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The joint adverse effects of aged nanoscale plastic debris and their co-occurring benzo[α]pyrene in freshwater mussel (*Anodonta anatina*)

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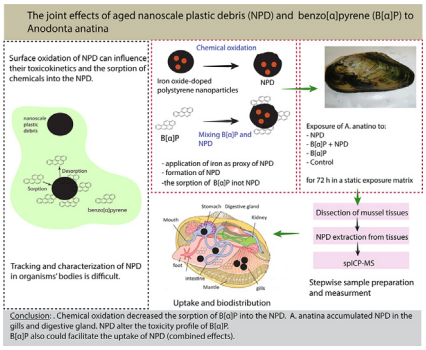
HIGHLIGHTS

- Chemical oxidation decreased the sorption of B[α]P into nanoscale plastic debris (NPD).
- After 72-h of exposure, *A. anatina* accumulated NPD in gills and digestive gland.
- Presence of B[α]P increases accumulation of NPD in the tissues of mussels.
- The mixture of NPD and B[α]P increased the activity of SOD and CAT enzymes.

GRAPHICAL ABSTRACT



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ABSTRACT

Although the presence of small-scale plastics, including nanoscale plastic debris (NPD, size <1 μm), is expected in the environment, our understanding of their potential uptake and biodistribution in organisms is still limited. This mostly is because of the limitations in analytical techniques to characterize NPD in organisms' bodies. Moreover, it is still debatable whether aged NPD can sorb and transfer chemicals into organisms. Here, we apply iron oxide-doped polystyrene nanoparticles (Fe-PS NPs) of 270 nm size to quantify the uptake and biodistribution of NPD in freshwater mussels (*Anodonta anatina*). The Fe-PS NPs were, first, oxidized using heat-activated potassium persulfate treatments to produce NPD (aged particles). Then, the sorption of benzo[*a*]pyrene (B[α]P), as a model of organic chemicals, into the aged NPD was studied. Chemical oxidation (i.e. aging) significantly decreased the sorption of B[α]P into the particles over 5 days when compared to pristine particles. After 72-h of exposure, *A. anatina* accumulated NPD in the gills and digestive gland. When exposed to the mixture of NPD and B[α]P, the number of particles in the gills and digestive gland increased significantly compared to the mussels exposed to NPD alone. Moreover, the mixture of NPD and B[α]P increased the activity of Superoxide dismutase and Catalase enzymes in the exposed mussels when compared to the control and to the NPD alone. The present study provides evidence that aged NPD not only could accumulate and alter the toxicity profile of organic chemicals in aquatic organisms, but the chemicals also could facilitate the uptake of NPD (combined effects).

1. Introduction

Large plastic debris in the environment gradually degrades into smaller pieces because of weathering, including ultraviolet radiation,

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wave actions, and acidic pH (Andrady, 2011), generating microplastics (1 μm < size <5 mm) and nanoscale plastic debris (NPDs <1 μm). It is reported that NPD can be taken up by organisms and transfer across different trophic levels (Abdolahpur Monikh et al., 2021a). Due to their small size and their similar chemistry to biogenic polymers, tracking and characterization of NPD in the environment organisms' bodies is difficult. Most of the available analytical techniques cannot directly analyze NPD in organisms' tissues and cells (Abdolahpur Monikh et al., 2021a) and in the complex matrices of environmental samples. As a result, the concentration of NPD in the environment is unknown and toxicokinetics studies on NPD have often applied fluorescently labelled polymeric nanoparticles as a model of NPD (Al-Sid-Cheikh et al., 2018). Fluorescent labels, however, come with drawbacks (Zhang et al., 2019). For example, the fluorescent dye not only modifies the surface of the particles, interfering with the interactions of the particles in biological systems, but the dye can also be released from the core particles (Deline and Nason, 2019). The detach dyes have completely different uptake kinetics and biodistribution compared to the particle-bound dyes (Salvati et al., 2011). Recently the application of metal-doped polymeric nanoparticles opened a new horizon for understanding the toxicokinetic of the particles in organisms' bodies (Koelmans, 2019). In this approach, the polymeric particles are doped with metals, which allows the application of the well-established mass spectrometry such as single-particle inductively coupled plasma mass spectrometry (spICP-MS) for characterization of the particles in biological matrices (Abdolahpur Monikh et al., 2021b). As the metals are doped inside the particles and as the surfaces of the particles are polymeric, the metallic part is unlikely to influence the interactions of the particles with cells and biomolecules (Koelmans, 2019).

Polymeric nanoparticles have been commonly used as a model of NPD to investigate the toxicokinetics and toxicodynamics of NPD. For example, it was reported that pristine polystyrene (0.05–0.5 μm) can induce oxidative stresses in exposed freshwater rotifer (Jeong et al., 2018). Polystyrene and polyethylene (20–1000 μm) can cause neurotoxicity in mussels, such as *Scrobicularia plana* (Ribeiro et al., 2017) and *Mytilus galloprovincialis* (Avio et al., 2015). Nevertheless, pristine polymeric nanoparticles cannot completely represent aged NPD in the environment. For example, the surfaces of NPD in natural conditions undergo photo/thermal oxidation, which can change e.g. physicochemical properties of the fragments such as molecular weight and oxygen content as well as functional groups (Lv et al., 2019). As a result, the interaction of NPD with cells and their uptake into organisms as well as their biodistribution and toxicity might be different when compared to pristine polymeric nanoparticles. On the other hand, it is still impossible, using current analytics, to find, gather and identify NPD in the environment for performing ecotoxicity tests. The alternative approach is to produce artificial NPD, with the best methodology currently available, by aging polymeric nanoparticles under controlled conditions and performing ecotoxicological experiments with these aged particles.

Surface oxidation of NPD not only can have a considerable influence on the toxicokinetics of NPD but also might influence the sorption of chemicals from the surrounding water into the NPD (Liu et al., 2019b). It was reported that NPD can act as vectors for transferring chemicals into organisms. This process, which is known as Trojan horse mechanisms (Abdolahpur Monikh et al., 2020; Rios et al., 2007) (also referred to as vectoring effects (Chae and An, 2017) has been reported recently for polystyrene nanoparticles, where the nanoparticles transferred silver ions into daphnids and altered the toxicity profile of silver. Hydrophobic chemicals have a high affinity for sorption into the hydrophobic surface of polymeric nanoparticles. Oxidation of the surface of NPD, however, can generate oxygen-containing groups on the surfaces, increasing the hydrophilicity of the fragments. As a result, the dispersion of the particles in water increases, and the sorption of hydrophobic chemicals into the NPD may decrease. Chemical oxidation of NPD in natural conditions might be a long process due to the relatively low oxidant concentration in aquatic environments. Persulfate treatments could be an alternative to oxidation in natural conditions as it was reported to effectively oxidize microplastics surfaces (Liu et al., 2019b). Although there are differences in initiators between persulfate treatment and aging in natural conditions, the oxidation pathways and products may be similar in both processes (Gardette et al., 2013). The application of persulfate for aging allows for the shortening of the aging time required in natural conditions.

Mussels are long-recognized sentinels as bioindicator species for different environmental contaminants (Farris and Van Hassel, 2006). It is obvious that the filter-feeding habit of mussels allows them to accumulate large amounts of pollutants from the surrounding water (Wang et al., 2021). Pollution is an important factor contributing to the global decline of freshwater mussel diversity (Lopes-Lima et al., 2017). Moreover, their living habitats as sediment-dwelling organisms make them also exposed to contaminants present in the sediments (Vidal-Liñán et al., 2010). These features make mussels an optimal candidate model to study ecotoxicology of NPD because NPD is expected to be present in water and sediment, depending on their chemistry (van Weert et al., 2019). It has been already reported that microplastics and NPD can be taken up by mussels (Wegner et al., 2012) through their gills and digestive tissues (García-Negrete et al., 2013; McCarthy et al., 2013). A recent study showed that polystyrene nanoparticles can biodistribute throughout the tissues of marine mussels and induce adverse effects in gills and digestive glands (Gaspar et al., 2018). Polystyrene nanoparticles could reduce the clearance rate, impaired the feeding, and inhibited the individual growth of the quagga mussel *Dreissena bugensis* (Pedersen et al., 2020). Nevertheless, the number of studies focusing on the adverse effects of NPDs in freshwater mussels is scarce. For example, the freshwater duck mussels *Anodonta anatina*, native and widespread in Northern Europe and Siberia, play an important role in the aquatic systems as they can utilize both native and also invasive fish as their hosts (Huber and Geist, 2019). These mussels have faced population declines in many countries and regions and have been the subject of some ecotoxicological studies (Bielen et al., 2016; Falfushynska et al., 2013, 2014). However, to the best of our knowledge, there is no study available to show how NPD might affect this species. It is important to understand if plastic pollution can contribute to the decline of this species.

The objective of this study was twofold. First, we aimed to understand whether the chemical oxidation of NPD influences the sorption of Benzo[*a*]pyrene (B[α]P) into the particles. Benzo[*a*]pyrene is an organic chemical from the family of polycyclic aromatic hydrocarbons, frequently found in the environment. Previous data indicate that B[α]P can induce sublethal toxicity to mussel (Banni et al., 2017). We hypothesized that oxidation of the particles decreases the hydrophobicity of the particles, in turn, reduces the sorption of B[α]P into the particles. Second, we aimed to quantify the uptake and biodistribution of aged NPD in freshwater mussels (*A. anatina*) and to understand whether the presence of NPD alters the toxicity profile of B[α]P in the organism. Our hypothesis is that the toxicity of B[α]P in the presence of NPD is different compared to the toxicity of B[α]P alone. The NPD were produced so that they had iron oxide particles in their structure, which were used as a proxy for tracing NPD in the organisms. We are aware that in natural conditions NPD are not doped with any metals. In this study, however, we used iron oxide nanoparticles as a proxy to facilitate quantification of NPD uptake and biodistribution in organisms, which is a recommended approach to shed light on the biological fate of NPD until a straightforward method is available (Koelmans, 2019).

2. Material and methods

2.1. Chemicals

All chemicals used were of analytical grade and purchased from Merck (Espoo, Finland, or Darmstadt, Germany) unless otherwise mentioned. Benzo[α]pyrene ≥96% (HPLC, CAS 50-32-8), was obtained from

Sigma-Aldrich and Merck (Germany). Spherical Fe-PS NPs (270 nm, PDI = 0.01), doped with iron oxide (>40%), were purchased from Microparticles (GmbH, Berlin, Germany). The density of Fe-PS NPs was ~2 g/cm^3. Water was deionized by reverse osmosis and purified by a Millipore Milli-Q (MQ) system (RiOs™ Essential 16 Water Purification System).

2.2. Study site and sample collection

Adult *A. anatina* (57–86 mm in shell length), were hand collected by wading and snorkeling on October 21, 2020, from Lake Viinijärvi, Eastern Finland (62° 68.1601′ N and 29° 20.6887′ E). The mean ± standard error of weight (38.64 g ± 1.8), shell length (73.16 mm ± 1.1), width (24.83 mm ± 0.5) and height (39.05 mm ± 0.54) of the organisms were measured. The mussels were transported in 50 L plastic tubs with lake water from the study site to the two large maintenance tanks at the Joensuu campus of the University of Eastern Finland. The tanks were equipped with continuous mechanical aeration, water supply and 2 cm of coarse sand. The water was changed in the tanks during the culture. Mussels were acclimatized for two weeks in these flow-through maintenance tanks, each containing 289 L of filtered (naturally) dechlorinated water at 6 °C (the mussels were collected in October when the water temperature was 6 °C). The mussels were fed two times per week with a suspension of 1:1 Shellfish Diet 1800© and Nanno 3600© (Reed Mariculture, USA). The water quality of the circulation system was monitored for relevant parameters e.g. oxygen, nitrite, nitrate, ammonium, pH, electrical conductivity, and salinity.

2.3. Oxidation of Fe-PS NPs using heat-activated potassium persulfate ($K_2S_2O_8$) treatments

The persulfate treatment was performed following the method described previously (Liu et al., 2019b). Briefly, pristine Fe-PS NPs were dispersed in 100 mM freshly prepared potassium persulfate ($K_2S_2O_8$) to reach a final concentration of 10 g/L of particles. The dispersion was mixed at 70 °C using a thermostatic water bath on a magnetic stirrer for 48 h. The 48 h was arbitrarily selected to minimize the possibility of the particle's degradation such as polymer release. Every 12 h, an equal amount of $K_2S_2O_8$ was added to the dispersion to compensate for the consumption of the oxidant. After 48 h, the dispersion was centrifuged (Multifuge 1S-R, Germany) at 4000 rpm for 20 min at 4 °C. The supernatant was removed, and the pellet was dispersed in 100 mL of MQ water. The oxidized particles were used as a model of NPD for further application. These particles will be referred to as NPD (or aged NPD) throughout the study.

2.4. Characterization of NPD in culture medium

The morphology, zeta potential and the surface chemical compositions of pristine Fe-PS NPs and NPD were characterized in MQ water and the mussel culture media. The particles were dispersed in MQ water (1 g/L) and sonicated using a bath sonicator (35 kHz frequency, DT 255, Bandelin electronic, Sonorex digital, Berlin, Germany) for 5 min and used as stock dispersion. The hydrodynamic size and the zeta potential were measured using a Zetasizer Nanodevice (Malvern Panalytical, Almelo, the Netherlands) and a (DynaPro-MSXTC). A transmission electron microscope (TEM JEM-2100F) operating at 80 kV accelerating voltage was used to image the Fe-PS NPs and NPD. The stability of the metals in the structure of the NPD was confirmed in our previous study for pristine Fe-PS NPs (Abdolahpur Monikh et al., 2020) and measured in the current study for NPD using the same method. To measure the agglomeration rate of the particles, the hydrodynamic size of the pristine particles and NPD was measured over 96 h in the mussel culture medium.

2.5. Preparing the stocks solutions

As a hydrophobic compound, B[α]P is a ubiquitous environmental contaminant (Banni et al., 2017; Orbea et al., 2002). Due to the high log K_{ow} (6.13), B[α]P is expected to have a high affinity to plastic surfaces. This chemical has been shown to produce oxidative stress in marine bivalves (Banni et al., 2017; Di et al., 2011). The stock of B[α]P was prepared by dissolving the chemicals in acetone (30%) as reported previously (Kontir et al., 1986), to reach the final concentration of 1 g/L. The dispersion of NPD was sonicated for 30 s and immediately used for providing the exposure media and for testing the sorption kinetic.

2.6. Sorption experiments

The sorption experiments were carried out using a batch adsorption approach (Abdolahpur Monikh et al., 2020) in the exposure medium, which was used to culture the mussels. Four different treatments were used to perform the sorption experiments (Fig. 1), including (1) control (mussel culture medium without any NPD and B[α]P), (2) the solution of B[α]P (5 mg/L), (3) the mixture of Fe-PS NPs (10 mg/L) and B[α]P (5 mg/L), (4) the mixture of aged NPD (10 mg/L) and B[α]P (5 mg/L). The concentration of B[α]P in the sorption experiment was selected based on some pre-tests to ease the measurement of the sorbed B[α]P into the particles over time. The rationale behind selecting the concentration of 10 mg/L of the particles was based on two reasons. First, the concentration should be low enough to represent the environmental conditions, as the concentration of NPD in the environment is expected to be low (Pittura et al., 2018). Second, the concentration of NPD must be high enough to be measurable by the existing analytical technique in organisms.

The treatments were prepared immediately after sonication of the particles (Fig. 1a). The samples were left at room temperature in closed glass vials in dark (Fig. 1b) for 5 days. Every day, aliquots (1 mL) of the samples were taken from the samples, for 5 days in total (Fig. 1c). The condition was kept the same during the 5 days of particle- B[α]P interactions. The samples were filtered through a 200 nm filter (25 mm Syringe Filter, Cellulose Acetate Membrane), assuming that the particles and the sorbed B[α]P are filtered out and only the dissolved B[α]P passes the filter (Fig. 1d). Since the sorption of PAHs to cellulose is not very extensive (Jonker, 2008), very low mount (<0.001%) of the B[α]P was sorbed to the filter. The concentration of B[α]P in the filtrate was measured over time using High-Performance Liquid Chromatography (HPLC-DAD) (Fig. 1e, and see S1, Supporting information).

2.7. Developing the exposure media

In the exposure medium, an aliquot of the B[α]P dissolution was dissolved in a mussel culture medium to reach the final concentration of 5 μg/L. The concentration of the acetone in the exposure medium was lower than 0.01% which has no toxicity effects on freshwater mussels (Milam et al., 2005). An aliquot of the NPD dispersion was added to the exposure medium to reach the final concentration of 10 mg/L. The concentration of the B[α]P was selected based on a literature review as it is shown that this concentration causes no mortality in mussels (Pittura et al., 2018).

Despite mixing the exposure media using aeration to minimize sedimentation, some of the NPD might attach to the sediment. To reveal the distribution of the NPD in the exposure matrix, both the water and sediment (coarse sand, see Section 2.8) were sampled to measure the number of the NPD (by using iron oxide nanoparticles as a proxy) in each sample using spICP-MS. For the details of sample preparation for the spICP-MS see S2, Supporting information. The presence of the background iron in the sediment and water might interfere with measuring the iron oxide in the NPD. To understand the concentration of the background iron in soil and sediment, some water and sediment samples were collected before the addition of NPD to the exposure medium.

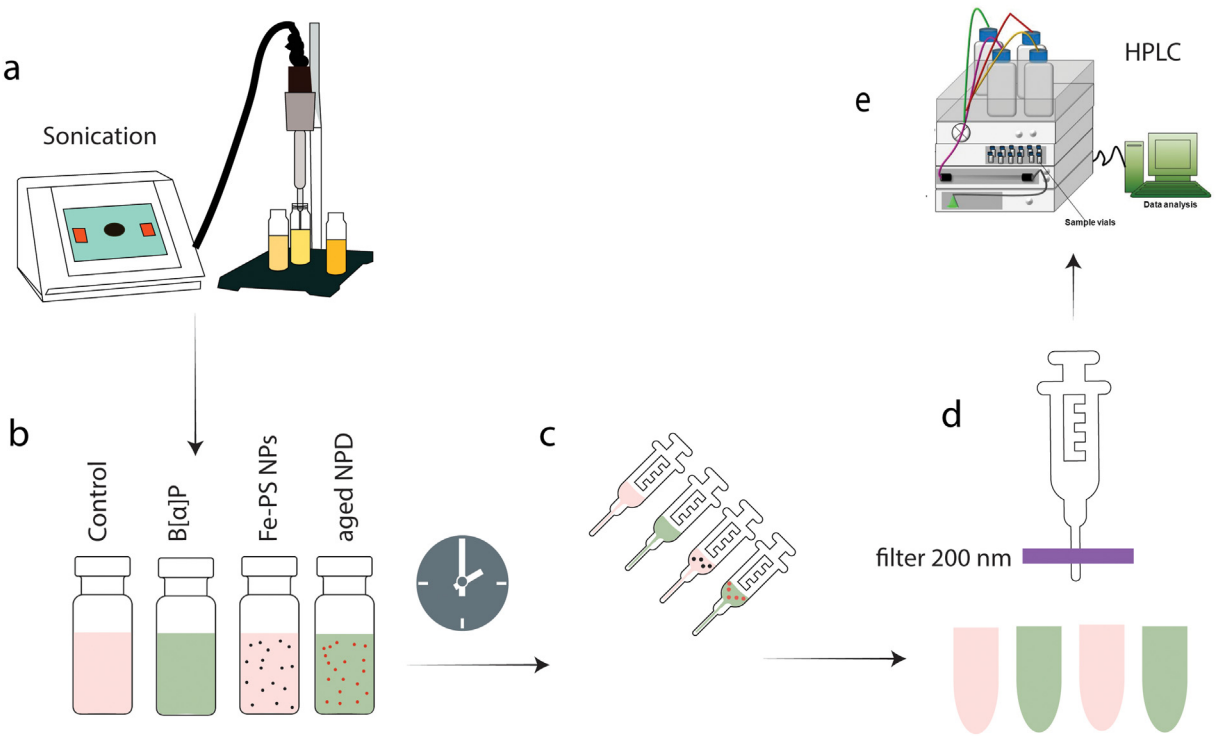

Fig. 1. Schematic presentation of the sorption experiment. a) The sonication process, which has been done immediately before the preparation of the samples. b) Preparing the samples, including control (mussel culture medium without any chemicals), the solution of benzo[α]pyrene (B[α]P), the mixture of iron oxide-doped polystyrene particles (Fe-PS NPs) and B[α]P, the mixture of aged nanoscale plastic debris (NPD) and B[α]P. c) Aliquots of the samples were taken and filtered through 200 nm filters (d). e) The concentration of the B[α]P was measured using a High-Performance Liquid Chromatography (HPLC).

The concentration of the background iron was measured using ICP-MS (S3, Supporting information).

2.8. Exposure of organisms

The exposure experiments were carried out at a constant temperature of 6 °C and a 12 h light/dark cycle. In total four exposure treatments were carried out, including treatment 1: control (unexposed: negative controls), treatment 2: B[α]P (5 μg/L) alone, treatment 3: NPD (10 mg/L) alone, and treatment 4: a mixture of NPD (10 mg/L) and B[α]P (5 μ/L). The concentration of B[α]P (5 μg/L) was selected based on literature review, which shows no lethal effects on freshwater mussels (Châtel et al., 2012). Three replicates were used for each treatment and three mussels were used per each replicate (n = 9 samples per treatment in total). The three adult mussels were randomly selected from the main mussel maintenance tanks. The mussels were placed in glass aquaria containing 6 L of the exposure medium and 2 cm of coarse sand (Beeztees, light aquarium gravel, 3–6 mm, European Community). The exposure media were continually aerated, not only for aeration purposes but also to mix the medium and assist the particles to stay in the dispersion phase for a longer time. The mussels in each treatment were exposed for 72 h in a static exposure system. The particles (hydrodynamic size) were monitored and the water parameters were regularly measured to keep the exposure conditions the same over the exposure time. The experiment was planned such that feeding during the exposure was avoided. The exposure medium has not been changed because this could disturb the sediment and influence the sediment-born exposure in the mussels. After 72 h, the exposure stopped, and the mussels were collected. There was no mortality during the experiment. The total body weight, shell length, shell width and mean shell height was measured. The mussels were dissected to isolate the digestive gland, gills, gonads, kidney, foot, and mantle. We did not euthanize the mussels because this can influence the animals' physiological responses. The dissection was performed by cutting the anterior and posterior adductor muscles. After that, the shells were gently opened, and the tissues were separated. Part of the digestive systems and the gills were separated for histopathological study. The rest of the tissues were stored at −80 °C until further processing.

2.9. Quantification of NPDs in mussel tissues

To quantify the uptake of NPD in organisms, we used iron oxide as a proxy of the NPD. Prior to analysis, tissues were placed inside a laminar flow cabinet and thawed. Gills, digestive gland, gonads, kidney, foot and mantle were digested separately, following modified methods outlined by Abdolahpur Monikh et al. (2020). Briefly, 5% tetramethylammonium hydroxide (TMAH) was added to the samples. The samples were put on a water bath at 70 °C for 1 h. This digestion method has been shown not to cause degradation in the polystyrene particles (Abdolahpur Monikh et al., 2021b). The extracted NPD from each tissue were analyzed using spICP-MS to measure the number of NPD in each tissue (see S2, Supporting information).

2.10. Enzymatic activity test

The activities of Superoxide dismutases (SOD) and Catalase (CAT) were measured as described below. Briefly, the digestive (100 mg ± 1) gland and gills (100 mg ± 1) were homogenized using a glass homogenizer in 10 vol (w:v) ice-cold 10 mM potassium phosphate buffer (pH 7.4) for 2 min at 3000 rpm. The homogenates were then centrifuged at 10000 ×*g* (Sorvall RC5C, Netherlands) for 20 min at 4 °C, and the supernatant was removed and kept at −80 °C for enzymatic assay. The SOD activity was determined at 420 nm by determining the rate of pyrogallol auto-oxidation for 3 min using Ultraviolet-visible spectroscopy (UV-3600, Shimadzu Corporation, Kyoto, Japan) following the method reported previously by Marklund and Marklund (1974). The

CAT activity, as measured by hydrogen peroxide consumption, was assayed at 240 nm using UV–vis following the method reported previously (Aebi, 1974). The activities of the enzyme were reported after normalization with the value obtained for the control (Abdolahpur Monikh et al., 2021a).

2.11. Histopathological studies

Gills and digestive gland samples were fixed in 6% neutral buffered formalin (Sigma-Aldrich, Espoo, Finland) for 24 h in clean tubes. The samples were then dehydrated using increasing concentrations of ethanol, cleared in xylene, and embedded in paraffin wax. Sections were cut at 7 μm, transferred to slides and placed in a 37 °C oven overnight to ensure the tissue fixation to slides. Slides dewaxed, rehydrated by descending concentrations of ethanol and stained with hematoxylin-Eosin (HE) (Sigma-Aldrich, Espoo, Finland). Histopathology examination of slides was performed using a Leica Dmi1 microscope with a Leica Mc 120 HD digital camera (Leica microsystems CMS GmbH, Wetzlar, Germany). Six slides were used for each treatment (24 slides in total for all treatments). We evaluate all the slides. The analysis and the choice were purely qualitatively based on the damages in the tissues and cell membrane.

2.12. Statistical analysis

For all concentrations reported, the respective background concentration of Fe (6 ± 0.5 μg Fe/L, $n = 10$) and iron oxide particles (810 ± 113 particle/L, $n = 10$) in non-exposed (control) organisms and exposure medium was subtracted. Raw data were analyzed using SPSS Statistics 25 and 27. Normality was tested using the Shapiro-Wilk test and homogeneity of variances by the Levene test. One-way analysis of variance (ANOVA) followed by Dunnett's test analysis was performed to compare the number of NPD between the tissues and between the treatments and control. ANOVA (with LSD or Dunnett's T3 post hoc tests) or non-parametric Kruskal-Wallis tests were used to compare enzymatic activity differences between control and different treatments. To compare the number concentration of NPD in the sediment and water, a *t*-test was used. All data are reported as mean ± SD and differences between mean values (biologically independent samples) were deemed statistically significant if $p < 0.05$. The total bioconcentration was calculated according to the following formula:

$$\textit{Total bioconcnetration} = \frac{\textit{Number of NPD in organism}}{\textit{number of NPD in exposure media}}$$

3. Results and discussion

3.1. Characterization of the NPD

The Fe-PS NPs were first characterized in terms of shape using TEM (Fig. 2a). The image shows that the particles are spherical in shape with a narrow size distribution. No differences could be observed in the shape and size distribution of the particles after aging e.g. because of chemical degradation (polymer release) (Fig. 2b). The hydrodynamic size and the zeta potential for the Fe-PS NPs and the NPD in the mussel culture medium are reported in Table S2 (Supporting information). The results showed that the aging process did not influence the hydrodynamic size (size ± SD, 345 ± 36 nm) of the particles however the absolute value of the zeta potential decreased from −35 for Fe-PS NPs to −21 for NPD (aged particles). A decrease in the absolute value of the zeta potential can influence the stability of the particles and lead to particle agglomeration. To test this hypothesis, we measured the hydrodynamic size of the particles over time (Fig. 2c). No homoaggregation was observed for the Fe-PS NPs as determined by the stable hydrodynamic size of the particles over time. However, the hydrodynamic size increased slightly for NPD over 96 h.

Our previous study showed that Fe-PS NPs were stable against dissolution and Fe release from the particles (Abdolahpur Monikh et al., 2020). The Fe ion release from the aged NPD was measured also in the current study and likewise, the results showed that the concentration of elementary Fe measured in the supernatant is stable over 72 h of exposure (S5, Supporting information), indicating no Fe release from the particles. We used UV–vis to determine the changes in the surface of the particles after aging (S6, Supporting information); however, we could not detect any considerable changes.

3.2. Distribution of the NPD in the exposure medium

Sedimentation of the particles over time can result in the sediment-mediated exposure of mussels to NPD. Herein, we monitored the number concentration of NPD in the water and sediment of the exposure medium (Table 1). The calculated nominal number of the particles in all treatments was 4.8×10^{11} particle/L (see the calculation in the S7, Supporting information). Our results showed that the number of particles in water is higher than the number of particles in sediment for all treatments, indicating the higher exposure of the organisms through the water. After 72 h, the number concentration of the NPD in the sediment increased significantly compared to that at 0 h. This showed that the particles do sediment over time and increased the possibility of sediment-mediated exposure to mussels. This is of paramount importance for the environmental risk assessment of plastics. For example, some types of NPD that have a higher density than water could be removed over time from the dispersion phase ending up in the sediment. This might increase the exposure of the sediment-dwelling organisms to these materials (van Weert et al., 2019) but may also decrease the bioavailability of the NPD due to attachment to the sediment's particles and organic matter.

3.3. Sorption of B[α]P into NPD

To test whether the presence of particles influences the concentration of B[α]P (due to sorption into the particles) and to understand how the aging process influences the sorption of the chemicals into the NPD, we performed a sorption experiment. The results are reported in Fig. 3. The concentration of the B[α]P in all the samples, even in the control without any particles, decreased over time. This could be due to B[α]P chemical degradation at room temperature as reported previously (Kot-Wasik et al., 2004). Despite using the same initial concentrations of B[α]P in all treatments, there was a variation in the concentration of B[α]P at time 0 between the treatments, which could be due to the partitioning (sorption-desorption) of B[α]P into the particles and the glass vials.

In the treatment with Fe-PS NPs, the concentration of the B[α]P was significantly (ANOVA, $p < 0.05$) lower than the concentration of B[α]P in the treatment without any particles after 72 h and 96 h. This clearly shows that B[α]P sorbed into the Fe-PS NPs as was expected due to the high hydrophobicity of B[α]P. Interestingly, the concentration of the B[α]P in the filtrates of the treatment containing aged NPD was, however, higher than the concentration of B[α]P in the filtrates of treatment contain Fe-PS NPs. These results support the hypothesis that the aging of NPD due to oxidation can reduce the sorption of hydrophobic chemicals into NPD. The oxidation processes could increase the hydrophilicity of NPD as previously reported for microplastics, where oxidation decreased the contact angles of microplastics rapidly and stabilized after 10 days (Liu et al., 2019b). This could be due to the generation of more hydrophilic groups (i.e., H—O) on the surface of the particles. Although decreasing the hydrophobicity of NPD and the subsequent decrease in the sorption of hydrophobic chemicals might minimize the Trojan horse effect on the chemicals, it is, however, must be considered that this can enhance the sorption of hydrophilic

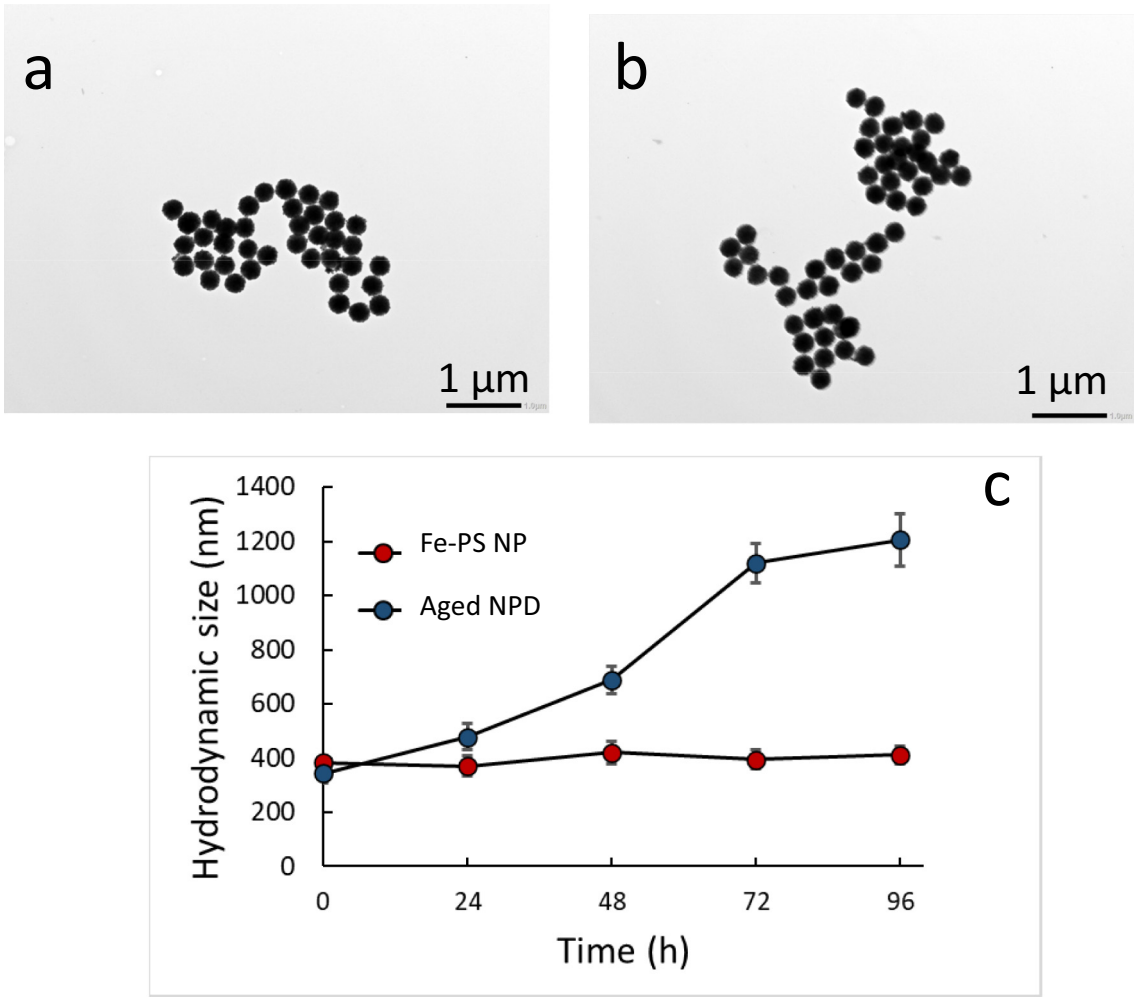

Fig. 2. Characterization of the iron oxide doped polystyrene particles (Fe-PS NPs) and the aged nanoscale plastic debris (NPD). a) The TEM image of Fe-PS NPs before aging (chemical oxidation). b) The TEM image of NPD after aging (chemical oxidation). c) The measured hydrodynamic size of the particles (Fe-PS NPs and aged NPD) in the mussel culture media over 96 h. The results are the average and SD of 10 samples.

chemicals, i.e. it may not decrease the hazard associate with the Trojan horse effect of NPD. Previous studies reported that sorption of hydrophilic compounds e.g. ciprofloxacin increases when the hydrophobicity of the plastic increases as a result of weathering (Liu et al., 2019a; Liu et al., 2019b; Zhang et al., 2019).

3.4. Quantification of NPDs in mussel tissues

The organisms were exposed to NPD and the uptake and biodistribution of the particles were quantified. There was no abnormality, such as abnormal behavior (changes in the feeding habit or swimming activities), in the mussels in any treatments during the exposures. We did not detect iron oxide nanoparticles in the control samples.

In general, the number concentration of the NPD in organisms' tissues (Table 2) was significantly lower than the number concentration of the NPD in sediment and water (Table 1). This indicated that no bioconcentration took place. Regarding treatment 2 (Table 2), where mussels were exposed to NPD alone, NPD were detected only in the gills and digestive gland, which was an expected result as these two organs are in direct contact with the exposure medium. A significantly higher number of NPD was detected in the digestive gland than in the gills. This suggests that NPD are trapped and transported to the mouth

Table 1
Number of NPD measured using spICP-MS in water and sediment of the exposure media at time 0 and after 72 h.

Treatment	Sample	Number of NPD at 0 h (particle/L)	Number of NPD at 72 h (particle/L)
Control	Water	810 ± 113	920 ± 195
	Sediment	9951 ± 1137	9670 ± 4081
	Measured total number	10,761	10,591
Treatment 2: NPD	Water	$3 \times 10^{11} \pm 2 \times 10^{10b}$	$1 \times 10^{11} \pm 3 \times 10^{10a}$
	Sediment	$5 \times 10^{6} \pm 1 \times 10^{6a}$	$5 \times 10^{10} \pm 1 \times 10^{10b}$
	Measured total number	3×10^{11}	2×10^{11}
Treatment 3: NPD & B[α]P	Water	$2.5 \times 10^{11} \pm 3 \times 10^{10}$	$1 \times 10^{11} \pm 4 \times 10^{10}$
	Sediment	$4 \times 10^{6} \pm 5 \times 10^{5}$	$5 \times 10^{10} \pm 1.8 \times 10^{9}$
	Measured total number	2.5×10^{11}	1.8×10^{11}

a,b show the significant difference ($p < 0.05$) between number of particles in different compartments (water and sediment). The results are the average and standard deviation of 3 samples. Degree of freedom is 5.

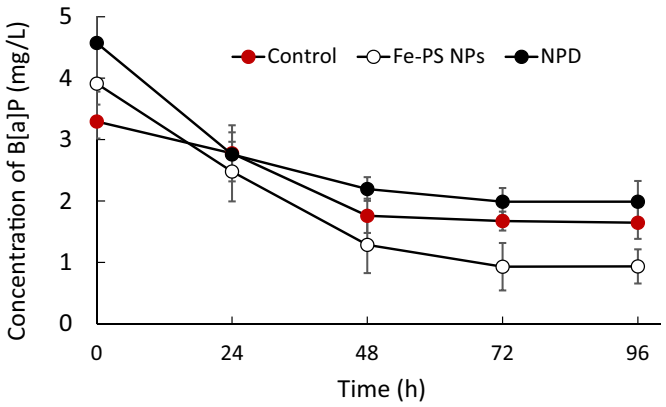

Fig. 3. The concentration of benzo[α]pyrene (B[α]P) after filtration over 5 days in the treatments measured by HPLC-DAD: no particles (B[α]P only), iron oxide doped polystyrene nanoparticles (Fe-PS NPs) and nanoscale plastic debris (NPD). The results are the average and standard deviation of 5 samples. Degree of freedom is 4.

by gills' cilia, and ingested and accumulated into the digestive gland of the mussel, but the NPD could not pass the gills and digestive gland to enter the organism's body. The observed accumulation of NPD in the digestive gland of *A. anatina* is in accordance with the results of Avio et al. (2015) and Merzel et al. (2020) for *Mytilus* and *Dreissena* mussels, respectively.

Regarding treatment 3 (Table 2), where the organisms were exposed to the mixture of NPD and B[α]P, NPD were detectable in gills, foot, and digestive gland, with a higher number in the foot followed by digestive gland and gills. The comparison between the number of NPD in organisms treated with NPD alone and in organisms treated with the mixture of NPD and B[α]P showed that the number of NPD in the latter case is significantly higher (Table 2). It is well known that B[α]P can induce lipid peroxidation and oxidative stress in cells, leading to cell damages (Telli-Karakoç et al., 2002). It is possible that B[α]P induces sublethal cellular damages to the digestive gland, gills, and foot of the mussel, which are in direct contact with the exposure medium, as observed previously in marine mussels (Banni et al., 2017). In turn, the presence of damages in cells facilitated the passage of NPD through cells and, consequently, the higher number of accumulated NPD in these tissues. This is an importing finding, indicating that a mixture of contaminants with NPD, even when the concentration of both the chemical and the NPD is low, could lead to combined effects. This type of combined effect has been previously observed for microplastics. For example, Na et al., 2021 reported that the addition of benzophenone-3 to polyethylene microplastic resulted in greater acute toxicity to *Daphnia magna* (EC50 = 0.99 mg/L) compared to microplastics (EC50 = 3.90 mg/L) or benzophenone-3 (EC50 = 2.29 mg/L) alone. Joint effects can be more hazardous for NPD as they have a smaller size than their microplastics counterparts, thus, they can easily penetrate through damaged cells. To test this hypothesis that whether oxidative stress and cellular damages mediated the higher NPD uptake in the organs, we measured the enzymatic activities and performed histopathological effects in the gills and digestive gland.

3.5. Enzymatic activity test

The SOD and CAT activity levels were analyzed in the digestive gland and gills (Table 3). The values obtained for the activity of the SOD and CAT are normalized by the value obtained for control and presented as percentages. The results indicated that after 72 h of exposure, the SOD activity in the digestive gland significantly increased in all treated groups in contrast to the control group. The maximum SOD activity of 167.3% was obtained for a treatment containing the mixture of NPD and B[α]P. The most significant inductions of CAT activity were attained in mussels exposed to a mixture of NPD and B[α]P followed by mussels exposed to B[α]P. Meanwhile, our measurements revealed no significant differences between mussels treated with NPD and the control ($p > 0.05$), except for the elevated SOD in the digestive gland.

The SOD and CAT activities in the gills were significantly ($p < 0.05$) enhanced after exposure to the mixture of NPD & B[α]P, compared to the control group. Exposure to NPD alone, however, did not result in a statistically significant increase of the activities of the enzymes in gills compared to the control (Table 3). These findings support our hypothesis that the presence of B[α]P can induce oxidative stress, and, in turn, this might increase the permeability of the cells to NPD. It was previously reported that oxidative stress can render fish susceptible to disease, when the balance between the generation and removal of reactive oxygen species is disrupted (Sheikhzadeh et al., 2012). This might also be the case for NPD, where exposure to chemicals causing oxidative stress can facilitate the penetration of NPD into organisms. In the study by Avio et al. (2015), the mussels, *M. galloprovincialis* were exposed for 4 weeks to 10 mg/L of low-density polyethylene microparticles (2.34×10^7 particles/L, size range 20–25 μm), to both non-contaminated and pre-contaminated with B[α]P (15 μg/g). In agreement with our study, they reported that microplastics were localized in hemolymph, gills, and especially digestive tissues. Avio et al. (2015) reported that polyethylene and polystyrene microplastics were shown to adsorb pyrene with a time and dose-dependent relationship. They showed that microplastics can transfer pyrene to exposed *M. galloprovincialis* and induce immunological responses, lysosomal compartment, peroxisomal proliferation, oxidative stress, and neurotoxic effects.

3.6. Histological changes in tissues

It was reported that mussels can take up microplastic and B[α]P via gills or digestive tubes from water and food (Von Moos et al., 2012; Speciale et al., 2018). The morphologies of the digestive gland of the control and treated mussels were investigated (Fig. 4). In the control group (Fig. 4a), the normal numerous digestive glandular tubules were surrounded by loose connective tissue and lined by the glandular epithelium was observed. In B[α]P (Fig. 4b) or NPD (Fig. 4c) treated groups, however, there were mild to moderate necrosis of the tubules with few inflammatory cells' infiltrations. In the mussels exposed to the mixture of NPD & B[α]P (Fig. 4d), severe necrosis and lysis of the digestive glandular tubules were observed with diffuse infiltration of inflammatory cells in the inter-tubular area. Our findings are in agreement with Yavaşoğlu et al. (2016) who reported some damages and disruption in digestive tubules in *Mytilus galloprovincialis* as a result

Table 2
Number of NPD measured using spICP-MS in tissues of the exposed organisms after 72 h and the calculated total bioconcentration.

Treatment	Gonad (particle/kg w.w)	Gills (particle/kg w.w)	Foot (particle/kg w.w)	Mantle (particle/kg w.w)	Kidney (particle/kg w.w)	Digestive gland (particle/kg w.w)	Total bioconcentration (L/kg w.w)
Control	ND	ND	ND	ND	ND	ND	
NPD	ND	$2.7 \times 10^3 \pm 642^a$	ND	ND	ND	$2 \times 10^4 \pm 2 \times 10^{3a}$	1.6×10^{-4}
NPD & B[α]P	ND	$6 \times 10^3 \pm 385^b$	$3 \times 10^5 \pm 4 \times 10^4$	ND	ND	$2.7 \times 10^4 \pm 2 \times 10^{2b}$	3.5×10^{-4}

a,b show the significant difference ($p < 0.05$) between number of particles in different treatments (mussels exposed to NPD and to the mixture of NPD and B[α]P). ND: not detected. The results are the average and standard deviation of 9 samples. Degree of freedom is 8.

Table 3
Enzymatic activities in samples treated with NPD relative to control, the mixture of NPD & B[α]P and B[α]P after 72 h exposure in Digestive gland and gills.

Tissues	Antioxidant enzyme activities	Control %	B[α]P %	NPD %	NPD & B[α]P %
Digestive gland	SOD	100^{a}	144.4 ± 32^{bc}	110.3 ± 1.3^{b}	167.3 ± 7.9^{c}
	CAT	100^{a}	116.4 ± 12.6^{b}	103.5 ± 8.8^{ac}	123.5 ± 20.4^{b}
Gills	SOD	100^{a}	112.8 ± 0.4^{b}	$109.2 \pm a7.3^{ab}$	133.2 ± 0.2^{c}
	CAT	100^{a}	112.3 ± 28.3^{ab}	103.2 ± 34^{a}	121.5 ± 27^{b}

Data are presented as means ± standard deviation (±SD) of 9 samples. a,b,c show the significant difference ($p < 0.05$) between different treatments (mussels in control, exposed to NPD and to the mixture of NPD and B[α]P). Degree of freedom is 8.

of exposure to contaminants. These are the first data showing how the NPD and combination of NPD and chemicals can induce histological responses in freshwater mussels. This is important for risk assessment and conservation biology because the amount of NPD in freshwater ecosystems are expected to be even higher than in marine ecosystems due to receiving discharges from urban and industrial wastewaters (Singh et al., 2021).

Similar observations of histological responses were documented in gills (Fig. 5). Histological sections from the gills of the control group showing a double layer of ciliated columnar epithelial cells arranged parallel to each other and separated by a narrow space (Fig. 5a). In the group exposed to B[α]P (Fig. 5b) or NPD (Fig. 5c), necrosis of the epithelial cells (arrowheads), edema (asterisks), and pigmentation (brown pigments) were observed. The severity of epithelial cell necrosis with loss of cilia (arrowheads), edema (asterisks), and pigmentation (brown pigments) was increased in the mussels exposed to the mixture of NPD and B[α]P. These observations support our hypothesis that NPD and B[α]P have combined effects, where the presence of B[α]P increases the uptake of NPD and the presence of NPD increases the effect of B[α]P. These findings are in agreement with a previous study, which has shown that exposure to B[α]P induces morphological changes such as epithelial cell detachment degradation, and sloughing (Speciale et al., 2018). This is the first study documenting histological changes in mussels' gills as a result of exposure to NPD and a combination of NPD and chemicals.

4. Conclusion

To date, most studies that tested the adverse effects of NPD on organisms applied polymeric nanoparticles as models of NPD. Our study shows that aging not only influences the uptake and biodistribution of the particles in organisms, but also decreases the sorption capacity of the particles for organic chemicals. In turn, this might alter the toxicity of the chemicals. For example, by decreasing the hydrophobicity of the particles due to aging, sorption of hydrophobic chemicals decreases

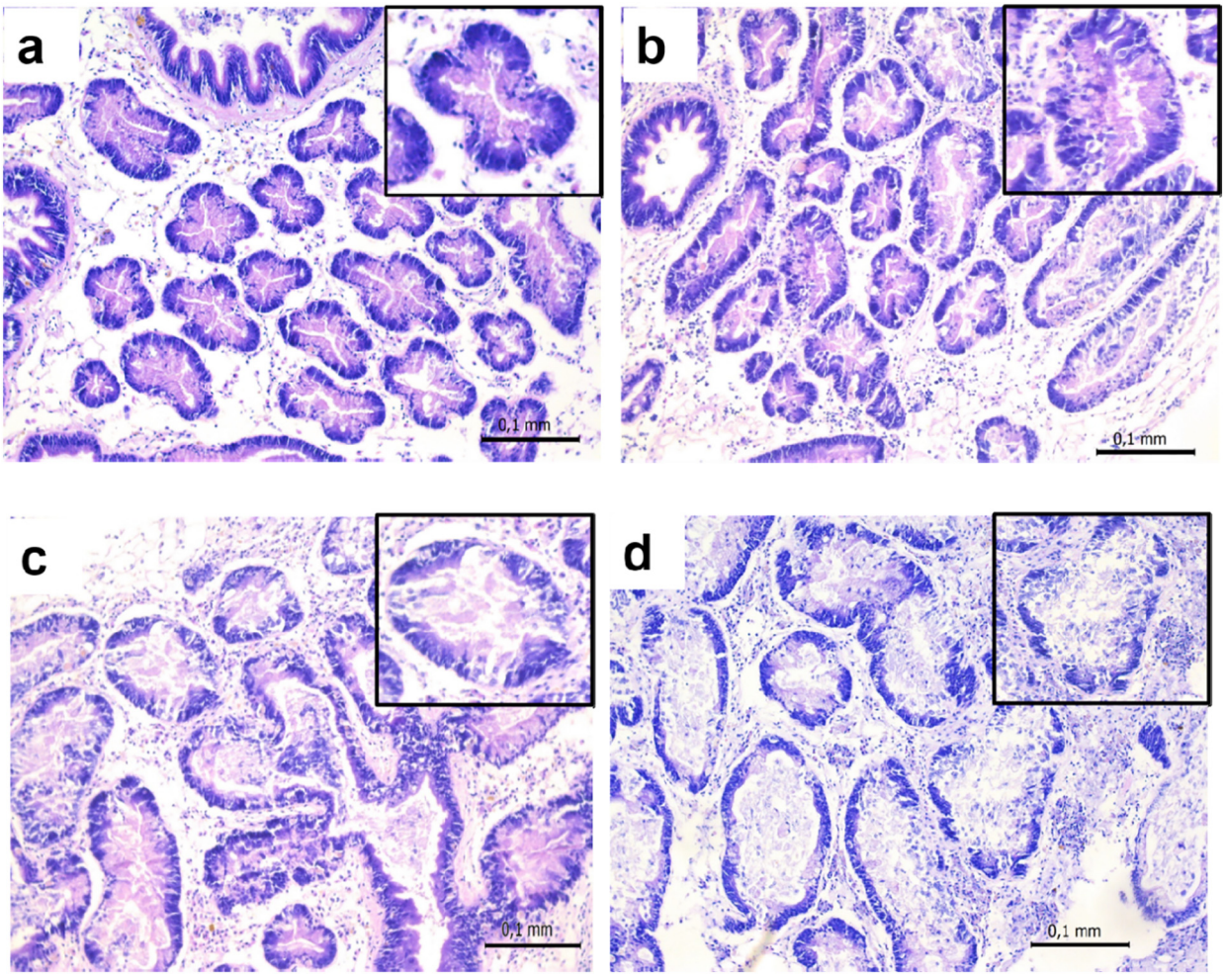

Fig. 4. Histological sections from the digestive gland of mussels in the control (a) and the treated groups including B[α]P (b), NPD and (c) the mixture of NPD and B[α]P (d). The inserts show an enlarged portion for the normality (a) and abnormalities (b, c and d) of the digestive glandular tubules under control and treated conditions, respectively.

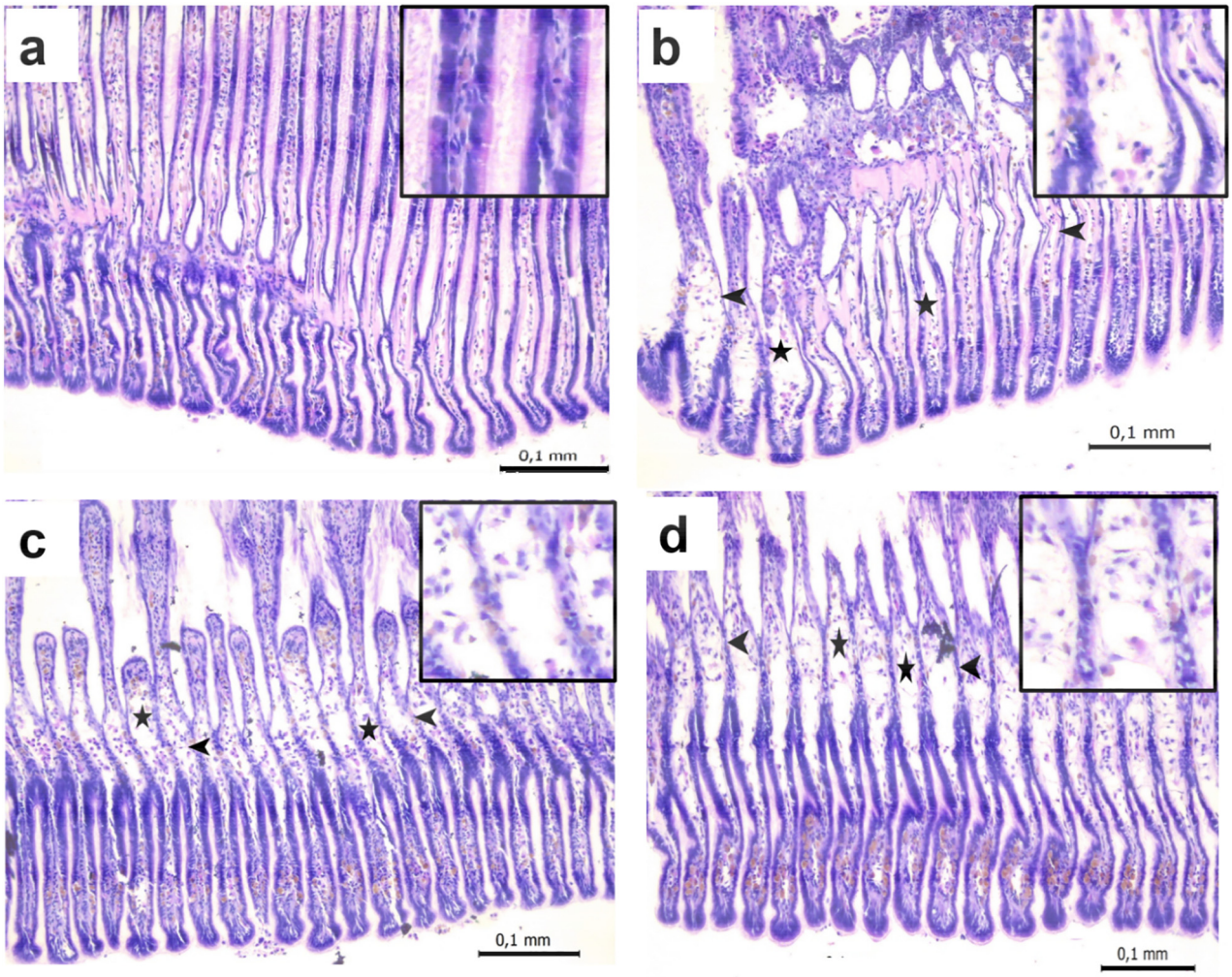

Fig. 5. Histological sections from the gills of mussels in the control (a) and the exposed organism to B[α]P (b), NPD and (c) the mixture of NPD and B[α]P (d). Necrosis (arrowheads), Edema (asterisks). The inserts show an enlarged portion for the normality (a) and abnormalities (b, c and d) of gill filaments under control and treated conditions, respectively.

which might influence the bioavailability and uptake of the chemicals. It is also possible that sorbed chemicals to particles follow different entry pathways compared to dissolved counterparts. Future studies may investigate how variation in the chemistry of the particles influences this sorption and the toxicity profiles of organic and inorganic chemicals. This work confirms the importance of applying aged NPD in ecotoxicological studies. With the aid of metallic nanoparticles as proxies, we showed that NPD could be taken up and accumulate in some tissues of freshwater mussels through water and sediment, especially the digestive gland and gills, which are in contact with the exposure medium. We conclude that NPD have the ability to accumulate in aquatic organisms. It is required to understand how the physicochemical properties of the particles influence their biodistribution in organisms. We show here that NPD and B[α]P have a combined effect where the presence of B[α]P increase NPD uptake, and the NPD can potentially increase the B[α]P toxicity. This work highlights the importance of understanding the combined effects of chemicals for environmental risk assessment, as this is a more environmentally realistic scenario.

Author contributions

O.A. wrote and reviewed the paper. O.A. and F.A.M., performed the exposure experiment and the sorption experiment. O.A. and R.K. and J.T. performed the mussels sampling and maintenance. S.K., F.A.M. and O.A. performed the chemicals and the particles measurements. O.A. performed the mussel's dissection, morphometric measurements, enzymatic activation test and preparation of the histological samples & histopathological examination. F.A.M., J.A. and R.K. contribute to the supervision and editing the paper.

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.scitotenv.2021.149196.

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