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Impacts of microplastic fibres on the marine mussel, *Mytilus galloprovinciallis*

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Abstract

Tumble dryer lint has been employed as a surrogate for synthetic and processed (microplastic) fibres discharged to the environment from laundering activities and exposed to marine mussels (*Mytilus galloprovinciallis*) in controlled experiments for a period of 7 d. A range of biological responses at different levels of organisation were subsequently determined, with copper employed concurrently as a positive control. Physiological changes were assessed from measurements of clearance rate, histopathological effects were evaluated from abnormalities in (or injuries to) gill and digestive gland tissues, and genetic damage was determined by measuring DNA strand breaks using the comet assay. With increasing lint concentration (over the range 56 to 180 mg L^{-1}) we observed a reduction in mean clearance rate, increasing extents of abnormality in both gills (e.g. deciliation and hypertrophy) and digestive gland (e.g. atrophy and necrosis), and an increase in damage to DNA. The precise causes of these effects are unclear but likely arise from both the fibrous material itself and from chemicals (e.g. additives and metals) that are mobilised from the polymers into seawater or the digestive tract. The latter assertion is consistent with an observed increase in the release of certain trace elements (e.g. zinc) into the exposure medium with increasing lint concentration. Although microfibre concentrations we employed are significantly greater than those typically encountered in the environment, the results indicate the potential for this type of material to exert a range of adverse effects on exposed marine animals.

Keywords: microplastics; fibres; lint; mussels; impacts; toxicity;

1. Introduction

Marine microplastics are conventionally classified as primary, where distinct particles like pre-production pellets and cosmetic microbeads enter the environment directly, or secondary, where small particles are formed in situ through the disintegration of larger plastic objects (Efimova et al., 2018). Synthetic microfibres also fall under the umbrella of microplastics but are not as readily classified because while some particles are secondary (e.g. fragments of fishing rope), others are derived ex situ from the gradual attrition of items via consumer and industrial usage (e.g. the laundering of clothing). Nevertheless, microfibres appear to represent one of, if not the most abundant form of microplastics in the marine environment (Watts et al., 2015; Barrows et al., 2018).

Perhaps the most significant source of both synthetic microfibres (e.g. polyethylene terephthalate, PET; polypropylene; acrylic) and processed microfibres (principally cotton whose polymeric structure is dominated by cellulose) to the environment is the laundering of clothes and other textile products. Here, estimates of detachment rates during washing range from 175 to 560 fibres per g or from 30,000 to 465,000 fibres per m^2 of garment (Belzagui et al., 2019). Given the size and density of these particles, the majority are likely to evade any water treatment process and enter the oceans where they have the propensity to be taken up into the food chain (Cesa et al., 2017). Accordingly, microfibres have recently been reported in a range of animals, including fish (Kumar et al., 2019), crustaceans (Abbasi et al., 2018), molluscs (Woods et al., 2018), birds (Le Guen et al., 2020) and seals (Perez-Venegas et al., 2018). Despite their ubiquity, however, there is a distinct lack of studies into the impacts of microfibres on marine life, including those related to physiology, habitat, food resources and delivery of pollutants (Zhang et al., 2019). Rather, the majority of studies focus on readily sourced and relatively uniform microspheres of a precise size and specific polymer construction (Van Cauwenberghe et al., 2015; Sussarellu et al., 2016; Chae et al., 2019; Seoane et al., 2019).

In the present study, we use established techniques to examine the physiological and histopathological impacts on and DNA damage to the filter-feeding marine bivalve, *Mytilus galloprovinciallis*, resulting from microfibre exposure. *Mytilus* species have commonly been used to monitor water quality in coastal areas due to their wide distribution, immobility, high tolerance of a range of environmental conditions and ability to accumulate a variety of contaminants (Phillips and Rainbow, 1993). As a source of synthetic and processed microfibres (which we hereafter refer to as microplastic fibres) that are representative of those derived from laundering and entering the environment via wastewater we use tumble dryer lint

(Turner, 2019a), a heterogeneous assortment of fibrous particles arising from the high temperature air-drying of washed clothes and other textiles.

2. Materials and methods

2.1 Reagents and materials

All chemicals and reagents used during the study were purchased from Sigma-Aldrich Ltd. (Gillingham, UK) unless otherwise stated. Seawater employed in the experiments (salinity = 34) was available on tap in the laboratory having been collected in bulk from Plymouth Sound and filtered to < 10 μm. Ultrapure water was obtained using a Milli-Q RG system (Millipore, Billerica, MA) and all glassware and plasticware were acid washed in a solution of 10% HCl (Fisher Scientific UK, trace analysis grade) for 24 h before use.

Lint was collected as a composite from a local, communal launderette in Plymouth. Large, visible impurities, including hair, paper and feathers, were removed manually before material was cut into smaller pieces and homogenised. Three ~ 10 mg portions from a ~ 100 g piece of lint selected for use in the study were examined under an Olympus SZX7 stereo-microscope with 8 x optical magnification and 100 random fibres were measured for length. Figure 1 illustrates a cutting of lint after removal of extraneous material and a selection of resulting microfibres viewed under the microscope.

A 1 g sample of lint was analysed in triplicate for elemental composition using a portable X-ray fluorescence (XRF) spectrometer (Niton XL3t 950 He GOLDD+) in a plastics mode for 120 s according to procedures outlined in Turner (2019a). The loss on ignition of three ~ 3 g portions of lint was determined by weight loss at 450 ^{o}C for 6 h in a Carbolite EAF 11/14 muffle furnace.

Adult *M. galloprovincialis* of average shell length ~ 5 cm were collected from a pristine site in Cornwall (Trebarwith Strand; 50 38’ 40” N, 4 45’ 44” W) during March 2019. The mussels were transported to the laboratory on ice before being allowed to depurate for two weeks in 55 L of seawater contained in a 75 L aquarium maintained at 15 ^{o}C and under a 12 h-12 h light-dark illumination regime, as described in detail elsewhere (Dallas et al., 2013). During depuration, mussels were fed three times a week with a suspension of *Isochrysis galbana* microalgae (~1.05 x 10^{6} cells mL^{-1}; Reed Mariculture, Campbell, CA), with a complete water change performed 2 h after feeding. No spawning occurred in the aquarium and the same batch of mussels was used throughout the experiments.

2.2. Exposure to Copper

Copper was employed to validate the methodology and end-points subsequently used in the lint exposures in accordance with protocols established in previous studies (Al-Subiai et al., 2011; Vernon and Jha, 2019). In duplicate, *M. galloprovincialis* were transferred to a series of twelve 2 L glass beakers each containing 1.8 L of seawater and maintained under the conditions described above for an acclimation period of 48 h. Three beakers (six mussels) were then exposed to one of four treatments for a period of 7 d: a control (no added Cu), and 18 µg L^{-1}, 32 µg L^{-1} or 56 µg L^{-1} of Cu (prepared from $CuSO_4.5H_2O$). A complete water change was undertaken on day 3, with added Cu concentrations renewed. At the end of the exposures, mussels were removed and clearance rate, degree of DNA damage and extent of tissue alteration due to disease determined (see below). A concentration of 32 µg Cu L^{-1} resulted in clear responses to all parameters and was employed as a positive control for the microfibre exposures.

Water quality was checked throughout the experiment by measuring dissolved oxygen concentration, pH and temperature with a HQ40D multimeter (Hach-Lange, Dusseldorf, Germany) and total ammonia using a 3893 Aquarium Test Kit (Hanna Instruments, Woonsocket, RI, USA). Dissolved oxygen was always above 90%, pH ranged from 7.8 to 8.4, temperature ranged from 13.0 to 15.8 ^{o}C and ammonia was always < 0.25 mg L^{-1}.

2.3. Exposure to microfibres

A preliminary exposure experiment using microfibrous lint at different concentrations established a suitable working range below which no observable effects were observed on *M. galloprovincialis* and above which significant lint aggregation could not be avoided. Mussels (*n* = 30) were transferred to individual 400 mL glass beakers each containing 350 mL of seawater and allowed to acclimatise for 48 h. Six mussels were exposed to one of five treatments: a control (no added lint), and 56 mg L^{-1}, 100 mg L^{-1}, or 180 mg L^{-1} of lint, or 32 µg L^{-1} of Cu. A magnetic stir bar (25 x 6 mm) was placed in each beaker, with beakers placed on separate magnetic stirrers at 400 rpm in order to disperse lint without disturbing mussels. Exposure proceeded for 7 d, with a complete water change on day 3 when lint or Cu concentrations were renewed and any lint that had stuck to the mussels removed using forceps.

Water quality was monitored throughout the exposures as described above, but in addition, water samples were taken for the determination of trace elements. Thus, 1.2 mL of seawater was added to an Eppendorf tube and the contents centrifuged at 1000 g for 5 min. A 1 mL

aliquot of supernatant was pipetted into a 10 mL Fisher Scientific centrifuge tube and diluted to 5 mL with 2% HNO_3. Samples were then analysed using an iCAP RQ inductively coupled plasma mass spectrometer (ICP-MS; Thermo Scientific, Hemel Hempstead, UK) calibrated with mixed, matrix-matched standards and operated under conditions described elsewhere (Turner, 2019b).

2.4. Determination of clearance rate

Clearance rate, CR, as a measure of physiological effects, was carried out as described in Canty et al. (2009). At the end of the exposures, individual mussels were placed in separate 400 mL glass beakers, each containing 350 mL of seawater, with three beakers without mussels serving as controls. Under magnetic stirring (described above), mussels were allowed to acclimatise and open their valves for ~ 20 min. A suspension of *Isochrysis galbana* was then added to each beaker to a concentration of ~ 12-13 x 10^3 algal cells mL^{-1}, and 20 mL samples of seawater taken with a syringe after a few seconds (T_0) and after 20 min (T_1). Samples were analysed on a coulter counter (Beckman Coulter Z2, US) fitted with a 100 µm aperture and set to count particles between 4.0 and 10.0 µm in diameter. The clearance rate of each mussel was calculated from the following equation (Coughlan, 1969):

$$CR = V(\ln C_0 - \ln C_1)/t_1 - t_0$$

where V is the volume of water and C_0 and C_1 are algal concentrations at t_0 and t_1, respectively.

2.5. DNA damage analysis

2.5.1. Collection of haemolymph samples

At the end of the clearance rate assay, mussels were removed from the beakers and placed on ice for haemolymph sampling as described in detail elsewhere (Al-Subiai et al., 2011; Vernon and Jha, 2019). Briefly, a blade was inserted midway towards the posterior end of the byssus thread and left to allow excess seawater to drain out. Haemolymph was extracted from the posterior adductor muscle of individuals using a 21-gauge hypodermic needle into a 1 mL syringe. A 250 µL aliquot of haemolymph-saline mixture was transferred to a siliconised Eppendorf micro-centrifuge tube and the contents centrifuged at 1000 g for 5 min. The resulting supernatant was removed and placed on ice until required.

2.5.2. Comet assay

For the determination of DNA strand breaks using the comet assay, standard procedures were adopted that have been described in detail previously (Al-Subiai et al., 2011; Vernon and Jha,

2019). Briefly, 400 µL of low melting point agarose (in DPBS) was added to each re-suspended cell pellet arising from centrifugation and 75 µL of the cell-agarose suspension was pipetted, in duplicate, onto slides pre-coated with 1% normal melting point agarose. Drops were then covered with 22 x 22 mm cover slips and checked under an inverted microscope for the presence of cells before slides were placed in a fridge (4ºC) for ~ 15 min to allow gels to set. Slides were placed in a slide rack which was subsequently transferred to a staining dish filled with chilled lysis buffer (2.5 M NaCl, 100 mM EDTA, 10 mM Tris, 1% N-lauryl-sarcosine, 1% Triton X-100, 10% DMSO, pH adjusted to 10 with NaOH) for 24 h at 4ºC. Slides were placed in an electrophoresis chamber (COMPAC-50) and chilled electrophoresis buffer (1 mM EDTA, 0.3 M NaOH) added. DNA was allowed to unwind under alkaline conditions for 20 min at 4 ºC (in the dark), followed by electrophoresis (300 mA, 15 V, 25 min). Slides were rinsed three times with neutralisation buffer (0.4 Triz, pH 7), washed with refrigerated ultra pure water and allowed to air dry for 24 h. Each slide was then coded and randomised to ensure scoring was unbiased ('blind'). Each replicate microgel was stained with 20 µL of 10 x Gel red (Cambridge Biosciences, UK), and 50 nuclei per microgel (100 per slide) were analysed using an epifluorescence microscope (DMR; Leica Microsystems, Milton Keynes, UK) at a magnification of 400 x and a Comet IV imaging system (Perspective Instruments, Bury St Edmunds, UK). Percentage of DNA present in the Comet tail (% Tail DNA) was considered to be the most suitable measure of DNA damage (Kumaravel and Jha, 2006).

2.6. Determination of histopathological changes

Gills and digestive glands from four individuals in each treatment were examined for potential histopathological changes as described elsewhere (Al-Subiai et al., 2011). Tissue was carefully removed from each animal and placed in a fixative (10% buffered formal saline) for 4 d before being placed in a histo-casette and processed in a TP 1020 Automatic Tissue Processor (Leica Microsystems, Wetzlar, Germany) for 24 h. Processed tissues were embedded in paraffin wax, sectioned (to 3-6 µm thickness) using a rotation microtome (R2125 RTS, Leica Microsystems), transferred to slides and stained with haematoxylin and eosin. Slides were examined under an Olympus Vanox-T light microscope and photographed using an Olympus Camedia C-2020 Z at 20 random locations, with identification of histopathological changes based on well-established effects (Sheir et al., 2010).

2.7. Statistical analysis

All statistical analysis was performed in SPSS (IBM SPSS Statistics 24). Normality was checked using a Shapiro-Wilk test before an appropriate parametric or non-parametric test was performed using an α-value for significance of 0.05.

3. Results

3.1. Microfibre characteristics

Table 1 provides information on the physical and chemical characteristics of the microplastic fibres contained in the lint used in the study. Thus, fibres range from about 20 to 750 μm in length and were always < 30 μm in thickness, and material is comprised of a highly combustible, organic-rich matrix. A variety of trace elements are present as inherent components of fibres (e.g. dyes or additives) or contaminants that have become associated with fibres before or during washing and drying. Of the elements measured in water samples at the end of the exposures, Zn was always detected and showed the clearest proportionality with the concentration of lint used (Table 2). Specifically, an increase in Zn concentration of about 3 μg L^{-1} was observed per 10 mg L^{-1} increase in microfibre concentration, presumably reflecting the fraction of the metal in lint that is soluble in seawater.

3.2. Physiological impacts and DNA damage

Table 2 also presents estimates of CR for the different microfibre exposures. The presence of material results in a reduction in mean CR compared with the controls, but the high variability amongst replicates means that differences among the exposures were not statistically significant.

Levels of DNA damage in mussels exposed to plastic microfibres are shown in Figure 2. The mean percentage tail damage (y) increases with increasing concentration of microfibres (x) according to the following regression equation: $y = 0.141x$ ($r^2 = 0.887$; $p < 0.01$), and there was a statistically significant increase in percentage tail DNA in mussels exposed to 100 and 180 mg L^{-1} of microfibres compared with the control (Tukey test). Percentage tail DNA also exhibited significant correlations with mean CR ($r = 0.391$) and percentage abnormality in gills (see below) ($r = 0.453$).

3.3. Histopathological effects

The histopathological effects of plastic microfibre exposure on mussel gills and digestive gland are illustrated in Figure 3 and are quantified in terms of percentage abnormality (injury) in

Figure 4. Regarding the gills, in the control mussels a 15% abnormality was observed, with the majority of samples exhibiting a well-preserved structure and ciliated epithelia covering the external surface. Abnormality increased progressively with increasing concentrations of microfibres to a value of 90% at the highest concentration employed (and in excess of abnormality effected by the addition of Cu). Specifically, hypoplasia (loss of cilia) was observed whenever lint was present and extensive hypertrophy (swelling) was evident at the highest exposure concentration. With respect to the digestive gland, about 75% of mussels in the control exhibited a normal round/ovular structure with a columnar epithelium liner and no evidence of haemocyte infiltration, necrosis or other damage. On exposure to microfibres, the principal change was atrophy, loss of definition of the digestive tubules and necrosis (diffuse nuclei and no clear distinction in some epithelial cells) with abnormality increasing with increasing concentration of fibres to about 87% at the highest exposure concentration (and, as above, in excess of abnormality effected by the addition of Cu).

4. Discussion

Synthetic microfibres are amongst the most abundant microplastics in the marine environment (Sanchez-Vidal et al., 2018) and significant quantities are known to be derived from laundering activities, either directly or indirectly (through waste water treatment plants) (McCormick et al., 2014; Henry et al., 2019). Consequently, microplastic fibres have been detected in a range of habitats (e.g. beaches, mangroves, deep sea; Browne et al., 2010; Nor and Obbard, 2014; Taylor et al., 2016) and in a multitude of aquatic organisms (e.g. fish, crustaceans, invertebrates; Rochman et al., 2015; Abassi et al., 2018). The accumulation of microplastic fibres by marine bivalves in particular has received recent attention because of the use of filter- and suspension-feeding animals as water quality monitors and the potential health implications of shellfish through human consumption. For example, De Witte et al. (2014) found samples of *M. edulis* along the Belgian coast and purchased from department stores contained between 0.26 to 0.51 fibres per g of tissue, with variations believed to reflect environmental availability. Li et al. (2015) report greater quantities of fibres (up to about 5 per g) in a variety of commercial bivalves from a fishery market in China, and Mathalon and Hill (2014) found about 30 and 70 fibres per individual in *M. edulis* collected from a Nova Scotian harbour and purchased from an aquaculture site, respectively.

Controlled laboratory exposures suggest that although filter-feeding bivalves reject regular-shaped particles (commonly microspheres) of 1000 μm or more in diameter, ingestion of microplastic fibres through the inhalant syphon and mouth to the digestive gland is far less

constrained provided that one dimension is within the size range that can be ingested (Ward et al., 2019). Experiments performed by Li et al. (2019) with a freshwater clam revealed a greater uptake of PET microfibres compared with other types of synthetic fibre suggesting that the physicochemical properties of fibres may be significant. Regarding PET microfibres, independent laboratory experiments have shown that although the majority of material exposed to *M. edulis* is rapidly rejected as pseudofaeces, 9% of fibres ingested and 1% egested (Woods et al., 2018). Consistent with our clearance rate observations using laundry dryer lint fibres, Woods et al. (2018) observed a reduction in mussel filtration rate when exposed to (PET) microfibres.

Histological analysis provides a well-established measure of structural and morphological changes in tissues arising from contaminant exposure and the effects on the gills and digestive gland illustrated in Figure 3 accord with those observed in other studies using mussels and polyethylene microparticles (von Moos et al., 2012; Bråte et al., 2018), as well as for metals and organic pollutants (Al-Subiai et al., 2011; Di et al 2017). That in at least one study microplastics were additive–free suggests plastic itself is partly responsible for tissue damage, with the possibility that specific chemicals mobilised from the polymer are able to accentuate any adverse effects (see below). Deciliation of the gills on exposure to microfibrous lint may also account for the reduction in clearance rate observed in *M. galloprovinciallis* through a decline in the ability to capture food and transport it to the mouth. This then impacts more widely on the physiological functioning of the whole organism, especially when food concentrations are low (Xu et al., 2016).

Our study has also demonstrated that microplastic fibres in laundry lint are, directly or indirectly, genotoxic to *M. galloprovinciallis*. As such, they may be implicated in a variety of pathological processes that could extend beyond the individual. Microfibres are likely to be accumulated in the digestive gland and gills but it is unclear whether they have the propensity to be transported into the circulatory system and translocated to other tissues and cause direct damage. With respect to more regularly-shaped, commercial microplastics, there is evidence of translocation to the haemolymph and haemocyctes of filter-feeding bivalves (Browne et al., 2008) and DNA strand breaks have been reported in some cases, with a toxic response being attributed to reactive oxygen species production and oxidative stress (Avio et al., 2015; Ribeiro et al., 2017). In the present study, an additional source of indirect toxicity is chemicals associated with microfibres that may be mobilised into seawater or the digestive tract of *M. galloprovinciallis*. Those associated with synthetic and processed microfibres include monomers, additives, dyes and catalytic residues (and any degradation products derived therefrom), as well as metals and organic compounds sorbed to the polymer. Specific toxic

chemicals that have been identified and quantified in common textiles include antimony, phthalate esters, chloranilin, formaldehyde and nonylphenol ethoxylates (Laursen et al., 2003). Although the determination a complex array of chemicals was beyond the scope of the study, we measured a suite of elements in the lint microfibres and their release into seawater during the experimental exposures. Among these elements, Zn was most readily detected and exhibited a clear increase with increasing concentration of fibres. While Zn itself may not be able to induce significant DNA damage at the concentrations measured (Bolognesi and Cirillo, 2014), the results are significant in that they demonstrate the potential for a cocktail of more harmful chemicals to be mobilised from plastic microfibres into the exposure medium or digestive tract.

Although the exposure concentrations employed in the present study are higher than those likely to be encountered in marine environment (typical plastic microfibre concentrations are < 0.1 mL^{-1}; Barrows et al., 2017), the findings are significant because the impacts of fibrous particles on the health of bivalves have not previously been reported. Moreover, laboratory experiments examining the processing of microfibres by bivalves to date have employed rather homogeneous particles of a single polymer from a well-defined source (e.g. a specific garment) that is not representative of the complex assortment of fibres discharged into the environment (Woods et al., 2018; Li et al., 2019). Thus, heterogeneous tumble dryer lint microfibres derived from laundering and drying have the potential to exert a range of adverse physiological and behavioural effects on marine mussels. While it has not been possible to attribute these effects to particles directly or to chemicals mobilised from fibres, previous studies using bivalves exposed to regularly-shaped microplastics (e.g. von Moos et al., 2012; Sussarellu et al., 2016) and the ready leaching of Zn from our microfibres suggest that both pathways are likely.

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Table 1: Physical and chemical characteristics of the lint microfibres used in the study. LOI = loss on ignition and errors represent one standard deviation about the mean of 3 analyses. Particle dimensions were derived from microscopic measurements of 100 fibres.

Ba, μg g^{-1}	244±26.2
Bi, μg g^{-1}	8.1±2.8
Br, μg g^{-1}	19.1±1.2
Cl, μg g^{-1}	3030±26.1
Cr, μg g^{-1}	184±20.0
Cu, μg g^{-1}	81.1±10.3
Fe, μg g^{-1}	548±12.8
Ni, μg g^{-1}	18.7±0.8
Pb, μg g^{-1}	9.8±0.6
Sb, μg g^{-1}	<35
Ti, μg g^{-1}	349±15.3
Zn, μg g^{-1}	1750±46.9
LOI, %	94.1±1.2
min. particle length, μm	22.0
max. particle length, μm	743
median particle length, μm	85.1

Table 2: Concentrations of aqueous Zn and clearance rate at the end of the exposures to lint microfibres (MF) or Cu (mean ± 1 sd; *n* = 6).

[MF], mg L^{-1}	[Zn], μg L^{-1}	CR, L h^{-1}
0 (control)	33.52 ± 9.24	1.89 ± 1.07
56	45.88 ± 8.49	1.21 ± 0.69
100	60.93 ± 20.26	0.75 ± 1.07
180	84.33 ± 38.18	0.78 ± 0.54
0 ([Cu] + control)	32.68 ± 9.83	1.81 ± 0.96

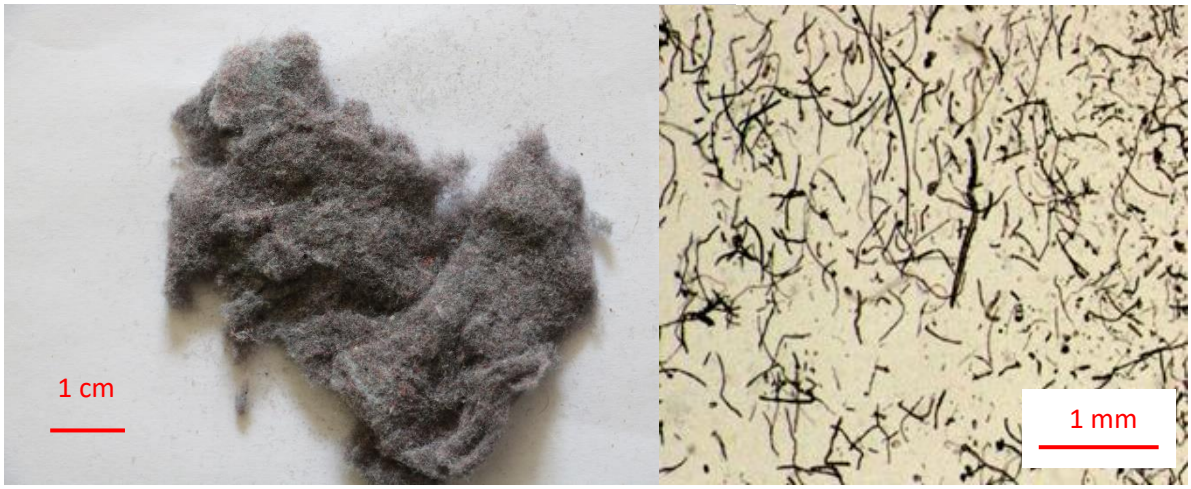

Figure 1: Illustration of a cutting of lint after manual removal of extraneous material and a microscopic image of resulting fibres.

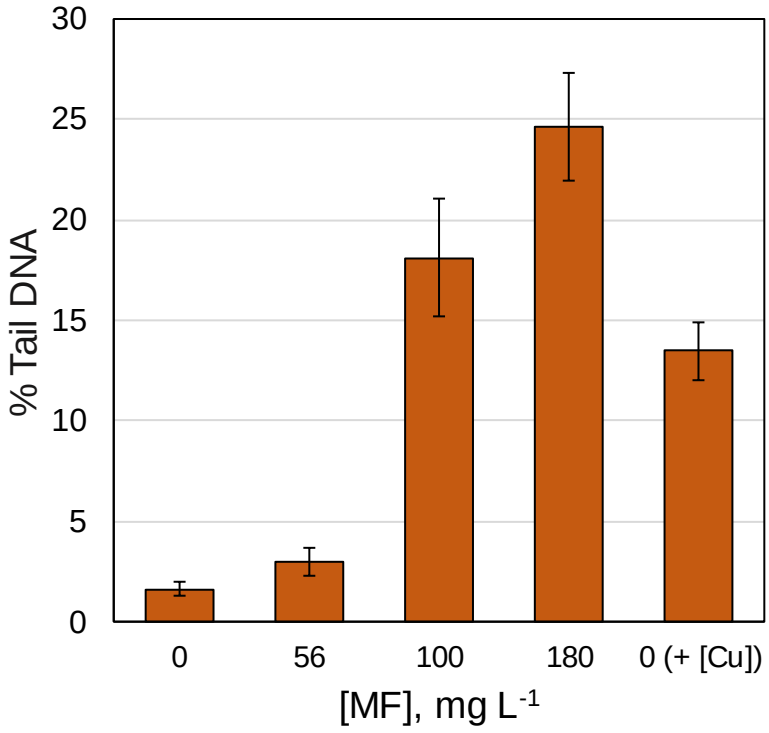

Figure 2. Induction of DNA damage in *Mytilus galloprovincialis* haemocytes following 7 d exposure to different concentrations of lint microfibres (MF). Values reported are means ± 1 sd (*n* = 6); [Cu] = 32 µg L^{-1}.

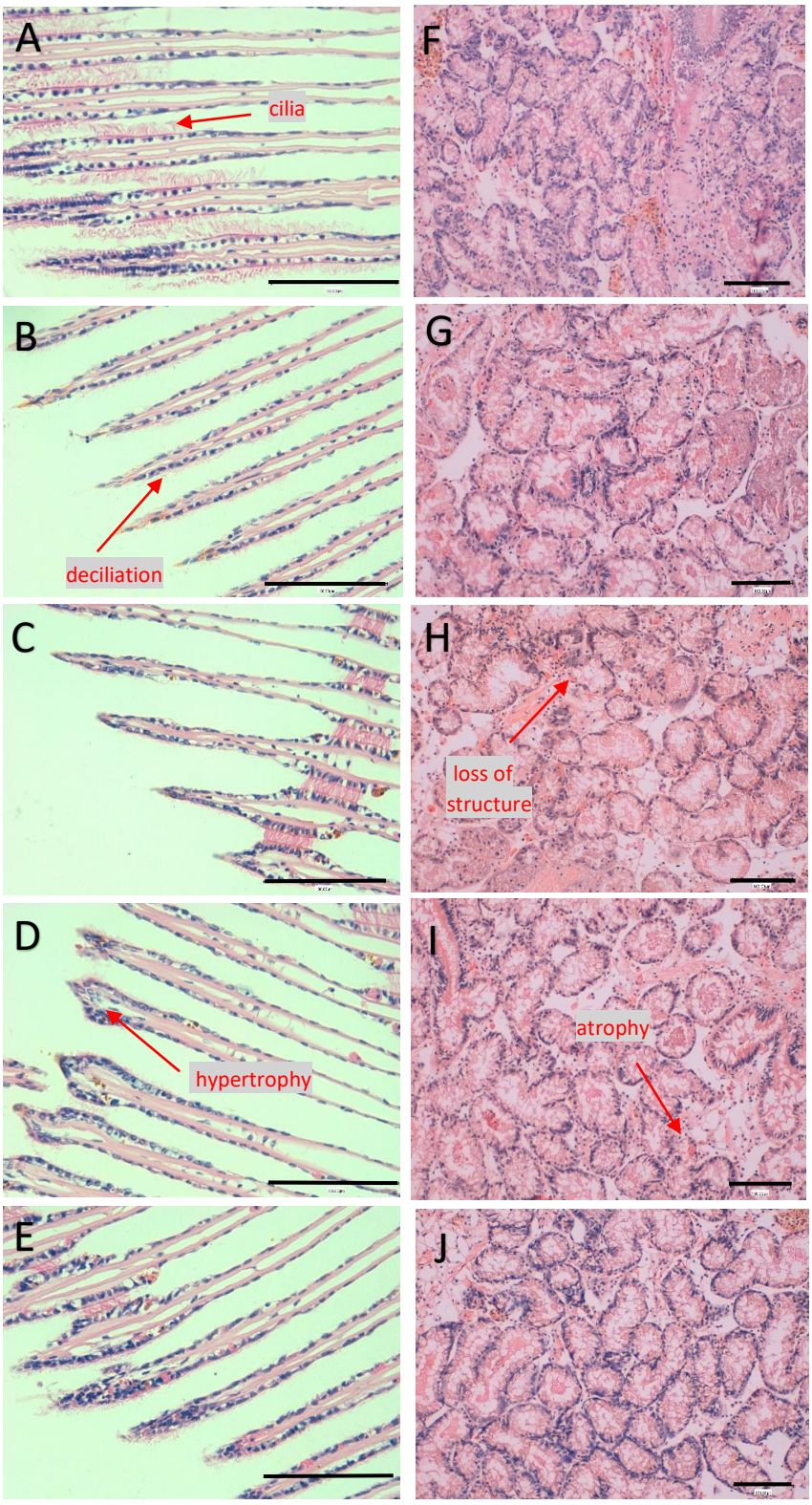

Figure 3. Histopathological effects of lint microfibre (MF) exposure on mussel gills (A-E) and digestive gland (F-J). Exposure conditions: A and F = control; B and G = 56 mg [MF] L^{-1}; C and H = 100 mg [MF] L^{-1}; D and I = 180 mg [MF] L^{-1}; E and J = control + 32 µg [Cu] L^{-1}. Scale bar = 100 µm.

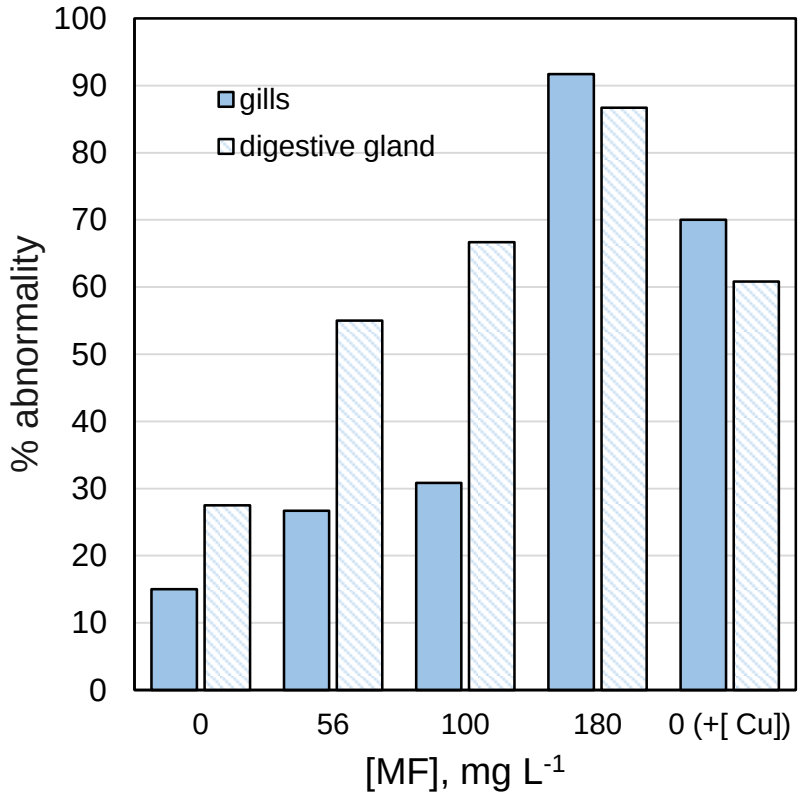

Figure 4: The percentage of *Mytilus galloprovincialis* exhibiting abnormal gills and digestive gland following 7 d exposure to different concentrations of lint microfibres (MF) (*n* = 120 for each treatment). Note that [Cu] = 32 µg L^{-1}.