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The sub-lethal impact of plastic and tire rubber leachates on the Mediterranean mussel *Mytilus galloprovincialis*☆

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ABSTRACT

Ocean contamination by synthetic polymers can represent a risk for the fitness of marine species due to the leaching of chemical additives. This study evaluated the sub-lethal effects of plastic and rubber leachates on the mussel *Mytilus galloprovincialis* through a battery of biomarkers encompassing lysosomal endpoints, oxidative stress/detoxification parameters, and specific responses to metals/neurotoxicants. Mussels were exposed for 7 days to leachates from car tire rubber (CTR), polypropylene (PP), polyethylene terephthalate (PET), polystyrene (PS) and polyvinyl chloride (PVC), containing organic additives and metals in the ng-μg/L range. The leachate exposure affected general stress parameters, including the neutral lipid content (all leachates), the lysosomal membrane stability (PS, PP, PVC and CTR leachates) and lysosomal volume (PP, PVC and TR leachates). An increased content of the lipid peroxidation products malondialdehyde and lipofuscin was observed in mussels exposed to PET, PS and PP leachates, and PP, PVC and CTR leachates, respectively. PET and PP leachates increased the activity of the phase-II metabolism enzyme glutathione S-transferase, while a decreased acetylcholinesterase activity was induced by PVC leachates. Data were integrated in the mussel expert system (MES), which categorizes the organisms' health status based on biomarker responses. The MES assigned healthy status to mussels exposed to PET leachates, low stress to PS leachates, and moderate stress to PP, CTR and PVC leachates. This study shows that additives leached from selected plastic/rubber polymers cause sub-lethal effects in mussels and that the magnitude of these effects may be higher for CTR, PVC and PP due to a higher content and release of metals and organic compounds.

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1. Introduction

The trend of global plastic production follows a clear exponential increase over time. Approximately 400 million tonnes of plastic were produced in 2019 (PlasticsEurope, 2020) and production levels are forecast to reach ~1800 million tonnes by 2050 (UNEP, 2016). Marine habitats are exposed to massive pressure by plastic materials, including discarded or entangled fishing nets, plastic bottles, lids, straws and bags, among others (GESAMP, 2016). Marine plastics are classified as macroplastics (>20 mm diameter), mesoplastics (5–20 mm), microplastics (MPs, 0.1 μm-5 mm) and nanoplastics (NPs, 1–100 nm) (Andrady, 2017; European Commission, 2019). Around 5 trillion MPs are thought to currently float in oceans, although their real abundance is likely underestimated (Eriksen et al., 2014; Lindeque et al., 2020). Other polymer-based materials also contribute to the presence of MPs in marine systems. Car tires, for instance, slowly degrade into micro-sized rubber fragments during use, flowing into estuarine/coastal waters at an estimated rate of 0.5 million tons per year ((Hann et al., 2018)Tian et al., 2021). MPs and NPs fall within the optimal prey size range for a variety of aquatic animals and are therefore available for ingestion (Capolupo et al., 2018; Wang et al., 2021).

Between 10 and 70% of a plastic items' mass is represented by chemical additives incorporated during production processes (Andrady and Rajapakse, 2019). These additives are used to impart desired properties to the final products and include fillers, antimicrobials, stabilizers, flame retardants, vulcanizers, pigments and metals, among others (Gunaalan et al., 2020; Hahladakis et al., 2018). Most additives are not covalently bound to the plastic polymer and so they can migrate to the material surface, potentially being released into the environment (Gewert et al., 2015; Paluselli et al., 2019; Schmidt et al., 2019). Additives have been found in effluents from waste water treatment plants, surface and marine waters (Hermabessiere et al., 2017; Schmidt et al., 2019). Bisphenol A (BPA) and phthalates, listed as potential endocrine disrupting chemicals due to their ability to impair hormone regulation in wildlife and humans (ECHA, 2018; UNEP, 2017), represent examples of widely investigated plastic additives (Balbi et al., 2016; Canesi and Fabbri, 2015). Less studied, though still widely used additives include ketones, such as acetophenone, which acts as co-polymerization catalyst and is described as a contaminant of surface waters with potential toxicity to fish (Api et al., 2018; Kolpin et al., 2002). Benzothiazoles, used as vulcanizing

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agent in tire rubber manufacture, are suggested as a marker of road run-off (Halle et al., 2020) and have been identified as toxic to aquatic species (He et al., 2011). Most of the cited studies addressed the environmental occurrence or effects of plastic/tire additives as individual compounds, while leachates comprising additive mixtures released by synthetic based materials into aqueous media have attracted attention only recently.

Leachates affect marine flora and fauna at different levels of biological complexity (reviewed by Gunaalan et al., 2020). The mixture of additives leached by crumb rubber granulate strongly affected the 48h survival of the marine copepods *Acartia* and *Calanus* sp. (Halsband et al., 2020). Growth and transcriptional changes were observed in the marine cyanobacteria *Prochlorococcus* exposed to leachates from high density polyethylene (HDPE) and polyvinylchloride (PVC) (Tetu et al., 2019). Leachates from PVC also caused high toxicity towards sea urchins (*Paracentrotus lividus*) larval growth and development (Oliviero et al., 2019), while polyethylene (PE) leachates impaired the ontogeny of the clam Meretrix meretrix (Ke et al., 2019). In our recent investigation, we found leachates from different plastics and car tire rubber (CTR) to contain a variety of metals and organic additives that cumulatively affected gamete fertilization, embryonic development, larvae motility and survival in the Mediterranean mussel *Mytilus galloprovincialis* (Capolupo et al., 2020). Increased byssal thread production was reported in the same species after exposure to PE leachates (Seuront et al., 2021). *Mytilus* sp. is widely used in aquatic toxicology because of its filter-feeding habits, wide geographical distribution, ease of acclimatization and rearing in laboratory condition and known responsiveness to anthropogenic stressors. It has been suggested as a global bioindicator of coastal microplastic pollution (Li et al., 2019) and is extensively used as model species for biomarker-based investigations (Viarengo et al., 2007). Biomarkers are "early-warning" signals of biological effects induced by toxicants at lower levels of biological organization (i.e. biochemical, cellular or physiological levels). Their use is currently recommended in risk assessments to characterize the sub-lethal alterations and the toxicological pathways at the basis of major environmental impacts in aquatic systems (ECHA, 2013; Khan et al., 2020; (OSPAR, 2014)).

To the best of our knowledge, only a few reports are available on the effects of plastic/rubber leachates with a well-characterized chemical composition, thus limiting the current knowledge on hazardous additives in aquatic environment. Furthermore, only apical endpoints were examined. To address these gaps, the present study aimed to evaluate the sub-lethal impact of leachates from CTR, polypropylene (PP), polyethylene terephthalate (PET), polystyrene (PS) and PVC microplastics on adult *M. galloprovincialis* through a multi-biomarker approach. The organic chemical and metal content of the leachates were previously identified by a combination of non-target and targeted chemical analysis (Capolupo et al., 2020). Selected biological endpoints encompass a battery of biomarkers of environmental stress validated in mussels (*Mytilus* sp.) sub-chronically exposed to anthropogenic stressors, including MP (Avio et al., 2015; Brandts et al., 2018; Cole et al., 2020), and included parameters of lysosomal functionality, oxidative stress, neurotransmission impairment and metal exposure.

2. Materials and methods

2.1. Leachate preparation

The preparation of the plastic leachates used in this study and the analysis of their chemical composition has been performed previously and details are provided in Capolupo et al. (2020). Briefly, leachates from PET, PS, PP, PVC and CTR were generated in seawater collected at 80 m depth in Trondheimsfjorden (Trondheim, Norway) from a location generally known to be quite pristine; samples (80 g plastics/L) were shaken in a rotational incubator (125 rpm) at 25 ± 2 °C for 14 d in the dark to avoid degradation of the plastic or plastic-associated chemicals through light exposure. A control comprising pure seawater without plastic was treated in parallel to the leachates to account for background levels of additive and other chemicals. Each sample, including the control, was subsequently filtered using a 0.2 μm Nalgene® membrane and

analysed for metals and organics content (Capolupo et al., 2020). ICP-MS and GC-MS data from Capolupo et al. (2020) with relevance for this study are reported in SM (Table S1 and S2). After preparation, leachates were stored at −20 °C and used within 6 months. At each use, aliquots of stored leachates were slowly defrosted at 4 °C in the dark and used within 24 h.

2.2. Mussel acclimation and experimental treatments

Adult specimens of the Mediterranean mussel *M. galloprovincialis* (5–6 cm length) were harvested at a farm located in a class "A" zone (854/2004/EC) off the NW Adriatic coast (COPRALMO aquaculture, Cesenatico, Italy) during the summer period. This period corresponds to the "sexual rest stage" and ensures a lack of inherent differences in the physiological status of males and females due to gametogenesis (Da Ros et al., 1985; Jarque et al., 2014). Collected organisms were rapidly moved to the laboratory and acclimated for 5 d at controlled physicochemical conditions in line with previous studies (Chalkiadaki et al., 2014; Nogueira et al., 2017). During this period mussels were maintained at 16 °C in multi-mesh filtered seawater (FSW) collected from the same area as the mussels to minimize the presence of organic matter and facilitate the organisms' acclimation. Mussels were then subdivided into groups of ten individuals and placed into vessels containing 10 L FSW, i.e. at a ratio of 1 mussel/L in line with basic requirements for experimental applications involving the use of *Mytilus* sp. (Canesi et al., 2007; Gonzalez-Rey et al., 2014; González-Soto et al., 2019). The exposure vessels were then spiked with a 1:1000 dilution of each of the five leachates. This dilution was chosen based on the chemical composition of leachates (Supplementary material, Tables S1 and S2) in order to expose mussels to concentrations of additives that are comparable to those commonly measured in marine environments (i.e. ng to μg/L levels) (Hermabessiere et al., 2017; Schmidt et al., 2019, 2020). To debate the effect of external variables on the selected endpoints, a control condition containing only FSW was run alongside the leachate treatments under identical conditions in terms of temperature (16 °C), photoperiod (12h:12h, light dark), constant aeration (>90% oxygen saturation), and feeding (Coral Diet Filtrator, Xaqua, Italy, according to the product specification). The exposure period was set at 7 days, which is acknowledged as the time necessary for *Mytilus* sp. to modulate cellular, biochemical or physiological parameters in response to external agents (Avio et al., 2015; Cole et al., 2020; Gonzalez-Soto et al., 2019). Leachate administration was renewed daily until the end of the experiment by adding 10 mL of the leachate stock solution (or FSW for control) to 10 L FSW, along with mussels feeding and water renewal. All conditions (including control) were carried out in triplicate (N = 3) for a total of 18 tanks and 180 animals employed.

After seven days of exposure, mussel tissues were dissected and immediately analysed for selected toxicological endpoints (haemolymph) or stored at −80 °C for subsequent analyses (gills, digestive gland). To reduce the likelihood of biases due to inter-individual variability, data from each replicate (vessel) were expressed as the mean (±SEM) level of endpoints from four to six randomly selected mussels, as described in detail in the below sections.

2.3. Biomarker analysis

2.3.1. Lysosomal membrane stability (LMS)

The lysosomal membrane stability (LMS) was assessed in mussel haemocytes, which are haemolymph cells modulating key functions in mussels, including tissue/shell formation, maintenance of homeostasis and immune responses (Auguste et al., 2021; Munari et al., 2020). The haemolymph was drawn from four mussel per replicate (vessel) and tested for LMS using the Neutral Red Retention Assay (NRRA), as described by Martínez-Gómez et al. (2015). The method is based on exposing haemocytes to a solution containing 0.01% Neutral Red (NR) acidophile dye (which migrates into lysosomes) and on the assessment of the Neutral Red Retention Time (NRRT), which is the time (min) where more than 50% of the lysosomes released the dye into the cytosol.

2.3.2. Cytochemical parameters

Lipofuscin (LF) and neutral lipid (NL) accumulation and the lysosome to cytoplasm volume ratio (LYS/CYT) were histologically assessed on cryo-sections of mussel digestive gland, which is a known target of many classes of organic and inorganic toxicants (Capolupo et al., 2017; Orbea et al., 2006). Glands of four mussels per replicate (vessel) were dissected, placed on aluminium supports for cryotomy (chuck) and rapidly frozen according to the UNEP/RAMOGE protocol (1999). All parameters were subsequently assessed according to Capolupo et al. (2016). Ten-μm sections were obtained using a cryotome and rapidly placed onto glass slides at −30 °C. LF was selectively stained by exposing the slides to Schmorl ferric-ferricyanide solution (1% $FeCl_3$ and 1% $Fe[KCN]_6$ at a 3 to 1 ratio) for 15 min. A solution containing 1% Oil Red O stain in 60% triethylphosphate was used to assess the NL content. For LYS/CYT assessment, lysosomes were highlighted by inducing the reaction between the lysosomal hydrolase N-acetyl-β-hexosaminidase and its specific substrate naphthol AS-BI N-acetyl-β-D-glucosaminide in digestive cells. To do so, gland sections were exposed to a polypep/naphthol solution for 20 min at 37 °C and to the diazonium dye Fast Violet B for 10 min in the dark at 25 °C. LF, NL and LYS/CYT were assessed using light microscopy at 40× magnification and five photos per each of the four glands on section were taken using a digital camera (AxioCam MRc, Carl Zeiss, Milan, Italy). Images were then analysed using the software Scion Image ver. 4.0.2.

2.3.3. Enzymatic and biochemical biomarkers

The activities of the enzymes glutathione S-transferase (GST), catalase (CAT) and acetylcholinesterase (AChE), and the content of malondialdehyde (MDA) and metallothionein (MT) were assessed on digestive gland and/or gills pooled from six mussels per each of the three replicates (vessel), for a total of eighteen organisms per experimental condition. Gills and digestive gland are the tissue mainly involved in pollutant uptake and metabolism, respectively, and were selectively screened for these endpoints based on previous toxicological evidence obtained in *Mytilus* sp. (reviewed by Viarengo et al., 2007). All parameters were assessed spectrophotometrically in samples obtained following tissue homogenisation and centrifugation performed at specific conditions (details reported in Table S3). GST and CAT were measured in both digestive glands and gills according to Capolupo et al. (2017). The GST activity was evaluated at 340 nm by following the reaction kinetics between the enzyme and the substrate 1-chloro-2,4-dinitrobenzene (CDNB) for 10 min (at 1 min intervals). Similarly, CAT activity was measured by tracking the decrease in the absorbance of samples at 240 nm for 2 min (at 10 s intervals) in the presence of 55 mM H_2O_2. The MDA content was assessed in mussel digestive glands according to Banni et al. (2007) by taking a single reading at 570 nm and quantifying by interpolating the data on a standard curve obtained using serial dilutions of the MDA precursor 1,1,3,3-Tetramethoxypropan (TMOP). AChE activity was determined in gills at 405 nm as described by Valbonesi et al. (2003). Samples were read at 405 nm for 10 min in the presence of 0.5 mM acetylthiocholine iodide and 0.33 mM 5.5′-dithiobis-2-nitrobenzoic acid (DTNB). The MT content was assessed in mussel digestive glands using the protocol described by Viarengo et al. (1997). Aliquots of homogenised samples were subjected to ethanol/chloroform precipitation to obtain a partially purified metallothionein fraction, which was read after interaction with DTNB at 410 nm.

2.4. Statistical analysis and data integration

Data were analysed using the statistical software packages 'R' and SigmaPlot 13 (Systat Software Inc. San Jose, CA, USA). After testing data for normal distribution and homoscedasticity using the Shapiro-wilk and Levene's tests, respectively, significant differences between each treatment group and control mussels were determined using one-way ANOVA followed by the Dunnett's post-hoc test. Pairwise correlations among the battery of biomarkers were analysed by using the Pearson's test. For all tests, the statistical significance was set at $p < 0.05$. Multivariate principal component analysis (PCA) was per-

formed to investigate the potential relationships among tested endpoints and leachates. Prior to performing the PCA, data from all measured biomarkers were expressed as percentage of variation compared to controls to debate the possible variability due to different basal levels, scale of observation and toxicological profile. Biomarker data were integrated using the Mussel Expert System (Dagnino et al., 2007), which assigns a unique Health Status Index (HSI, in a scale from A to E) to performed treatments based on biomarker modulation(s) and toxicological profiles (more details are reported in section S1).

3. Results

3.1. Effects of plastic leachates on lysosomal biomarkers

The effects induced by tested additive leachates on the lysosomal membrane stability (LMS), unsatured neutral lipid content (NL) and lysosome to cytoplasm volume ratio (LYS/CYT) measured in *M. galloprovincialis* are shown in Fig. 1. The LMS measured in control mussels' haemocytes was 110 ± 10.4 min, i.e. within NRRT thresholds for good health status reported in the UNEP/RAMOGE manual (1999) (~90 min) (Fig. 1A). Average NRRT
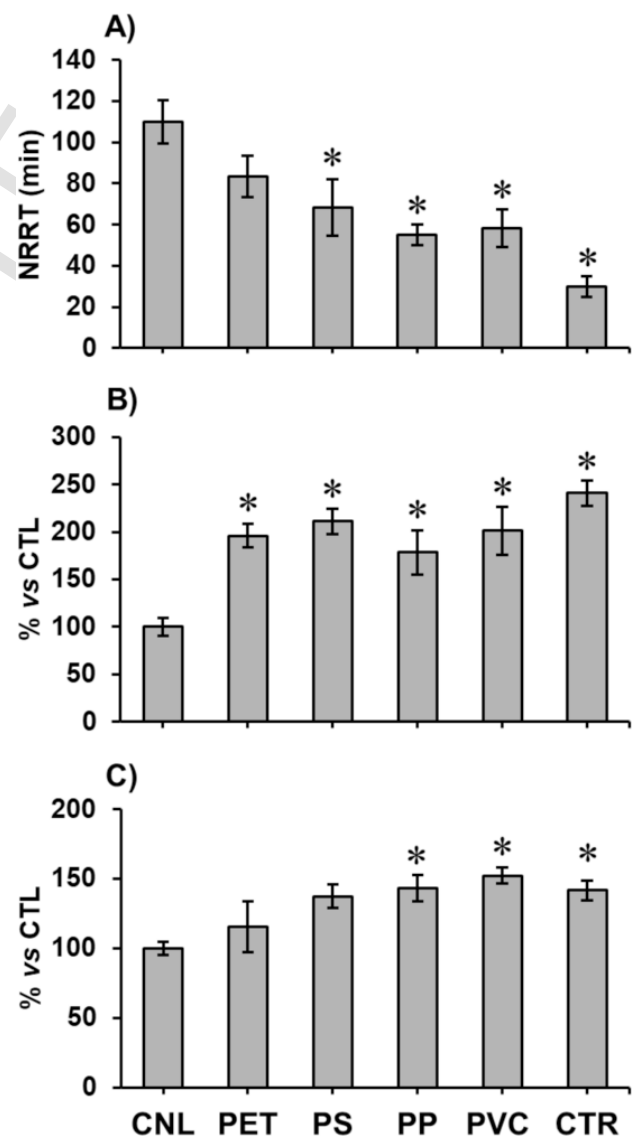
Fig. 1. Lysosomal biomarkers of general stress. Parameters measured in mussel lysosomes after 7 days of exposure to plastic leachates. A, lysosomal membrane stability (LMS); B, Unsatured neutral lipid content (NL); C, Lysosome to cytoplasm volume ratio (LYS/CYT). Data represent the mean ± SEM (n = 3). Asterisks indicate significant differences with respect to the control (CNL) ($p < 0.05$, One-way ANOVA, Dunnett's *post-hoc* comparison).

levels measured following exposure to the leachates were ranked as CTR < PVC < PP < PS < PET. A significant LMS reduction *versus* control was observed in the haemocytes of mussels exposed to PS ($p = 0.034$), PP ($p = 0.006$), PVC ($p = 0.009$) and CTR ($p < 0.001$), while no statistically significant difference was observed in mussels exposed to the PET leachate (Fig. 1A). All leachates induced a significant NL accumulation in mussel digestive cell lysosomes, with increases compared to control ranging between 1.78-fold and 2.41-fold for PP ($p = 0.030$) and CTR leachates ($p < 0.001$), respectively (Fig. 1B). A significant LYS/CYT increase compared to control samples was also observed in the digestive gland of mussels exposed to PP (1.43-fold increase, $p = 0.036$), PVC (1.52-fold increase, $p = 0.012$) and CTR leachates (1.42-fold increase, $p = 0.045$) (Fig. 1C). On the other hand, no statistically significant change in LYS/CYT levels was observed in the digestive gland of mussels exposed to the PS and PET leachates (Fig. 1C).

3.2. Effects of plastic leachates on oxidative stress/detoxification biomarkers

A significant LF accumulation compared to control samples was identified in the digestive gland of mussels exposed to the PP (2.01-fold increase, $p = 0.029$), PVC (2.13-fold increase, $p = 0.015$) and CTR (3.5-fold increase, $p < 0.001$) leachates (Fig. 2A). No significant change in LF levels were observed in mussels exposed to the PS and PET leachates (Fig. 2A). MDA content was significantly higher compared to the control in mussels exposed to the PET (2.04-fold increase, $p = 0.003$), PS (1.86-fold increase, $p = 0.007$) and PP leachates (1.94-fold increase, $p = 0.013$), while no significant MDA change was noted following exposure to the PVC and CTR leachates (Fig. 2B). GST activity showed remarkably lower basal levels in digestive glands compared to gills (Fig. 2C). In digestive gland, a significant increase in GST activity compared to the control was observed in mussels exposed to the PP leachates (1.2-fold increase, $p = 0.048$), while no significant difference was observed for the PET, PPS, PVC and CTR leachates (Fig. 2C). In gills, a significantly increased GST activity was noted only in mussels exposed to the PP (1.49-fold increase, $p = 0.011$) and PET leachates (1.42-fold increase, $p = 0.030$) (Fig. 2C). No significant change was observed in CAT activity measured in gills and digestive gland of mussels exposed to any of the tested leachates compared to the control (Fig. 2D).
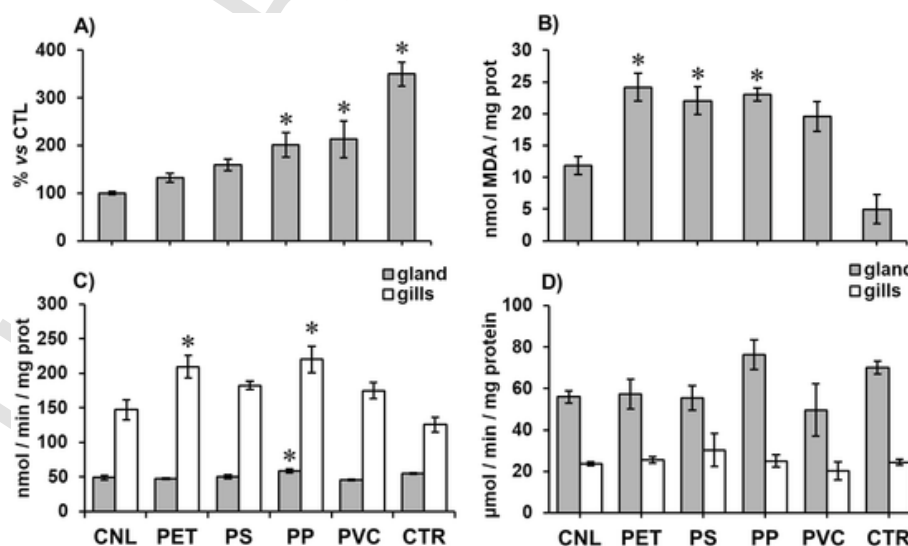
3.3. Effects of plastic leachates on biomarkers of exposure

A significant AChE down-regulation was noted in mussels exposed to PVC leachates (31.93% decrease, $p = 0.015$), while no change was observed in those exposed to the PET, PS, PP and CTR leachates (Fig. 3A). No difference was observed between the average MT content measured in mussels exposed to all tested leachates and those maintained under control conditions (Fig. 3B).

3.4. Multivariate statistics and data integration

To explore the potential relationships between obtained data from different biomarkers, a pairwise correlation analysis was performed by means of the Pearson's coefficient calculation (Table 1). LMS was significantly and inversely correlated with LF, NL and LYS/CYT. In digestive glands, a significant positive correlation was observed between GST and CAT activity. MDA content was significantly and positively correlated with GST activity in gills of exposed mussels (Table 1). The MES output integrating biomarker data in a single Health Status Index (HSI) for each treatment is reported in Table 2. The system assigned a healthy condition (HSI = A) to mussels exposed to control conditions and PET leachates. A low stress condition (HSI = B) was assigned to PS leachates, while moderate stress condition (HSI = C) was identified in mussels exposed to PP, PVC and CTR leachates. LMS was chosen as guide parameter based on observed alterations and on its known representativeness of the mussel health status (Viarengo et al., 2007).

Fig. 4 shows the biplot generated following the principal component analysis (PCA) carried out on the whole set of biomarker data measured following leachate exposure. The two principal components explained 66.4% of the total variance. MDA and GST measured in gills (GSTg) were scored in the PC1 < 0/PC2 > 0 domain; AChE activity, LMS, MT, LYS/CYT and NL were clustered in the PC1 > 0/PC2 > 0 domain, while LF, GST and CAT measured in the digestive gland (GSTdg and CATdg) were scored in the PC1 > 0/PC2 < 0 area. Finally, CAT activity measured in the gill was scored in the PC1 > 0/PC2 < 0 domain. As to treatments, control and PS were scored in the PC1 < 0/PC2 > 0 domain; PP and CTR were scored in the PC1 > 0/PC2 > 0. PVC was under the domain of PC1 > 0/PC2 < 0, while PET fell under the PC1 < 0/PC2 < 0 domain.

Fig. 2. Oxidative stress/detoxification parameters. Effects induced by plastic leachates on A, lipofuscin content (LF); B, Malondialdehyde content (MDA); C, glutathione S-transferase activity (GST) in both gills and digestive gland; D, catalase activity (CAT) in both gills and digestive gland measured in mussels after 7 days of exposure. Data represent the mean ± SEM (n = 3). Asterisks indicate significant differences with respect to the control (CNL) ($p < 0.05$, One-way ANOVA, Dunnett's *post-hoc* comparison).
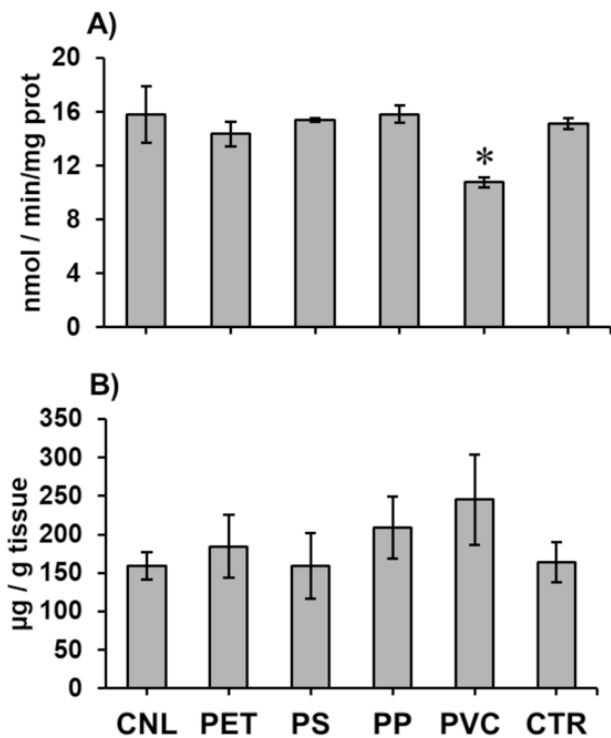
Fig. 3. Biomarkers of exposure to neurotoxicants/metals. Activity of acetyl-cholinesterase (A) and metallothionein conten (B) measured in mussels after 7 days of exposure to plastic leachtaes A, acetylcholinesterase activity (AChE), B, metallothionein content (MT). Data represent the mean ± SEM (n = 3). Asterisks indicate significant differences with respect to the control (CNL) ($p < 0.05$, One-way ANOVA, Dunnett's *post-hoc* comparison).

4. Discussion

The data reported here show that the 7-day exposure to selected thermoplastic and rubber leachates is linked to the onset of sub-lethal alterations in *M. galloprovincialis*, including lysosomal morpho-functional changes, lipid peroxidation, pro-oxidant conditions and neurological disorders. The magnitude of the observed effects tended to change depending on the polymers tested, apparently mirroring the different composition and concentrations of leached additives. Lysosomal endpoints were the most impacted of the tested parameters and were subsequently identified by the MES data integration as a major factor involved in the overall impairment of mussel fitness by leachates.

Evidence of LMS reduction was found in the haemocytes of exposed mussels, with CTR leachates inducing the highest lysosomal membrane destabilization (NRRT = 32.5 ± 9.01 min), followed by PVC, PP and PS exposed to CTR, PVC, PP and PS leachates. LMS in haemocytes is the most representative biomarker of general stress in marine bivalves (Martínez-Gómez et al., 2015; Viarengo et al., 2007). Its reduction reflects an impaired integrity and functionality of the lysosomal compartment and represents a predictive indicator for cell and tissue injuries in *Mytilus* spp. (Moore et al., 2006). The LMS results are in line with our previous findings on mussel haemocytes exposed *in vitro* to increasing concentrations of the same leachates (0.6–100% of total leachate solution) (Capolupo et al., 2020), corroborating the suitability of using cellular bioassays to estimate the *in vivo* response of mussels to chemical mixtures. Chemical data reported by Capolupo et al. (2020) also suggest possible relationships between LMS trends observed in this study and the presence of specific classes of organic and inorganic additives in leachates. All tested leachates, except PET, showed significant enrichment of BPA, which is known for its potential to affect LMS in *M. galloprovincialis* at relatively high concentrations (Canesi et al., 2005). Benzothiazole, showing a 500-fold enrichment in the CTR leachate, was reported to adversely affect the lysosomal functionality and the permeability of the lysosomal membrane (De Wever and Verachtert, 1997). Moreover, Pb, Zn and Cu, which showed relatively high concentrations in CTR, PP and/or PVC leachates, are known to affect LMS in marine mussels, especially when present as a mixture (Giamberini and Pihan,

Table 1
Pearson's correlation analysis among biomarkers measured in mussels (*M. galloprovincialis*) after seven days of exposure to selected plastic leachates. For each pairwise correlation, the corresponding coefficients and *p*-values (in italic) are reported. Statistically significant correlations ($p < 0.05$) are bold.

	LF	NL	LYS/CYT	GSTdg	GSTg	CATdg	CATg	MDA	AChE	MT
LMS	**−0.93**	**−0.858**	**−0.857**	−0.497	0.135	−0.507	0.057	0.244	0.182	−0.248
	0.00723	*0.0288*	*0.0293*	*0.315*	*0.799*	*0.304*	*0.915*	*0.641*	*0.73*	*0.636*
LF		0.734	0.669	0.454	−0.451	0.491	−0.203	−0.574	−0.127	0.0879
		0.0966	*0.147*	*0.366*	*0.369*	*0.323*	*0.7*	*0.234*	*0.811*	*0.869*
NL			0.735	0.171	−0.0199	0.193	0.19	−0.0195	−0.267	0.13
			0.0962	*0.746*	*0.97*	*0.715*	*0.718*	*0.971*	*0.609*	*0.805*
LYS/CYT				0.271	0.113	0.221	−0.126	0.104	−0.465	0.579
				0.604	*0.832*	*0.674*	*0.812*	*0.844*	*0.353*	*0.228*
GSTdg					0.104	**0.97**	0.264	−0.17	0.646	−0.194
					0.844	*0.00132*	*0.614*	*0.748*	*0.166*	*0.713*
GSTg						0.123	0.217	**0.941**	−0.016	0.423
						0.816	*0.679*	*0.00514*	*0.976*	*0.404*
CATdg							0.139	−0.189	0.592	−0.139
							0.792	*0.72*	*0.216*	*0.793*
CATg								0.272	0.642	−0.663
								0.602	*0.169*	*0.151*
MDA									−0.172	0.419
									0.744	*0.409*
AChE										−0.791
										0.0609

LMS, lysosomal membrane stability; LF, lipofuscin content; NL, neutral lipids content; LYS/CYT, lysosome to cytoplasm volume ratio; GSTdg, glutathione S-transferase activity in digestive gland; GSTg, glutathione S-transferase activity in gills; CATdg, Catalase activity in digestive gland; CATg, Catalase activity in gills; MDA, malondialdehyde content. AChE, acetyl-cholinesterase; MT, metallothionein content.

Table 2
Output of the Mussel Expert System (MES) assigning a unique health status Index (HSI) to mussels exposed to selected plastic leachates based on biomarker modulation.

Biomarkers	Toxicological profile		Control	PET	PS	PP	PVC	CTR
Cell level								
LMS [GP]	Decreasing	AF	1.00	0.76	0.62*	0.5*	0.53*	0.27*
		AL	NV	NV	–	–	–	–
LF	Increasing	AF	1.00	1.33	1.59	2.01*	2.13*	3.5*
		AL	NV	NV	NV	+ +	+ +	+ + +
NL	Bell-shaped	AF	1.00	1.96*	2.11*	1.78*	2.01*	2.41*
		AL	NV	+	+ +	+	+ +	+ +
GSTdg	Bell-shaped	AF	1.00	0.97	1.03*	1.2	0.93	1.12
		AL	NV	NV	NV	NV	NV	NV
GSTg	Bell-shaped	AF	1.00	1.42*	1.24	1.49*	1.19	0.85
		AL	NV	+	NV	+	NV	NV
CATdg	Bell-shaped	AF	1.00	1.03	0.99	1.36	0.89	1.25
		AL	NV	NV	NV	NV	NV	NV
CATg	Bell-shaped	AF	1.00	1.08	1.28	1.05	0.86	1.03
		AL	NV	NV	NV	NV	NV	NV
MDA	Increasing	AF	1.00	2.04*	1.86*	1.94*	1.65	0.42
		AL	NV	+ +	+	+	NV	NV
AChE	Decreasing	AF	1.00	0.91	0.97	1.00	0.68*	0.96
		AL	NV	NV	NV	NV	–	NV
MT	Bell-shaped	AF	1.00	1.16	1.00	1.31	1.54	1.03
		AL	NV	NV	NV	NV	NV	NV
Tissue level								
LYS/CYT	Increasing	AF	1.00	1.16	1.37	1.43*	1.52*	1.42
		AL	NV	NV	NV	+	+	NV
HSI			**A**	**A**	**B**	**C**	**C**	**C**
			Healthy	**Healthy**	**Low stress**	**Moderate stress**	**Moderate stress**	**Moderate stress**

LMS, lysosomal membrane stability; NL, neutral lipid content; LF, lipofuscin content; GSTdg, glutathione S-transferase activity analysed in digestive gland; GSTg, glutathione S-transferase activity analysed in gills; CATdg, catalase activity analysed in digestive gland; CATg, catalase activity analysed in gills; MDA, malondialdehyde content; AChE, acetylcholinesterase activity; MT, metallothionein content; LYS/CYT, lysosome/cytoplasm volume ratio; GP, guide parameter; HSI, health status index. AF thresholds for increasing/bell-shaped biomarkers: 'NV' (no variation) = AF < 1.2; '+' = AF > 1.2; AF thresholds for decreasing biomarkers: NV = AF > 0.8; '-' = AF < 0.8. $_*$, $p < 0.05$ vs control, one-way ANOVA followed by the Dunnett's test.

1997; Hietanen et al., 1988). The lysosomal membrane destabilization is a recurring effect of PS micro- and nano-plastic exposure in marine invertebrates, including mussels (Canesi et al., 2015; Capolupo et al., 2021; Fabbri et al., 2020), oysters (Thomas et al., 2020) and sea urchins (Marques-Santos et al., 2018). Although not directly investigated, the data reported here indicate that this effect could be caused by chemicals associated with the plastic particles and not by the physical particles themselves.

Two further lysosomal biomarkers of general stress were evaluated in the digestive gland of exposed mussels; the unsaturated neutral lipid content (NL) and the lysosome to cytoplasm volume ratio (LYS/CYT). NL accumulation is a generalized biomarker of lipidosis and is thought to result from either an increased cytosolic lipid content or a decrease in fatty acid processing (Viarengo et al., 2007). In this study, all the plastic leachates significantly enhanced the NL content with respect to the control. NL alterations have previously been identified in mussels exposed to organic pollutants in field conditions (Capolupo et al., 2017) and to increasing BPA concentrations in experimental settings (Canesi et al., 2007). Similarly, field exposure to metals generally enriched in tested leachates, such as Zn, Cu, Cd and Pb, were found to be related to NL disorders in mussels (Donnini et al., 2007; Fokina et al., 2013).

The increase of the lysosomal volume is an advanced physio-pathological condition in the hepatopancreas of mussels, and is thought to precede highly hazardous processes to the viability of digestive cells and digestive gland functions (Orbea et al., 2006). In this study, mussels exposed to CTR, PP and PVC leachates showed LYS/CYT levels significantly higher than in controls. As reported by Capolupo et al. (2020), these leachates contained several chemical additives known to induce lysosomotropic effects in mussels. As an example, biomonitoring studies highlighted high LYS/CYT levels in specimens of *M. galloprovincialis* showing increased tissue levels of Cu and Zn

(Capolupo et al., 2017;(Etxeberria et al., 1995) , which were present in the highest levels in PP and CTR/PVC leachates, respectively. The Pearson's correlation analysis highlighted significant relationships between LMS, NL and LYS/CYT. This phenomenon is in line with previous observations (Capolupo et al., 2017; Martin-Diaz et al., 2009) and highlights the generalized effect induced by leached additives on lysosomal parameters of general stress.

The overproduction of reactive oxygen species (ROS) is a well-documented adverse effect of pollutants (Regoli and Giuliani, 2014). Cellular ROS-mediated alterations are multiple and include, among others, the peroxidation of the lipid bilayer composing cell membranes (Moore et al., 2008). Lysosomal autophagic processes provide an effective sequestration of lipid peroxidation intermediate and final products, such as MDA and LF (Viarengo and Nott, 1993). These parameters tend to accumulate in mussels in response to metals, such as Cu, Cd, Ag and Hg (Gomes et al., 2011; Maria and Bebianno, 2011), and organics, including some known for their use as polymer additives, such as 47- BDE and other endocrine disrupting chemicals (Canesi et al., 2008; (Gu et al., 2020). The results reported here outline a mutual relationship between MDA accumulation, observed for PET, PS and PP leachates, and LF increase, noted in mussels exposed to PP, PVC and CTR. In particular, the data suggest a different timing of LPO induction among tested leachates, with a relatively high intensity for those leachates inducing accumulation of final LPO products (i.e. LF in PP, PVC and CTR) and moderate peroxidation in the case of sole MDA up-regulation (i.e. PET and PS). This would also explain the low MDA content observed in CTR leachate-exposed mussels as a consequence of its depletion due to LF formation. LF levels were significantly correlated to LMS observed in mussel haemocytes. This relationship has also been observed elsewhere (Donnini et al., 2007; Franzellitti et al., 2014) and further confirms the mechanistic links between lysosomal disorders occurring in different tissues and the onset of pro-oxidant effects in exposed organisms.
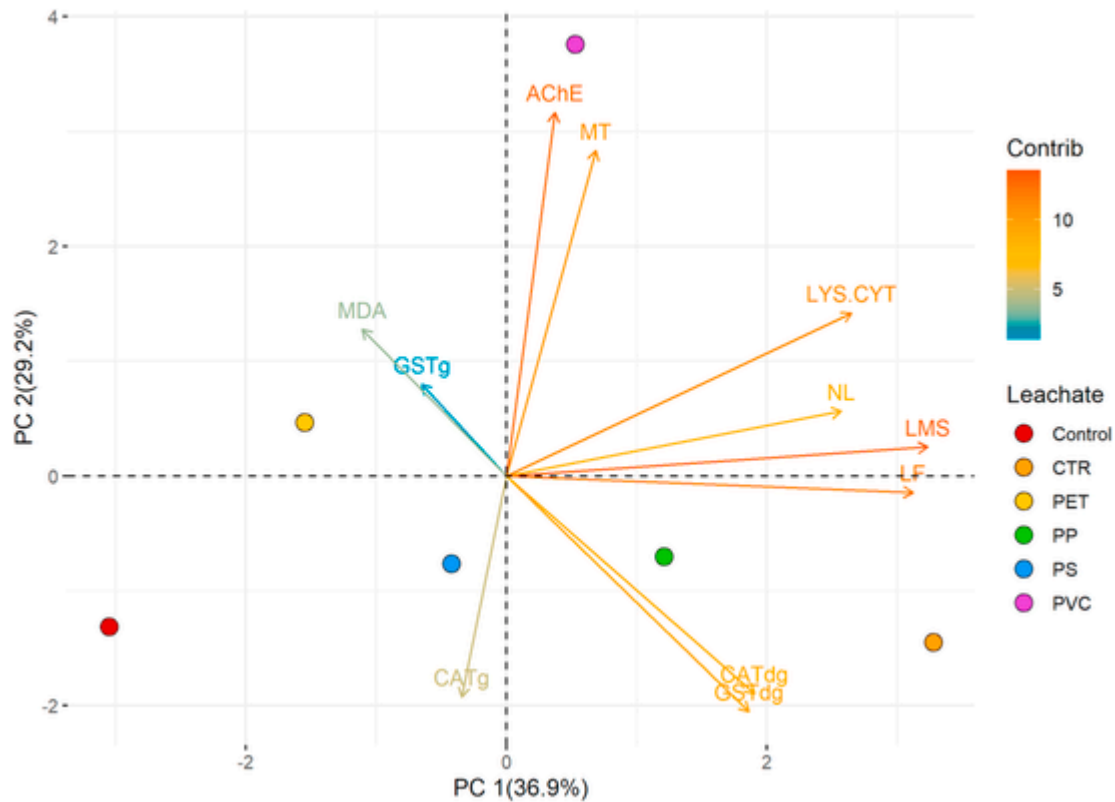
Fig. 4. Principal Component Analysis (PCA). Biplot showing the PCA output obtained on the entire set of biomarker data measured in mussels (*M. galloprovincialis*) exposed to plastic polymer leachates for 7 days.

To screen the possible effects of leached additives on the mussel antioxidant defence mechanisms, the activity of two enzymes having high substrate specificity for ROS, i.e. CAT and GST, were measured in gills and the digestive gland of exposed mussels. Results showed that basal GST activity is higher in mussel gills than in digestive gland, while CAT activity had opposite trend. These relationships are in line with previous observations on marine mussels (Capolupo et al., 2016; Saènz et al., 2010), and suggest the different role played by these enzymes depending on tissue. In particular, the GST-mediated inactivation of ROS and xenobiotics is expected to be highly expressed in tissues directly exposed to physicochemical fluctuations from the surrounding environment, such as gills (Soldatov et al., 2007). Conversely, a higher basal activity of CAT, which catalyses the hydrogen peroxide decomposition to water and oxygen, is expected in the digestive gland, where peroxidation processes are frequently modulated in response to toxicant exposure (Orbea et al., 2006). Leachates from PP and PET significantly increased the GST activity in digestive glands, while PP is the only leachate up-regulating the GST in the gills of exposed mussels. Considering the relatively low organic additive content in PET-derived leachates, observed changes may be attributed to the pro-oxidant behaviour of leached metals. For example, Co, which showed maxima in the PET leachate along with Sb (Table S2), is known to be a strong GST activator in mammalian models (Daido and Aniya, 1994). Metals showing high levels in the PP leachate, such as Cu and Zn, are also known to induce phase II metabolism reactions mediated by GST activation in *Mytilus* spp. (Canesi et al., 1999; Capolupo et al., 2017; Vidal-Liñan et al., 2010). Additionally, a significant GST activation was reported in mussels following exposure to BPA (Canesi et al., 2007), suggesting that the observed activation by the PP leachate might result from a synergistic activity of both organic and inorganic additives.

AChE is the most commonly used biomarker of neurotoxicity in aquatic organisms (Rank et al., 2007; Rickwood and Galloway, 2004). Its inhibition induces a protraction of the nervous stimulus, which represents the mechanism of action of many neurotoxic formulations, such as pesticides (Bocquene and

Galgani 1998; Zinkl et al., 1991). In the present study, we found evidence of AChE inhibition in mussels exposed to PVC leachates. A study performed by Juhel et al. (2017) found BPA elicited inhibitory effects on AChE on mussels (*Perna viridis*) when administered in a mixture with other emerging contaminants, such as carbamazepine and atrazine. Further evidence obtained in the past decade has shown that metals, including Zn, which was highly leached by CTR and PVC, act as AChE inhibitors, the effect being visible at very low concentrations (Frasco et al., 2005).

Notwithstanding the presence of metals in the prepared leachates, none of the performed treatments altered the intracellular levels of MT compared to control mussels. MT are cysteine-rich proteins which tend to increase in fish and invertebrates tissues after exposure to metals for which they possess binding affinity (e.g. Zn, Cd and Cu) (Viarengo et al., 1997). Zn and Cu, two of the most enriched metals in the tested leachates, have previously been described as MT up-regulators in marine bivalves (Capolupo et al., 2017; Donnini et al., 2007). Evidence obtained in mussels and oysters suggest that concentrations of around 40.8 μg/L Cu (Viarengo et al., 1997) and >500 μg/L Zn (Piano et al., 2004) are necessary to stimulate MT synthesis following 3–7 days of exposure. Considering that we exposed mussels to a 1:1000 leachate dilution to create a more environmentally realistic exposure scenario, it can be speculated that the final metal concentrations, i.e. less than 1 μg/L for most elements and between 0.05 and 6.4 μg/L for Zn, were not sufficient to up-regulate the MT-mediated scavenging activity in mussel tissues.

The MES data integration ordered the health status alterations induced by the exposure to selected leachates as CTR = PVC = PP > PS > PET. This output is in line with the modulation of biomarkers of general stress, detoxification and neurotoxicity and reflects the concentrations of organic and inorganic additives measured in tested leachates (Capolupo et al., 2020). The CTR leachate, which elicited major effects on lysosomal parameters, shows the highest enrichment of n-cyclohexylformamide, phthalide and bisphenol A, as well as relatively high levels of Mn and Zn. The PVC leachate, which is the only additive mixture inducing AChE inhibition, showed the highest Zn and Co con-

centrations and a significant enrichment of organics, including phthalide. Finally, the PP leachate showed the highest levels of acetophenone, Cu and Pb (Capolupo et al., 2020). The different effects induced by leachates on the tested endpoints were also confirmed by the PCA output. According to their spatial distribution, data from the PVC and CTR leachates showed the highest difference relative to control levels, followed by PP, PS and PET. The overall variations among leachates seem mainly related to the changes observed in lysosomal and lipid peroxidation endpoints, notably LMS, LYS CYT, NL and LF. A further marked contribution to the overall PVC score was caused by AChE activity and MT, with both showing the highest levels in PVC leaches-exposed mussels. These variations confirm the stress degree identified by the MES output for selected leachates and provide further clues on the mechanisms underlying their overall impact on the fitness of marine mussels.

5. Conclusions

This study demonstrates for the first time that the complex additive mixtures leached by different polymer types into seawater may selectively alter the physiological fitness of marine mussels, providing further insights on the environmental implications of the oceanic contamination by plastic litter. Among the examined biomarkers, LMS, NL and LF showed the highest changes, suggesting that leached organic and inorganic additives may pose specific risks to the functionality of the lysosomal system. Data also highlight the onset of pro-oxidant conditions in the mussel digestive gland, apparently resulting from the release of metals and organic from the tested polymers. In some cases, these alterations were associated to an increase GST activity, which denotes the onset of phase II metabolism reactions involved in the biotransformation of ROS and toxicants.

From our experimental trials, CTR, PVC and PP materials generate the most harmful leachates to mussels. These outcomes, however, should be interpreted with caution, since the additive chemical composition and toxicity may vary greatly among commercial products of the same polymer type, depending on environmental factors and/or on the used elution method. It thus seems prudent to recommend that toxicity studies conducted with plastic materials should include a chemical characterisation of the test materials and the chemicals that might be leaching from them over the exposure period. In addition, analysis of the uptake and accumulation of the target chemicals from the leachates in the tissues of organisms would strengthen interpretation of results in future studies. Sub-lethal toxicity data reported here were obtained after sub-chronic exposure of mussels using physiological parameters validated as "early-warning" signals of higher dysfunctions. To gain a more coherent insight on the impacts of plastic and rubber additives at environmentally realistic conditions, further efforts are necessary to define the effects of leachates under prolonged exposure and/or in association with environmental stressors (e.g. pH, salinity or temperature fluctuations).

Author statement

Marco Capolupo: Conceptualization, laboratory and data analysis, Writing – original draft; Kuddithamby Gunaalan: Methodology application, Formal analysis, graphical representations; Andy M. Booth: chemical interpretation, draft correction, Resources; Lisbet Sørensen: chemical interpretation, draft correction; Paola Valbonesi: methodological application; Elena Fabbri: Conceptualization, Supervision, draft correction, Resources

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.envpol.2021.117081.

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