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Weathering impacts the uptake of polyethylene microparticles from toothpaste in Mediterranean mussels (*M. galloprovincialis*)

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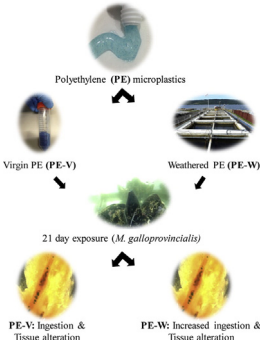
HIGHLIGHTS

- Polyethylene (PE) from consumer products can enter the environment via effluent.
- *M. galloprovincialis* was exposed to PE for 21 days, both virgin and weathered PE.
- Ingestion, tissue alteration, growth and genotoxicity were assessed.
- Weathering did impact PE ingestion of *M. galloprovincialis*.
- Ingestion caused histopathologic alteration in gills and digestive system.

GRAPHICAL ABSTRACT



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ABSTRACT

Mediterranean mussels (*Mytilus galloprovincialis*) were exposed over 21 days to polyethylene (PE) particles (0.01 mg ml^{-1}; 50–570 μm) isolated from toothpaste. PE was deployed in the Outer Oslofjord (Norway) for 21 days, before exposing the mussels to both virgin (PE-V) and weathered PE (PE-W) particles. The mussels ingested both types of particles, but significantly more weathered particles were ingested than virgin ($p = .0317$), based on PE dosed by weight (mg ml^{-1}) but not when considering particle number (PE-V: 1.18 ± 0.16 particles ml^{-1}; PE-W 1.86 ± 0.66 particles $ml^{-1;}$). PE particle ingestion resulted in structural changes to the gills and digestive gland, as well as necrosis in other tissues such as the mantle. No differences were found regarding the degree of tissue alteration between PE-virgin and PE-weathered exposures. This current study illustrates the importance of using weathered particles in microplastic exposure studies to reflect the behaviour of plastic particles after entering the marine environment. The observed tissue alterations demonstrate the potential adverse effects to mussels exposed to microplastic particles.

1. Introduction

Microplastic particles (<5 mm; Arthur et al., 2009) can enter the marine environment following direct release from certain consumer products, such as those intended for personal care and categorised as primary microplastics, or from the breakdown of larger plastic items. Primary microplastic particles from consumer products can pass through wastewater treatment plants (WWTPs) and contribute to the overall environmental load of microplastic particles (Fendall and Sewell, 2009). Polyethylene (PE) microbeads are regularly used in personal care products and studies estimate that the average person in the

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US typically uses 2.4 mg of microbeads per day (Gouin et al., 2011) with Europeans averaging up to 17.5 mg per person per day (Gouin et al., 2015). If these estimations are representative, then approximately 263 t year^{-1} are released into sewer systems by American citizens (Gouin et al., 2011) and 4360 tons by those in Europe (for 2012) (Gouin et al., 2015). Toothpaste is a product that can contain up to 1.8% PE microbeads (by weight) and similar PE particles have been shown to occur in WWTP effluent (Carr et al., 2016). WWTPs are, however, reported to efficiently (>98%) remove microplastic particles when comparing influent and effluent concentrations (Murphy et al., 2016). Despite the efficient removal of microplastics by WWTPs, the volume of effluent discharged from WWTPs daily can still be high. Extrapolation of data from 17 different WWTPs in the US suggests that >4 × 10^6 microparticles day^{-1} are being released from each WWTP (Mason et al., 2016), while Rochman et al. (2015) estimated a total emission from US WWTPs into aquatic habitats to be 8 trillion microbeads per day. A study from Glasgow, UK, estimates that a large WWTP with a population equivalent of approximately 650,000, releases >65 × 10^6 microplastic particles every day (Murphy et al., 2016). PE is the most produced polymer worldwide, and PE microplastics, together with polypropylene, are often the most dominant polymer found in seawater, as summarised by Phuong et al. (2016). However, existing analytical methods cannot distinguish between PE in the environment from primary sources and secondary break-down products.

Plastics entering the ocean become coated over time with an organic corona (Galloway et al., 2017). This organic corona builds up initially through attachment of a bacterial biofilm often referred to as "slime" or "conditioning film", followed by the colonising of invertebrates (Dunne Jr., 2002; Ye and Andrady, 1991; Artham and Doble, 2009; Andrady, 2011). In addition to biofilm formation, many abiotic processes also impact plastics in the marine environment such as physical stress, UV-radiation, shifting temperatures, salinity and oxidisation (Andrady, 2011; Kukulka et al., 2012; Jahnke et al., 2017). All these processes are often referred to as "weathering", "ageing" or "conditioning". Weathering of PE in the field has been found to alter the surface properties and morphology (ter Halle et al., 2017) and therefore it is of importance to study weathered PE to better mimic an environmental realistic scenario. For example biofouling can result in an increase in the density of buoyant microplastic particles, such as PE, and thereby cause them to sink (Cole et al., 2011), potentially making the plastic more available to also other marine organism not only in the pelagic zone. A recent exposure study by Vroom et al. (2017) did find increased microplastic ingestion in zooplankton following weathering of polystyrene (PS), showing that this process ageing can impact ingestion rates of microplastics.

Since blue mussels (*Mytilus* spp.) are widely distributed, sessile and filter large volumes of seawater (active feeding: 3 l h^{-1}; Famme et al., 1986); they are seen as sentinel organisms for costal pollution monitoring, as recently reviewed by Beyer et al. (2017). Mussels might also be suitable as indicators for microplastic contamination, as both M. *edulis* and *M. galloprovincialis* have been suggested for monitoring microplastic contamination in the marine environment by ICES (2015) (OSPAR, 2015). Findings from a recent field study also supports the use of blue mussels as a sentinel species for microplastic pollution; M. *edulis* from the North Sea was found to accumulate microplastics, as the levels of microplastics were 1000-fold higher within the mussels than the surrounding water and sediment, with an average of 37,000 microplastics per kg d.w. (Karlsson et al., 2017).

Phuong et al. (2016) reported that in general, most microplastic exposure studies are not conducted with the most environmental relevant plastic polymers, as nearly all previous exposure studies have used spherical fluorescent polystyrene (PS). This is mainly due to these particles being standardised in size and pre-made, as well as being easy to detect since they are florescent. In addition, previous marine bivalve studies have been conducted with levels being very high, with exceptions from Paul-Pont et al. (2016), as well as studies using only virgin microplastics.

Despite increased knowledge of microplastics being found in marine organisms, little is understood of how microplastic pollution might affect marine wildlife. Laboratory studies have shown uptake and evidence of adverse effects from microplastic exposure in marine mussels (both *M. edulis* and *M. galloprovincialis*). In these experimental studies microplastics were found in whole mussels analysed (Van Cauwenberghe et al., 2015) but also more specifically in association with the gills (von Moos et al., 2012; Avio et al., 2015; Paul-Pont et al., 2016; Kolandhasamy et al., 2018), haemolymph (Browne et al., 2008; Avio et al., 2015), digestive tract (Browne et al., 2008; von Moos et al., 2012; Avio et al., 2015; Paul-Pont et al., 2016; Kolandhasamy et al., 2018) and Kolandhasamy et al., 2018 also found microplastics in association with gonad, mantle, adductor muscle, visceral and foot. Furthermore, particle uptake was found to impact the marine mussels. von Moos et al. (2012) found inflammatory response and histological alterations in the digestive gland to HD-PE exposure (up to 80 μm; 2.5 mg ml^{-1}) and Van Cauwenberghe et al. (2015) reported an increase in respiration following PS exposure (10, 30 and 90 μm; total of 110 particles ml^{-1}) Co-exposure of microplastics and contaminants have also been conducted in marine mussels; Avio et al. (2015) found accumulation of pyrene at the same sites as microplastic accumulation as well as several cellular effects (PE and PS; 100–1000 μm; 1.5 mg ml^{-1} + pyrene) and Paul-Pont et al. (2016) found effects of PS alone by e.g. increased haemocytic mortality while the co-exposure lead to e.g. increased histopathological damages (2 and 6 μm; 3.2 × 10^{-5} mg ml^{-1} + fluoranthene).

The main objective of this study was to determine if *M. galloprovincialis* could ingest PE particles from toothpaste, using both "virgin" (PE-V) and "weathered" microplastics (PE-W). In addition, the study aims to obtain information on PE particles from toothpaste as well as sub-lethal effects on *M. galloprovincialis* following ingestion.

2. Materials and methods

2.1. Particle extraction and characterisation

PE was extracted from Colgate® Max Fresh® toothpaste (Colgate-Palmolive, UK). The smallest PE fragments in the toothpaste were 50 μm, therefore the toothpaste was filtered through a 50 μm mesh. This was then rinsed with tap-water until foaming had stopped and rinsed with filtered (0.22 μm) reverse osmosis (RO) water before being transferred into a beaker containing RO water (0.09 mS/m). The PE particles floated on the surface while other white non-water-soluble substances >50 μm, sank. Carr et al. (2016) also observed these two non-water-soluble particle types when isolating microplastic particles from toothpaste; polyethylene and white unidentified particles that were suggested by Carr et al. (2016) being the mineral mica. The top layer consisting of PE was scraped off and dried overnight at 50 °C before measuring the dry weight (dw). The isolated particles were confirmed as PE polymer by Fourier Transform-Infra Red spectrometric analysis (FT-IR Nicolet™ iS™50) with a library match of 97.05% (Polyethylene, Mn 1400) (spectrum attached in SI). In addition, FT-IR analysis was also performed on PE-V and PE-W particles that were collected from the digestive system of blue mussels post exposure. On average, one tube of toothpaste (100 ml) rendered approximately 100 mg (d.w) of extracted microplastics. All the extracted PE from all of the tubes were pooled in one falcon tube to reduce effect of any differences in PE particles from the different toothpaste batches.

The size distribution of the particles was determined by counting 400 random particles of both PE-V and PE-W using a microscope equipped with image analysis software (Cell^D, Olympus, Greece). Both the length and width of the particles were measured (particles had irregular shape) and the largest of these dimensions (μm) was selected as the defined size of the particle (Fig. 1).

Particle concentration was determined by adding 3 mg of PE (PE-V and PE-W was done separately) to a glass slide with a cover slip before

counting the total number of microplastic particles in the 3 mg of PE-V or PE-W. This number was then extrapolated to the particle density of 0.01 mg ml^{-1} PE. For both PE-V and PE-W, five replicates (5 glass slides with 3 mg of PE) were counted and 2 technical replicates were analysed (one slide counted twice). The particle densities were unexpectedly found to be different; the particle concentration of PE-V from 0.01 mg ml^{-1} exposure being 1.18 ± 0.16 particles ml^{-1} while for PE-W being 1.86 ± 0.66 particles ml^{-1}.

2.2. Particle weathering

For the experimental procedures, approximately 3 g (dw) of particles were weathered in a closed 50 μm mesh bag and placed in an outdoor wave simulator basin with continuous intake of sea water (top layer) from the outer Oslofjord (Drøbak, Norway) for 21 days.

2.3. Sample collection

Mediterranean mussels (*M. galloprovincialis*) (mean length 5.7 ± 0.67 cm at time 0) were collected at the beginning of September 2014 from Tarragona (Fangar Bay, Spain) at a depth of 3–5 m. The mussels were transported to the animal-holding facilities at the ICM-CSIC (Barcelona, Spain; 41° 23′ 7″ N; 2° 11′ 46″ E) in a thermally insulated container. They were acclimatised for 20 days in a 600 l tank supplied with a continuous circulation of filtered sea water (50 μm), taken from a collector located at 300 m from the coastline and at 10 m depth (salinity 38 ppt; local seawater temperature 22 ± 1°C).

2.4. Experimental design

Following acclimation, 270 mussels were selected and randomly transferred to 20 l glass tanks (30 mussels per tank) containing 15 l filtered sea water provided with continuous aeration through ceramic air stone diffusers, located in the middle of the tank. As mussels were sampled (removed from the tanks), the concentration of microplastic particles was kept constant to 0.01 mg ml^{-1}, by adjusting the seawater volume at 0.5 l per mussel. Temperature was held constant at 22 °C and the photoperiod was set at 12 h light: 12 h dark with an exposure period of 21 days. Mussels were exposed to either PE-V or PE-W at a concentration of 0.01 mg ml^{-1}; in addition, three glass tanks contained a control group with no PE. Each of the three groups were performed in triplicate (PE-V A, B, C and PE-W A, B, C and Control A, B, and C). Special care was taken during the experiment to avoid possible cross-contamination with glass lids covering the tanks. At time zero, 150 mg of polyethylene (nominal concentration) was weighed out in a falcon tube, then 50 ml of seawater was added to the tube. Next, the particles and the seawater were mixed by vigorously shaking the tube, then added to a total of 15 L of seawater with thorough rinsing of the tube. The falcon tubes were checked under a stereomicroscope to see that all particles were gone. The exposures were performed using a semi-static system with manual removal of seawater and re-dosing of the particles three times a week. Between the re-dosing the tanks were rinsed thoroughly with clean seawater. Several attempts were done to measure the actual exposure concentration in the exposure medium during re-dosing, which was accomplished by filtering all exposure medium and analysing the filters. However due to too high levels of organic matter, the filters kept clogging. Consequently, the actual measurement of the exposure concentration was not possible. Taking water sub-samples was not considered a suitable method since microplastic particles are non-water-soluble and have a patchy distribution within the water column. Therefore, the exposure levels used in this study are unfortunately only based on nominal concentrations.

Eight hours prior to every water change, mussels were fed *ad libitum* with algae mix (Easy Reefs®; Cádiz, Spain), containing 100% marine phytoplankton, which consisted of *Phaeodactylum tricornutum* (33.3%), *Nannochloropsis gaditana*. (33.3%) and *Tetraselmis chuii* (33.3%). Mussels were sampled on day 0, 5, 9 and 21 by carefully cutting the byssus threads. Mussels used to study ingestion were only sampled at the end of the exposure study (day 21). Sample preparation was as follows, haemolymph was immediately collected for micronuclei analysis, whole mussels were immediately fixed in 4% paraformaldehyde for histology (see Section 2.6) and individual whole mussels for ingestion analysis were carefully removed from their shell and placed in falcon tubes before being frozen at 20 °C (see Section 2.5). All samples were weighed (total weight) and the shell length measured. In addition, for

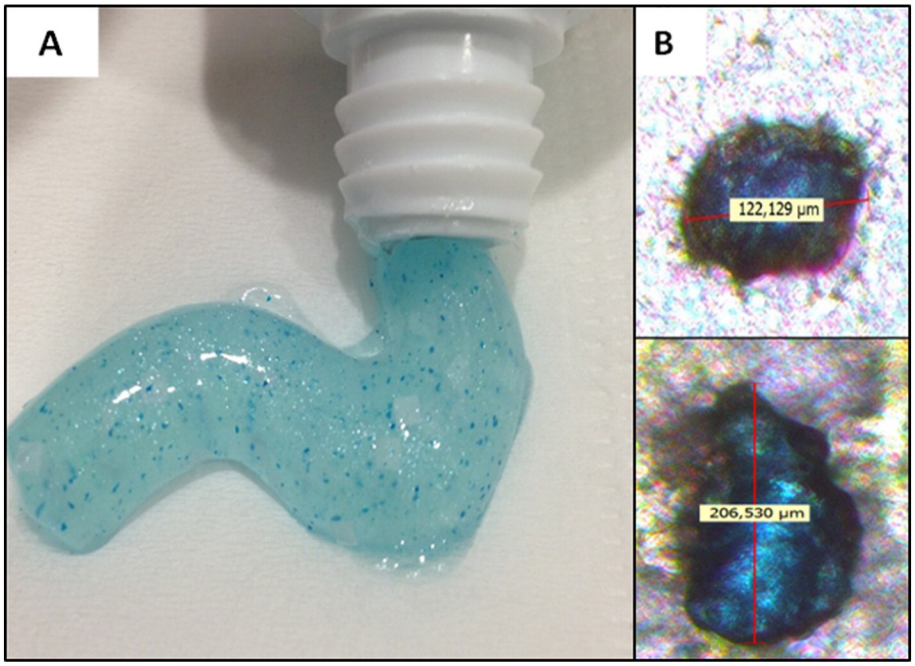

Fig. 1. A: Colgate® Max Fresh® toothpaste B: Examples of size measurements of PE particles.

a subset of individuals the wet weight (ww) of the mussel tissue and the shell weight were also assessed to calculate the condition index (n = 12 for each group). There was no mortality during the study in any of the nine exposure tanks.

2.5. Ingestion determination

To detect the uptake of PE particles following exposure at day 21, two methods were utilised. Visual assessment of the intestine (10 individuals per treatment) and KOH-digestion of whole mussels (5 individuals per treatment). For the visual assessment, whole mussels were removed from their shells, the byssus filaments and foot removed, and carefully subjected to visual inspection using a stereomicroscope (UNITRON Z10, U.S.). The outer part of the whole individual was inspected for the possible presence of PE particles. When present, these particles were thoroughly washed away to avoid possible cross contamination. The digestive gland and the gut were removed from the rest of the tissues and the gut content released into a clean glass beaker. The gut content was examined under a stereomicroscope and all microplastic particles were counted manually under the microscope on a grid pattern. The size of randomly chosen ingested particles were measured in a sub-sample (number of particles measured = 57). The remaining tissues were cut into pieces and examined to make sure all microplastics were detached from the tissue. The chopped tissue was then homogenised with a magnetic stirrer plate (IKA C-MAG HS 7, GERMANY) for 1 h at 500 rpm and dried out in a heating cabinet at 50 °C. For the KOH-treatment of the whole blue mussels, the method was modified from Dehaut et al., 2016. Dehaut et al. (2016) found no impact on PE from this alkaline digestion method. Whole mussels were removed from their shells and the byssus filaments and foot was removed, before checking for outside contamination. Each individual was weighed before being added to glass jars with 10% KOH (10 times w/w), then placed in an incubator (New Brunswick™ Innova® 44/44R) at 60 °C with 140 rpm for 24 h. Then the dissolved blue mussel individual was filtered on a glass microfiber GF/D filter (pore size 2.7 μm) and PE-particles were counted under a stereomicroscope at 10× magnification using a grid pattern.

2.6. Micronuclei assay, histology and condition index

Micronuclei formation in haemocytes, gill and digestive gland histology were measured in the mussels from day 0, 5, 9 and 21. Micronuclei formation is widely used in mussels providing a measure of genotoxicity following exposure to environmental contaminants (Majone et al., 1987; Bolognesi et al., 1999; Barsiene et al., 2004; Brooks et al., 2012; Brooks et al., 2015). Haemolymph was collected by carefully lifting the mussel shell with a scalpel and draining any excess water before extraction. For this operation, a needle attached to a syringe pre-filled with PBS buffer (100 mM PBS, 10 mM EDTA) was inserted into the sinus near to the posterior adductor muscle and used to extract the haemolymph at an approximate final dilution 1: 1 PBS: haemolymph. About 4–5 droplets of the dilution were added to a cover a glass slide and left to settle for 15 min in a humid chamber at room temperature. The haemocytes were then fixed with 1% glutaraldehyde for 5 min, rinsed with PBS, dried for 2 h, and stored until further processing. Samples were stained with 1 μg ml^{-1} bisbenzimide (33,258 Hoechst) for 5 min in the dark, rinsed with distilled water, mounted in glycerol-McIlvaine buffer (1:1) and kept in the dark until further image analysis with a fluorescent microscope (excitation filter 355 nm and barrier filter 465 nm). For each treatment group, 1000 cells with intact cytoplasm were scored. Micronuclei frequency was expressed as the number of micronuclei per 1000 cells scored (Barsiene et al., 2006). Histological analysis provides a measure of the structural and morphological changes in target tissues following chemical exposure and is well-established in biological effects studies (Au, 2004). To perform such an analysis, the whole mussels were immediately fixed in 4% paraformaldehyde for 48 h (6 mussels per treatment from day 0, 5, 9 and 21). Following fixation, the samples were rinsed with running water, and dehydrated with increasing concentrations of ethanol. Prior to paraffin embedding, individuals were longitudinally cut in half with a scalpel and the byssus threads and foot were removed since they could interfere with the process of sectioning, before each half was individually embedded in paraffin. Samples were finally cut at 5–7 μm thick (RMC microtome MR2, USA) and stained with haematoxylin-eosin. Condition index was calculated using following formula: (wet meat weight/total weight) × 100 (Freeman, 1974).

2.7. Statistical analysis

All the statistical analyses were performed by using GraphPad Prism 5 (GraphPad Software, Inc., USA). All data were checked for normality using D'Agostino-Pearson omnibus test and "F-test to compare variance" to check for homogeneous variance. Non-parametric tests (Mann–Whitney) or the non-parametric one-way ANOVA (Kruskal-Wallis) and otherwise one-way parametric ANOVA were performed. For all the tests, a level of significance of $p < .05$ was used. All results are given "mean ± S.D", if nothing else is specified.

3. Results and discussion

3.1. Particle characterisation

Since there are currently no automated processes to count irregular microplastics varying in size and shape, particle characterisation was performed manually. This is a drawback of using microplastics isolated from consumer producers in exposure studies. The blue PE particles (Fig. 1 A and B) were fragments with a size range of 50–590 μm (Fig. 2) with an uneven surface, as visualised by scanning electron microscopy (Supplementary S1). The virgin PE particles had an average size of 247.7 ± 95.1 μm, while the weathered PE particles had an average size of 241.2 ± 103.7 μm. No significant difference in size between the two PE particle types (Mann-Whitney p = .067) was found.

For PE particle dosing, the same weight per volume was added to both exposure studies (0.01 mg ml^{-1}). However as seen in Fig. 3, there were significantly more PE-W particles per weight than for PE-V (p = .0057; Mann-Whitney). Since particle dosing was done based on weight as most mussel exposure studies are conducted, it gave an unexpected difference in number of particles added to each of the two exposure systems, with on average 57% more PE-W being added (mean 1.86 particles ml^{-1}) than PE-V particles (mean 1.18 particles ml^{-1}).

As far as we know, the highest reported field concentrations of microplastics in seawater, reported as particles per m^3, are from the North Pacific corresponding to a maximum concentration of 0.0092 particles ml^{-1} (Desforges et al., 2015). Despite the exposure concentration in this current study being relatively low compared to most blue mussel exposure studies, with exception of Paul-Pont et al., 2016, it is important to note that the exposure concentration is still >100 times higher than natural waters from the North Pacific. In other marine species, such as oysters (Sussarellu et al., 2015) and marine phytoplankton (Long et al., 2017), environmental relevant concentrations of microplastics have been used; 2.3×10^{-5} mg ml^{-1} and 3.96×10^{-6} mg ml^{-1} of PS, respectively. However, there are uncertainties within the microplastics field regarding "true" environmental concentration. For example, the limit of detection in microplastic studies is typically circa 150 μm, so for smaller microplastics there may be an underestimation of the environmental load. Recently, there has been indications that a reduction in the limit of detection results in more microplastics being detected in the environment (Maes et al., 2017). A recent study also found that only 11% of the identified microplastics in mussels were >100 μm (Phuong et al., 2016).

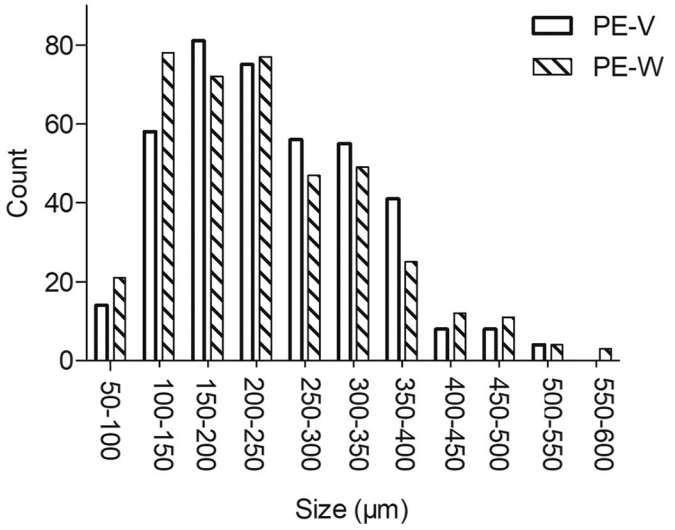

Fig. 2. Size distribution of PE-V and PE-W particles. Number of particles measured equals 400 for both PE-V and PE-W.

3.2. Ingestion of PE particles

This current study illustrates that *M. galloprovincialis* can ingest primary polyethylene particles from toothpaste (Fig. 4) with a size range between 62 and 383 µm. To our knowledge, this is the first-time PE particles from consumer products have been shown to be ingested by any organism.

Interestingly, *M. galloprovincialis* ingested significantly more PE-W than PE-V particles per weight PE added (Fig. 5A and B). However, when standardising ingestion based on particle concentration (Fig. 5C and D), by reducing the ingested particles found in PE-W mussels with 57%, no significant difference was found.

When comparing the "visual gut particle counting method" with the digestion of the whole mussels using 10% KOH, more particles were found using the KOH-method. This is probably due to the presence of microplastic in organs other than the gut and intestine of the mussels. A recent publication by Kolandhasamy et al. (2018), using the same KOH method as in this current study, found the highest proportions of microplastics in the stomach and intestine, but in addition they also found microplastics adherence in gills, gonad, mantle, adductor muscle, visceral and foot. These findings illustrate that KOH-digestion of either the whole mussel or individual parts of the mussel is a better approach than visually going through the stomach and intestine.

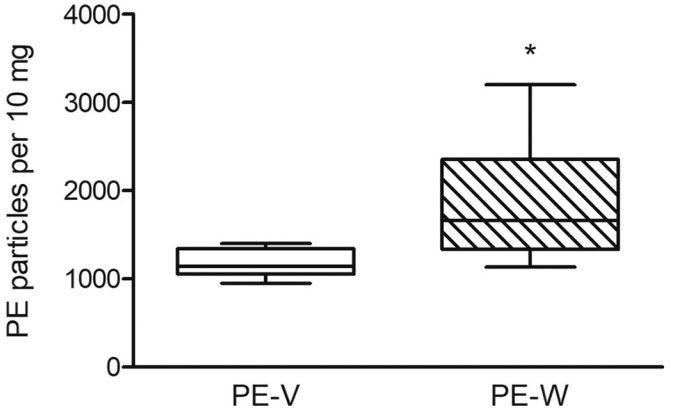

Fig. 3. Number of PE particles per 10 mg PE. PE-V (virgin polyethylene; n = 9) and PE-W (weathered polyethylene; n = 10). * indicates a significant difference (p = .0057, Mann-Whitney test).

It was evident from a visual assessment that the weathered PE particles were better mixed with seawater than the virgin PE particles.

The increased ingestion of PE-W contra PE-V particles could be related to several factors, or a combination; density, hydrophilicity and early biofilm formation. Since clean PE has a density between 0.915 and 0.965 (g/cm^3), depending on whether it is low or high-density PE (Klyosov, 2007), PE is positively buoyant in seawater. Biofilm formation on PE polymers has been found after one (Lobelle and Cunliffe, 2011) and two weeks in seawater (Eich et al., 2015) and this can affect both density and hydrophilicity. When studying field samples, ter Halle et al. (2017) found that weathered PE was chemically degraded compared to raw PE; for example as shown by an increase in the carbonyl index, also referred to as ageing index, which is considered to be a measure of degree of oxidation of polymers (Guadagnoa et al., 2001). The carbonyl index was measured in the 1780–1600 cm^{-1} region which are from ketone, carboxylic acid and ester functional groups. Additionally, another study found that weathering made the particles significantly more hydrophilic after 2 weeks (Lobelle and Cunliffe, 2011), measured by investigating the behaviour of sterile seawater on the surface of the polymer (Bodour and Miller-Maier, 1998). Weathering of PE in seawater can result in oxidation at the PE surface (Gewert et al., 2015), which again can eventually result in the incorporation of polar moieties (i.e. carboxylic acids, alcohols, aldehydes, and ketones) (Bodour and Miller-Maier, 1998; Ruvolo-Filho and Marconcini, 2007; Holmes-Farley et al., 1985) into the polymer surface that would result in a net increase in hydrophilicity (Inagaki et al., 1990). FT-IR analysis of the PE-W particles showed a clear peak (absorption band) at 1632 cm^{-1} which was not seen on the PE-V particles (Supplementary S2). This is most probably a signal from protein attachment, as the same signal has been reported on PE by Bonhomme et al. (2003) following biofilm attachment. The absorption band at 1632 cm^{-1} has also been ascribed to poly-β–L-Ala (Barth, 2007) which is an amino acid. The region of 1600–1800 cm^{-1} region in IR spectra can also stem from C=O formation which can be a result of photo-oxidation (Gardette et al., 2013). Despite the FT-IR spectra indicating the presence of a biofilm on the weathered PE, no visible biofilm formation was observed by SEM imaging. Other more sensitive methods such as DNA analysis or the analysis of nitrogen as a proxy for biomass, a method used by Morét-Ferguson et al. (2010), could be a more suitable method for detecting early biofilm formation.

If any early biofilm formation on PE-W is the cause of the increased ingestion based on exposure mass, it could be due to increased nutritional value of the weathered PE particles, since it is known that molluscs do not only sort particles based on size, but also on the quality of the organic matter (Tammes and Dral, 1956). Size independent selection of natural particles has been found in another marine mussel, the *Lithophaga simplex*. Their selection of particles was suggested to be based on the nutritional quality of the particles (Yahel et al., 2009). Despite being found earlier and in this study, that fouling increases the ingestion of microplastics for different marine organisms (Rassoulzadegan et al., 1984; Vroom et al., 2017), this appears not always to be the case. Kaposi et al. (2014) found that the sea urchin larvae *Tripneustes gratilla* did not ingest PE beads that had been "weathered" in the laboratory, but they did ingest virgin microplastics. The authors suggest that the reason for this was due to the increased size of the PE particles; that the weathered particles became too big for the larvae to ingest. This illustrates that by weathering microplastics prior to exposure studies, one avoids both any under- and overestimation of microplastic ingestion.

For this study, it is also an important finding that the increased ingestion was not significantly different when taking into consideration the "extra" particles being added to the PE-W exposure. Therefore, to give only weight per volume when doing exposure studies is not sufficient. If it is the total load of plastics based on weight or based on particle number that is the most environmentally relevant measure, is not sure. This difference also illustrates that it is not sufficient to characterise

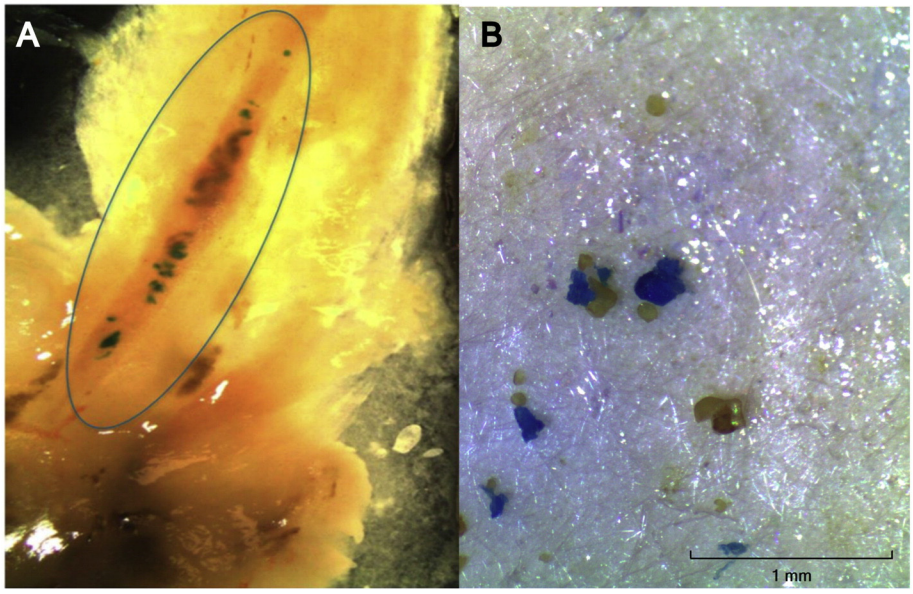

Fig. 4. A: Stereomicroscope picture of polyethylene particles from toothpaste inside the gut of a blue mussel B: Examples of Stereomicroscope picture of polyethylene particles on GF/D filter after KOH-digestion of whole mussel with scale bar.

microplastic particles only prior to "weathering", it must be conducted also for microplastics post-weathering.

3.3. Effects of PE ingestion

Ingestion of both virgin and weathered PE caused tissue alteration in *M. galloprovincialis* (Fig. 6). For the gills, control mussels exhibit properly developed frontal and lateral cilia and strong contacts between adjacent gill lamellae, while for exposed mussels it was observed a general decrease, and in some cases a disappearance in the number of contacts between adjacent filaments. Several other histopathological features were observed for the gills in treated mussels such as thickening and disorganisation of the epithelium and infiltration of haemocytes within intermediate and frontal epithelia. The digestive gland of control

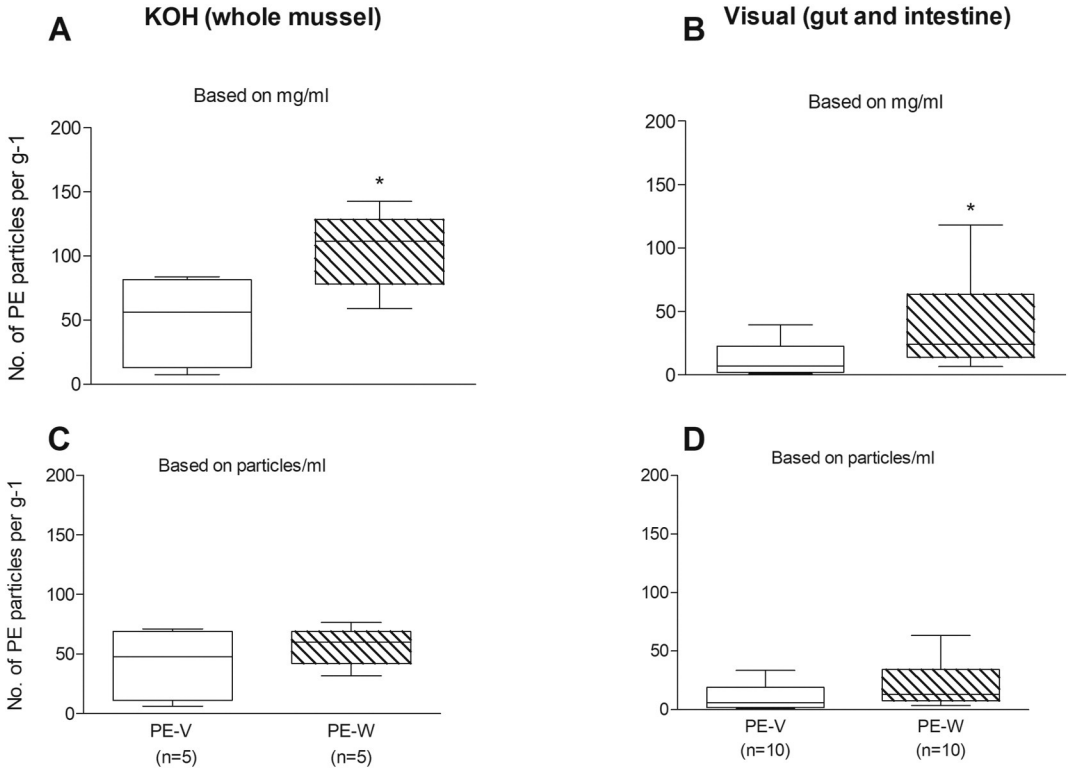

Fig. 5. Number of PE particles in blue mussels per gram mussel tissue (w.w) exposed to PE-V (white box-plot) and PE-W (dashed box-plot). n = number of mussel individual analysed A: PE particles identified using KOH treatment on whole mussels standardised towards mass exposure (mg PE ml^{-1}). * indicated a significance level of p = .0317 (Mann–Whitney). B: PE particles identified visually in gut and intestine standardised towards mass exposure (mg PE ml^{-1}). * indicated a significance level of p = .047 (Mann–Whitney). C: PE particles identified using KOH treatment on whole mussels standardised towards particle exposure (PE particles ml^{-1}) D: PE particles identified visually in gut and intestine standardised towards particle exposure (PE particles ml^{-1}).

mussels had ciliated epithelial cells, while for exposed mussels the digestive cells had a squamous shape and a clear decrease in the amount of cilia. In addition, haemocytic aggregates were present in exposed animals.

These histopathological changes although not being quantitative, do illustrate that marine mussels are impacted by microplastics at the current exposure levels, which are between realistic and high exposure concentration. These alterations are in accordance with other microplastic exposure studies finding tissue alterations in both *M. Edulis* (von Moos et al., 2012) and *Mytilus* spp. (a "species complex" of *M. edulis* and *M. galloprovincialis*; Paul-Pont et al., 2016). von Moos et al., 2012 also found haemocytic aggregates, also called granulocytoma formation, in the digestive gland of the exposed mussels that was strongly linked to a reduction in lysosomal membrane stability (LMS). LMS is commonly used as a stress response biomarker in mussels (Moore, 1985). Haemocytic aggregates have also been found in mussels following exposure to pyrogenic PAH (Aarab et al., 2011). Paul-Pont et al., 2016 found hemocytic infiltration in the stomach and digestive gland of exposed mussels, but this impact was higher for the co-exposure of fluoranthene and PS, than for PS exposure alone.

When mussels are feeding they transport particles through the inter-filamentary canals of the gills to the mouth and down the oesophagus (Riisgård et al., 2011), making the gills the most likely uptake route for microplastic particles. Since no significant effect was found on the condition index (control: 15.63 ± 3.00 PE-V: 15.35 ± 1.85, PE-W: 15.89 ± 2.64; time 21; Two-way ANOVA: p-value. 8755), it is unclear if these structural changes in the gills did affect their feeding capacity.

It is also unclear if these structural effects on the gills are due to particle exposure in general, or if it is due to PE particles in particular. The optimal test would be to use a reference particle in the same size range as the plastic particles. For example, sand could be used as a reference material to see if this induced the same structural changes in the gills of the mussels. Mussels are constantly exposed to natural particles in the marine environment, so it is important to asses if microplastic particles are causing more structural changes than natural particles alone.

The digestive gland is the main organ for xenobiotic biotransformation (Livingstone et al., 1992) and it has therefore been extensively used for toxicity assessment. In our study, it was observed that the digestive gland in the exposed specimens had decreased intestinal content with a reduced accumulation of debris. In addition, there were changes in the morphology of the epithelial cells from the digestive tubules, that tended to become thinner. Haemocytes are the most important internal protection system in bivalves, and they are also a part of the digestive system, by ingesting particles of nutritional value that are too big to enter the cells (Gosling, 2015). In addition, it is worth mentioning the presence of extensive areas of haemocytic aggregates in several tissues such as the mantle and the gonads leading in some cases to severe tissue necrosis (results not shown).

Despite the histological alterations observed in this current study, no significant effects were seen for either PE-V or PE-W particles on condition index or micronuclei (MN) formation (data not shown) for any of the time points (Kruskal Wallis, $p > .05$). Condition index provides a measure of the nutritional status and the general health of the mussel,

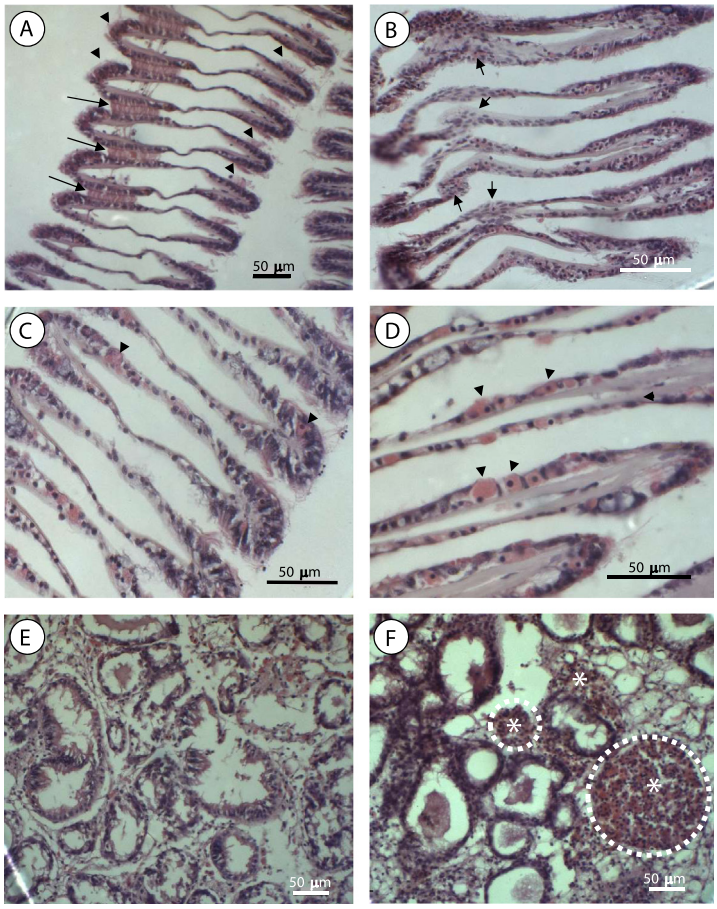

Fig. 6. Photomicrographs of haematoxilyn-eosin stained sections of gills and digestive glands from control (A, E) and microplastic-exposed specimens (B, C, D, F). Control mussels (A) exhibit properly developed gills with frontal and lateral cilia (arrowheads) and strong contacts between adjacent gill lamellae (arrows). Treated mussels (B–D) show thickening and disorganisation of the gill epithelium (arrows) and infiltration of haemocytes within the epithelial cells (arrowheads). Note the absence of contacts between adjacent lamellae and the hypoplasia of frontal and lateral cilia. (E) Control mussel showing the typical columnar ciliated epithelial cells in the digestive tubules. In exposed animals (F) the digestive cells show a squamous shape, a decrease in the amount of cilia and the presence of haemocytic aggregates (encircled and asterisk).

and is frequently used in pollutant monitoring studies (Brooks et al., 2015; Barsiene et al., 2006; Crosby and Gale, 1990). The absence of any effect on the condition index is in line with similar studies where bivalves have been exposed to microplastics, for example, M. *edulis* exposed to PE fluff (von Moos et al., 2012) and *C. gigas* exposed to PS (Sussarellu et al., 2015). For MN all groups were below 3.9 MN per 1000 cells set as background assessment criteria (BAC) by ICES (Davies and Vethaak, 2012). Any genotoxic effect from the weathered particles on the mussels would be expected to come from chemicals associated with the plastic particles. Since no increase in MN formation was observed for any of the particles, it could be assumed that the chemicals, if present, were not at a concentration sufficient to illicit a response.

The tissue alterations observed in our study shows the potential for adverse effects on mussels when they ingest primary microplastics particles. However, we find it important to note that since the ecotoxicological evaluation of microplastics is relatively new, there is at present no official guidelines on how to perform a well-executed exposure study for regulatory purposes.

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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.2018.01.141.

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