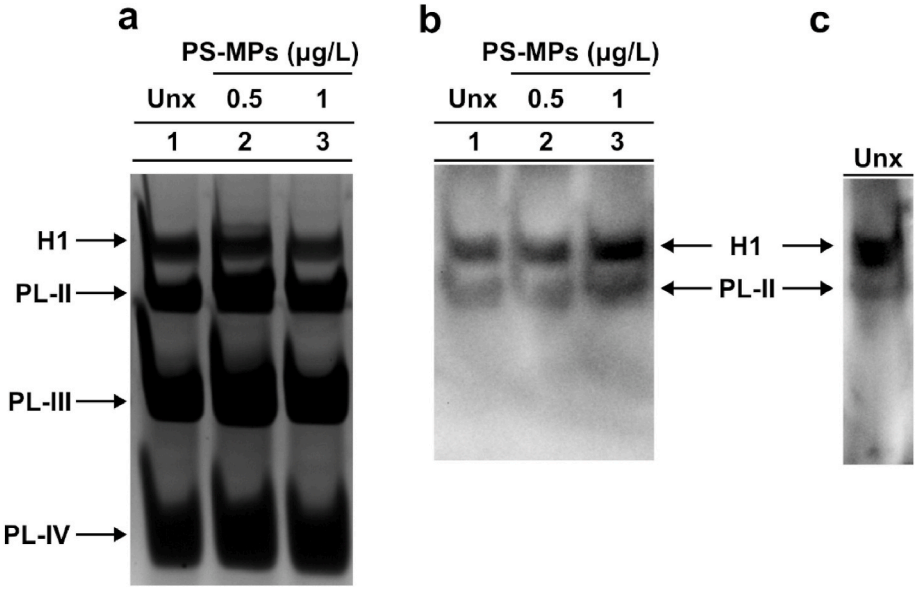

Fig. 8. Western blotting of PLs protein from *M. galloprovincialis* unexposed (Unx) and exposed to PS-MPs. (a): 11.2 % acetic acid-urea polyacrylamide gel (AU-PAGE); (b) anti-PAR immunoblotting; (c) anti-H1 histone immoblotting.

micrococcal nuclease to sperm chromatin in unexposed and exposed conditions. Given the increased DNA binding capacity of PLs from mussels exposed to PS-MPs, particularly at 1 μg/L PS-MPs, changes in PLs-DNA binding were also examined using increasing concentrations of NaCl to test the release of PLs from sperm nuclei in exposed and unexposed mussels. This analysis showed that after exposure of the mussels to 1 μg/L of PS, all the PLs were released to a very much lower extent than in the unexposed condition. This was the starting point for the investigation of possible poly(ADP)-ribosylation of PLs. In fact, immunoblot analysis with anti-PAR antibodies showed poly (ADP)-ribosylation of PL-II which was extremely pronounced under exposure conditions of 1 μg/L PS-MPs. Finally, it is possible that the simultaneous poly(ADP)-ribosylation of PL-II and histone H1 may have an effect on the binding of PLs to DNA, as, recently, a first evidence of poly(ADP)-ribosylation of this protein was obtained by studying the effects of chromium (VI) on the male reproductive system of *M. galloprovincialis* [48]. In the present paper, in addition to PL-II also histone H1 is endogenously poly(ADP)-ribosylated (Fig. 8b and c). This evidence further convinces us of the hypothesis that this modification has the effect of altering the binding of both proteins to DNA, favouring the sperm chromatin decondensation [48,49] or produce a different sperm chromatin organization. Therefore, a mechanism of gametic plasticity occurring under the stress condition produced by exposure of mussels to the 1 μg/L dose of PS-MPs could be suggested by all the results obtained in this work. Numerous reports have shown that sperm become resistant to stress following parental exposure. In the particular, the literature has reported that environmental stress can induce differing sperm phenotypes, some of which dependent on adaptive gamete plasticity. Indeed, gametic plasticity in response to stress could be a common feature of external fertilisers (such as the majority of marine invertebrates, many fish and amphibians). This is consistent with some work reported in the literature [23,24,50–53]. and less frequently in internal fertilisers, but this needs to be verified. While there would appear to be a mechanism of gametic plasticity following exposure of mussels to the higher dose of microplastics tested in this work, molecular alterations in the DNA-binding properties of PLs could pose a problem at fertilisation for this organism. In fact, PLs from mussels exposed to the highest dose of microplastics showed a different type of DNA binding. From sperm nuclei release experiments, EMSA assays and protection assays, the binding of PLs to DNA appears to be stronger than that of PLs from unexposed mussels. So much so that PLs from mussels exposed to this dose of microplastics were released at much lower levels than under unexposed conditions. This behaviour of PLs, which appear to be more closely bound to DNA, could represent a significant issue at fertilisation, as it is well known that sperm DNA must lose the sperm nuclear basic proteins in order to bind to the oocyte DNA. PLs play a crucial role in DNA compaction of sperm of this organism, but result a target of various pollutants, including microplastics, which are able to alter the DNA binding of PLs, presumably by determining conformational changes in these proteins. Therefore, although further studies are needed to better understand the toxicological and molecular mechanisms of action of this type of microplastics on the components of *M. galloprovincialis* sperm chromatin, the fact remains that the highest dose of these PS-MPs tested in this work could be extremely harmful to the reproductive health of *M. galloprovincialis*. In addition. In addition, *M. galloprovincialis* proves to be an excellent bio-indicator for environmental pollution, even in the case of microplastics. On the other hand, the evaluation of the presence of microplastics in mussels has been proposed as a parameter to evaluate the health of the marine ecosystem [25].

CRediT authorship contribution statement

Carmela Marinaro: Writing – review & editing, Writing – original draft, Visualization, Software, Investigation, Formal analysis, Data curation. **Giulia Scarciello:** Writing – original draft, Investigation. **Anna Rita Bianchi:** Writing – review & editing, Writing – original draft, Investigation, Data curation. **Bruno Berman:** Investigation, Data curation. **Teresa Chianese:** Writing – original draft, Visualization, Investigation, Formal analysis. **Rosaria Scudiero:** Validation, Supervision, Resources. **Luigi Rosati:** Writing – review & editing, Writing – original draft, Supervision, Resources, Methodology. **Anna De Maio:** Writing – review & editing, Writing – original draft, Validation, Supervision, Resources, Project administration, Methodology, Conceptualization. **Gennaro Lettieri:** Writing – review & editing, Writing – original draft, Visualization, Software, Methodology, Investigation, Formal analysis, Data curation. **Marina Piscopo:** Writing – review & editing, Writing – original draft, Validation, Supervision, Resources, Project administration, Methodology, Conceptualization.

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.

Data availability

Data will be made available on request.

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