Italian Journal of Food Safety

https://www.pagepressjournals.org/index.php/ijfs/index

eISSN 2239-7132

Ital J Food Saf 2026 [Online ahead of print]

Please cite this article as:

Di Natale A, Nalbone L, Condò R, et al. **Bioaccumulation and depuration dynamics of polyester microfibres in *Mytilus galloprovincialis***. *Ital J Food Saf* doi:10.4081/ijfs.2026.12818

Submitted: 27-12-2025
Accepted: 03-03-2026

Bioaccumulation and depuration dynamics of polyester microfibres in *Mytilus galloprovincialis*

Alessandra Di Natale,[1] Luca Nalbone,[1] Raffaele Condò,[2] Rachele Castaldo,[2] Mariacristina Cocca,[2] Filippo Giarratana,[1] Antonio Panebianco[1]

[1]Department of Veterinary Sciences, Annunziata University Campus, Messina; [2]Institute of Polymers, Composites and Biomaterials, National Research Council of Italy, Pozzuoli (NA), Italy

Correspondence: Alessandra Di Natale, Department of Veterinary Sciences, Annunziata University Campus, Viale Giovanni Palatucci SNC – 98168 Messina, Italy.
E-mail: alessandra.dinatale@studenti.unime.it

Key words: food safety, microplastics, microfibers, *Mytilus galloprovincialis*, mussel depuration.

Contributions: Alessandra Di Natale: investigation, methodology, validation, data curation, formal analysis, writing – original draft. Luca Nalbone, Filippo Giarratana, Antonio Panebianco: supervision, project administration, writing – review and editing. Raffaele Condò, Mariacristina Cocca, Rachele Castaldo: writing – review and editing, resources.

Conflict of interest: the authors declare that they have no competing interests, and all authors confirm accuracy.

Ethics approval and consent to participate: not applicable.

Availability of data and materials: the datasets used and/or analysed during the current study are available upon reasonable request from the corresponding author

Funding: this article was supported by the project “Biological Invasions Resolved through Adaptable, Versatile, and Engaging Nature-Based Solutions” (BRAVE), funded under the HORIZON EUROPE – Partnership Biodiversa+ program (call 2023NbS: “Naturebased solutions for biodiversity, human well-being and transformative change – BiodivNBS”), Project no. Biodiversa2023-634, CUP J43C24001020008.

Declaration of Generative Artificial Intelligence and Artificial Intelligence-Assisted Technologies in the Writing Process: no generative artificial intelligence or artificial intelligence-assisted technologies were used in the preparation of this manuscript.

Abstract
The aim of this study was to assess the bioaccumulation and depuration dynamics of *Mytilus galloprovincialis* following exposure to varying concentrations of polyester microfibers in a controlled laboratory setting. Specimens sourced from a depuration center in Messina, Italy, were acclimatized for seven days in filtered seawater at a temperature of 15°C. The mussels were then exposed to polyester microfibers (≤200 μm in length and 18 μm in diameter) at concentrations of 100, 10, 1 and 0.1 microfibers per milliliter for 24 hours. Three replicate tanks were prepared for each concentration, each containing ten mussels, totaling thirty individuals per concentration. Controls were included to monitor potential environmental contamination. Following exposure, the mussels were transferred to depuration tanks containing filtered, oxygenated seawater. Microfibers egested in feces and pseudofeces were collected at 24 and 48 hours, filtered onto 0.45 μm cellulose membranes, and counted using stereomicroscopy. After 48 hours, the mussels underwent alkaline digestion with 10% potassium hydroxide to quantify the retained microfibers. The results revealed no significant correlation between the concentration of exposure and the number of microfibers egested or retained. These findings suggest that the bioaccumulation and depuration of microfibers in *M. galloprovincialis* are influenced by factors other than concentration alone, such as particle size, morphology, and spatial configuration. Using environmentally relevant microfibers at ecologically plausible concentrations rather than virgin plastic fragments at unrealistic doses offers a more accurate representation of microplastic bioaccumulation and depuration mechanisms in mussels. Finally, background microplastics were also detected in control samples. This is significant as it indicates pre-existing contamination of the organisms and further highlights the pervasive presence of microplastics in the environment, as well as the potential for human exposure through the food chain.

Introduction
In recent decades, the widespread presence of microplastics in the environment has received increasing international attention and concern. Their persistence in environmental compartments and potential for bioaccumulation pose significant risks to ecosystem integrity, food safety, and human health (Miller *et al.*, 2020). Plastics are a diverse group of around one hundred polymers, with polyethylene, polystyrene, polypropylene, polyurethane, polyvinyl chloride and polyethylene terephthalate being particularly prevalent (He *et al.*, 2022). The plastic fragments are categorized by size as macroplastics (>20 mm), mesoplastics (5-20 mm), microplastics (5 mm-1 μm) and nanoplastics (<1 μm) (Lassen *et al.*, 2015; Winiarska *et al.*, 2024). Over time, these materials undergo physical (*e.g.*, photodegradation), chemical, and mechanical degradation, fragmenting into microscopic particles and generating a "*synthetic sand*" that can disperse widely throughout ecosystems. The environmental distribution of microplastics depends on the density and physical characteristics of the fragments. This is particularly pertinent in marine environments, where microplastics can be easily ingested by living organisms due to their small size and morphological similarity with food particles. Bivalve mollusks are particularly exposed to ingestion, because, owing to their filter-feeding behavior, they continuously filter suspended particles from the surrounding water (Sharifinia *et al.*, 2020; Ding *et al.*, 2022). Consequently, they are widely regarded as effective bioindicators for assessing food safety risks related to microplastic contamination along the food chain (Wesch *et al.*, 2016; Oliviero *et al.*, 2019; Mateos-Cárdenas *et al.*, 2019; Li *et al.*, 2019; Volgare *et al.*, 2022; Kazour and Amara, 2024a, 2024b; Nalbone *et al.*, 2024). This study focused on *Mytilus galloprovincialis*, a filter-feeding bivalve mollusc belonging to the Mytilidae family (Gosling, 1992), with a filtration capacity of approximately 0.2–2.5 L h^{-1} per standard individual (1 g dry weight) (Denis *et al.*, 1999). Various investigations conducted in

different geographical areas have demonstrated its attitude to behave as a good marine pollution indicator (Marrone *et al.*, 2011) and its ability to capture and bioaccumulate microplastics, underscoring its ecological relevance within the food-web (Catarino *et al.*, 2018; Webb *et al.*, 2019; Pazos *et al.*, 2020; Nalbone *et al.*, 2021; Zhao *et al.*, 2022). It is a key species in global and Italian shellfish farming, with an annual production of approximately 100,000 tons and per capita consumption exceeding 1.7 kg/year (FAO and WHO, 2023; EUMOFA, 2024). The common practice of consuming the whole organism further amplifies its importance from a food safety perspective. Against this background, the aim of this experimental study is to evaluate the depurative capacity of *M. galloprovincialis* when exposed to controlled contamination with polyester microfibers (PET MFs). Understanding the interactions between MFs and bivalves is important, as microplastic accumulation may affect functional processes such as filtration, with potential implications for the ecosystem services provided by mussels. While other studies have investigated long-term exposure scenarios (Christoforou *et al.*, 2020), the present study focuses on short-term uptake and depuration dynamics, in timeframes closer to those typical of aquaculture and food production practices. Specifically, the study seeks to quantify changes in MF load within mussels by assessing bioaccumulation in the digestate and retained particles on the filters used for water filtrations, which act as indicators of dispersed and non-bioaccumulated particles, relative to the initial exposure dose. Most existing studies use virgin plastic fragments at unrealistically high concentrations rather than fibers (the most common form of microplastic in the environment). By contrast, the use of MFs at ecologically plausible concentrations represents a more realistic experimental approach and provides more representative data for investigating mechanisms of bioaccumulation and depuration in mussels. Due to their relevance as pollutants, PET MFs were chosen for this study. This is a synthetic polymer widely used in textiles and contributes significantly to environmental contamination, particularly during laundering processes (De Falco *et al.*, 2018). The resilient nature of these particles allows them to persist in both freshwater and marine environments, where they can remain for prolonged periods and exert long-lasting environmental effects (Iroegbu *et al.*, 2021; Šaravanja *et al.*, 2022). Their use in this study is important as it enables the investigation of a topic that has not yet been sufficiently explored in the scientific literature.

Materials and Methods

Sampling and maintenance of mussels

Approximately 2 kg of live *M. galloprovincialis* (shell length along the longest axis: 4.7-7.5 cm) were obtained from a depuration center in Messina (Italy). The specimens were placed in plastic nets protected with aluminum to prevent contamination and were then transported in a refrigerated container to the Microplastics in Food Analysis Laboratory (“PLASTOFF Lab”) - Animal-Origin Food Inspection Unit, Department of Veterinary Sciences (Messina, Italy). In the laboratory, the mussels were acclimated for seven days in a seawater tank containing water that had been filtered through a 110 mm-diameter filter (LLG LABWARE, Meckenheim, Germany) with a pore size of 1.6 µm. The tank was maintained at a temperature of 14°C using a chiller (RESUN CL-280, Shenzhen, China) and the seawater was continuously aerated and circulated with the aid of a pump (LNICEZ, Shenzhen, China). A skimmer and a filter were also present (Hydor, Ferplast S.p.A., Castelgomberto, Italy). The light/dark cycle was 12 h, and the mussels were fed once daily with a microalgae suspension (Coral Fito marine supplement, AGP, San Giovanni in Persiceto, Italy). The mussels were then rinsed to remove any external residues and transferred to contamination tanks for 24 h (as reported in section 2.3).

Preparation of polyester microfibers for contamination

PET MFs were obtained by cryogenic milling. PET fabrics were first cut into small pieces (approximately 1×1 cm) using steel scissors. The samples were then immersed in liquid nitrogen and milled using an ultracentrifugal mill (ZM200, Retsch GmbH, Haan, Germany) equipped with an 80 µm grinding mesh and operated at 14,000 rpm. After milling, the resulting PET MFs were analyzed by optical microscopy to determine their size distribution, and by FTIR spectroscopy to verify that the polymer chemical structure was not degraded during the fragmentation process. The PET MFs (≤200 µm, Ø 18 µm) were suspended in filtered seawater by vortexing to obtain nominal concentrations of 100, 10, 1, and 0.1 MF/mL. The PET density (~1.36 g/cm³) enabled dispersion without the use of surfactants (as is the case with other types of polymers). A suspension of MFs in 10 mL of filtered seawater was initially prepared with a known weight of MFs. The suspension was then subjected to serial tenfold dilutions, and MFs were enumerated under a stereomicroscope (LEICA M205C, Germany) to determine the initial concentration and calculate the target contamination dose for the exposure tanks.

Experimental mussel contamination

Two pilot systems were set up, each comprising a 60 L main tank containing approximately 5 L of water and two 10 L inner tanks (with 4 L of filtered seawater and 1 oxygenator each) for contamination and depuration. The system was designed so that the cooling applied to the main tank was transmitted, by convection, to the inner tanks, thereby ensuring a uniform temperature. This configuration made it possible to maintain homogeneous thermal conditions across all sections of the system, while avoiding the use of an additional refrigeration pump which, otherwise, could have generated turbulence and caused the continuous resuspension of MFs in the water, potentially interfering with their capture by mussels. Moreover, the use of a single cooling unit resulted in a significant reduction in energy consumption. The water in the main tank was kept at a temperature of 15°C using a recirculation pump (60 L/h) connected to a chiller (RESUN CL-280, Shenzhen, China). After acclimatization, groups of ten mussels were secured to polylactic acid (PLA) supports (to maintain all mussels in the center of the water column) using a cyanoacrylate adhesive, applied externally (no observable adverse effects on mussel behavior were detected during the experiment), and exposed to MFs for 24 h. Three replicates were conducted per concentration (30 individuals per replicate). For each replicate, two mussels were also collected in parallel from the acclimation tank (*i.e.*, prior to exposure). These specimens were subjected to the same analytical procedures applied to the contaminated and subsequently depurated mussels, to verify the possible presence of pre-existing contamination before the start of the experimental process. These individuals constituted the control group (total n=6).

Water analysis

After 24 and 48 h of depuration, the tank bottoms were siphoned to collect the MFs egested by the mussels in the feces and pseudofeces. The water samples were then filtered through 5 µm cellulose ester filters (Ø 47 mm, Merck Millipore, Ireland) using a vacuum filtration system (Biosigma, Cona, Italy).

Mussel analysis

Mussel soft tissues and intra-valvular liquid were collected and weighed. They were then digested with a 1:40 (g wet weight/mL) ratio of 10% potassium hydroxide (KOH, Sigma-Aldrich, Missouri, USA) for 48 h at 42°C in a shaking incubator (Vdrl Asal 711/CT, Bioltecnical Service, Italy), according to Nalbone *et al.* (2024). The digestates were then filtered using 5 µm cellulose ester

filters (Ø 47 mm; Merck Millipore, Ireland) using a vacuum filtration system (Biosigma, Cona, Italy). The filters were then retrieved using flat-nose steel tweezers and placed on glass Petri dishes (Ø 60 mm, Normax, Portugal). Filters from water and digestates were examined under a stereomicroscope (Leica M205C) to count and identify MFs. To ensure accuracy, each filter was divided into four quadrants and analyzed separately.

Quality control

To minimize environmental contamination, all equipment and surfaces were cleaned with ultrapure deionized water that had been filtered through sterile 0.45 µm cellulose ester filters (Ø 47 mm; Merck Millipore, Burlington, MA, USA). Contamination and depuration aquaria were cleaned immediately before use and filled with freshly filtered seawater. Sample preparation occurred in a laminar flow hood, with white filters placed near the workspace during processing. Mussel vitality was monitored daily by evaluating reactivity, valve closure, absence of abnormal odors, and tissue integrity (Palese and Palese, 1991). Non-vital specimens were excluded. Surface foam formation was also monitored; persistent or excessive foam prompted checks of water quality and organism status.

Statistical analysis

Data from the three independent replicates are expressed as the mean ± standard deviation. The number of MFs retained by mussels after a 24-h exposure period was calculated as the sum of those egested at 24 and 48 h, plus those extracted from the mussels, divided by the number of individuals. The number of MFs retained in mussels after 24 h of exposure, the number of particles egested in the water samples after 24 h and 48 h of depuration, and the residual MFs within mussels after 48 h of depuration were compared among the exposure groups to evaluate whether exposure concentration influenced the organisms' accumulation and depuration capacity. Normality was tested using the Shapiro–Wilk test. Differences among groups exposed to varying concentrations were assessed using a one-way ANOVA with a Dunnett T3 post hoc analysis. Differences in MF egestion after 24 and 48 h were analyzed using two-tailed t-tests. Statistical significance was set at $p<0.05$ and the data were analyzed using GraphPad Prism 9.1.1 (San Diego, CA, USA).

Results

On average, FTIR spectra confirmed the chemical integrity of the PET MFs, showing no significant alterations or functional group shifts after cryogenic milling (Figure 1).

At an exposure concentration of 0.1 MF/mL, mussels retained 1.87±0.47 MFs after 24 h. During depuration, 3.67±1.53 MFs were recovered from the water after 24 h, increasing to 9.67±9.07 after 48 h, although variability among replicates was high. The residual load within mussels at 48 h was 0.53±0.58. At 1 MF/mL, retention after 24 h exposure reached 3.33±0.25 MFs. Waterborne MFs measured 14±3.61 after 24 h depuration and 12.67±3.21 after 48 h. The residual number within mussels at 48 h was 0.67±0.15. At 10 MF/mL, mussels retained 4.27±2.29 MFs after exposure. Depuration yielded 8±5.29 MFs at 24 h and 32.33±29.14 at 48 h. Residual MFs within mussels at 48 h were 0.23±0.25. At 100 MF/mL, retention after exposure was 5.13±1.44 MFs. Recovered MFs were 15.67±7.57 at 24 h and 31.33±7.51 at 48 h.

Statistical analysis revealed no significant differences among the tested groups. No contamination by the experimental MFs was detected in the control group. However, microplastics that had not been added experimentally were observed, suggesting the presence of background particles that were not related to the experimental treatment.

Discussion

The results of this study suggest that there is no linear correlation between the concentration of PET MFs to which mussels are exposed and the quantity of MF accumulated by the mussels or egested into the depuration water, after either 24 or 48 h (Figure 2A). This suggests that the accumulation and egestion of MFs by *M. galloprovincialis* does not follow a linear, dose-dependent model. This lack of correlation may be due to physiological factors unique to mussels, such as the presence of selective filtration mechanisms that prevent the accumulation of undesirable particulate matter. The mechanisms underlying this phenomenon lie in the structure and function of the mussel's gills. The gills, which consist of ciliated filaments arranged in two rows on either side, form the core of its filtering system. Water flow is generated by ciliary action (Riisgård and Larsen, 2001) and suspended particles are retained, selected and transported to the mouth via mucociliary processes (Ward *et al.*, 1998; Rosa *et al.*, 2018). This process is species-specific: in mussels, for example, it only occurs on the palps (Milke and Ward, 2003), and any particles that are not ingested are encapsulated in mucus and expelled as pseudofaeces (Ward *et al.*, 2019). The selectivity of rejection or ingestion is influenced by parameters such as size (*e.g.*, larger particles of 100-600 µm tend to be rejected as pseudofaeces due to anatomical constraints), morphology, and surface characteristics (Ward and Shumway, 2004; Rosa *et al.*, 2018; Ward *et al.*, 2019). From a morphological perspective, the GESAMP Report (2019) categorizes microplastics as fibers (thin filaments often derived from synthetic textiles or fishing nets), pellets (small spheres or cylinders used as raw materials), fragments (irregular pieces resulting from the breakdown of larger items), films (thin sheets, such as packaging), foams (expanded materials, such as polystyrene) and granules (spherical or oval particles used in cosmetics or detergents). Previous studies (Van Cauwenberghe *et al.*, 2015; Ward *et al.*, 2019) support the hypothesis of selective rejection, showing that filter-feeding bivalves do not passively ingest all particles present in the environment. This selection is based on factors such as particle shape (*e.g.*, long, thin fibers) and the muscular movements of the gills, which adjust the distance between adjacent folds. Particles with a high length-to-width ratio are more likely to be rejected. Additionally, both generic surface characteristics (*e.g.*, electric charge, wettability and hydrophobicity) and particle-specific features interact with the mucus covering the filtering structures, influencing whether particles are rejected or ingested. Particles ranging from several hundred to 1000 µm are efficiently captured but are more likely to be expelled as pseudofeces. For the MFs tested in this study (<200 µm), mussels effectively transfer only very small particles (<10 µm) into the digestive tract, where they can either reach different organs or be eliminated as feces. The observed accumulation plateau is consistent with this selection process. Indeed, the quantity of MFs accumulated in mussels was similar across all tested concentrations, including the lowest (0.1 MF/mL, ~2 MFs per mussel), supporting the hypothesis of a physiological limit regulating MF uptake. While the absence of statistically significant differences in egestion rates after 24 and 48 h contrasts with some literature, the present data show a higher number of particles released after 48 h (e.g., ~32 MFs per mussel at 10 MF/mL) compared to 24 h (~8 MFs per mussel at 10 MF/mL), although this difference is not statistically significant (Figure 2B). This discrepancy could be explained by several hypotheses. Firstly, other mechanisms may regulate the amount of ingested particles, such as a reduction in egestion rates. In other words, there may be a physiological limit to the depurative capacity of mussels, whereby egestion speed stabilizes once a saturation threshold is reached, reducing time-related differences. Secondly, the timing of particle rejection may be influenced by the organism's metabolism, with some mussels possibly limiting energy expenditure (given that the specimens were not fed) and consequently reducing depuration activity. This would result in the temporary retention of some MFs before they are subsequently expelled. It should also be noted that the selection process is neither perfectly efficient nor immediate, and some particles

may be retained for variable periods before expulsion. Much may also depend on the surface and specific characteristics of the plastic particles (PET MFs, in this case), and the differences observed in other studies could be due to the use of different types of microplastics, which have distinct properties and are subject to different selection processes by mussels. Another important factor to consider is the potential presence of underlying pathologies (*e.g.*, parasitic or microbial) which could affect the depuration capacity of mussels. Finally, it cannot be excluded that the lack of statistical significance may be due to underestimation. Individual fibers can usually be distinguished (Figure 3A); however, some fibers may entangle, forming aggregates that are difficult to quantify under the microscope and resulting in lower total counts (Figure 3B). Additionally, a limited sample size could have reduced the statistical power of the analysis, potentially masking real differences between the two time points considered. One final consideration is the presence of background microplastic contamination, morphologically and chemically distinct from the PET MFs used in this study. These were detected on the digested tissue filters and in the water filters (Figure 4). While this finding is only marginally relevant to the primary objective of this study, it is significant as it suggests pre-existing contamination of the organisms. This finding once again highlights the pervasiveness of microplastics in the environment and underscores the need for further research into depuration mechanisms in relation to food safety.

Conclusions

The results of the present study suggest that the accumulation and removal of MFs in mussels may not be directly proportional to the exposure concentration. Other factors, such as particle size, shape and spatial arrangement, are more likely to influence these processes. Since most studies use virgin plastic fragments, often at unrealistic concentrations, rather than fibers, which are the most widespread form of microplastic in the environment, using textile-derived PET MFs enhances the ecological relevance of this study. This methodology provides more representative data with which to investigate bioaccumulation and depuration mechanisms in mussels, enabling biological responses to be assessed under ecologically plausible conditions. While this study contributes to our understanding of MF accumulation and egestion mechanisms in mussels, further research could strengthen and expand upon the observations reported herein. In particular, longer-term experiments and the inclusion of histological analyses could help reduce the effect of individual variability and provide a more detailed understanding of the tissue-specific localization and potential translocation of MFs within the organism.

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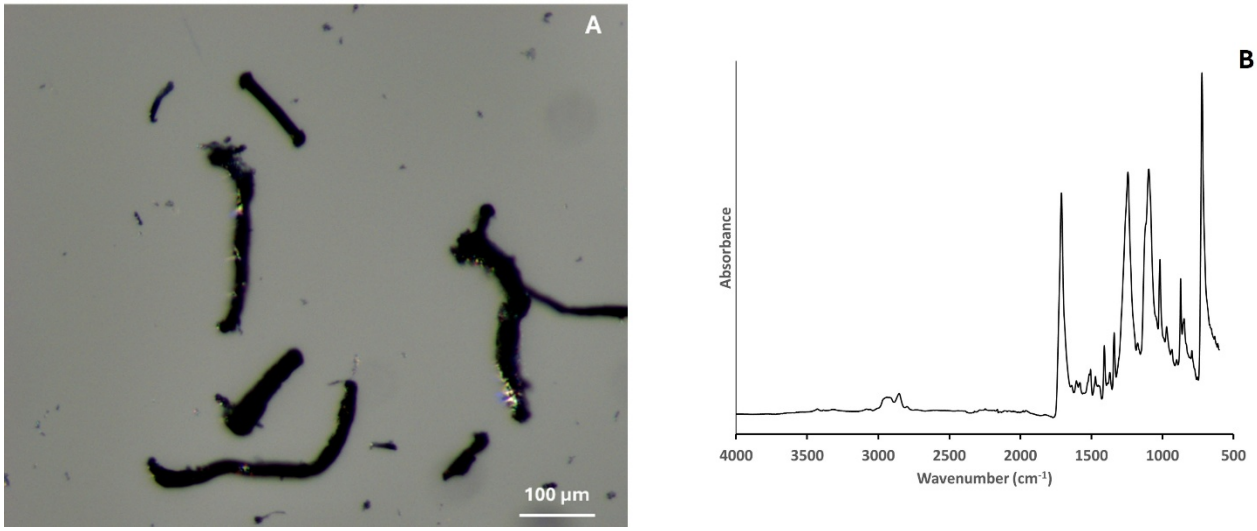

Figure 1. PET MFs under optical microscope (A) and their FTIR spectrum (B).

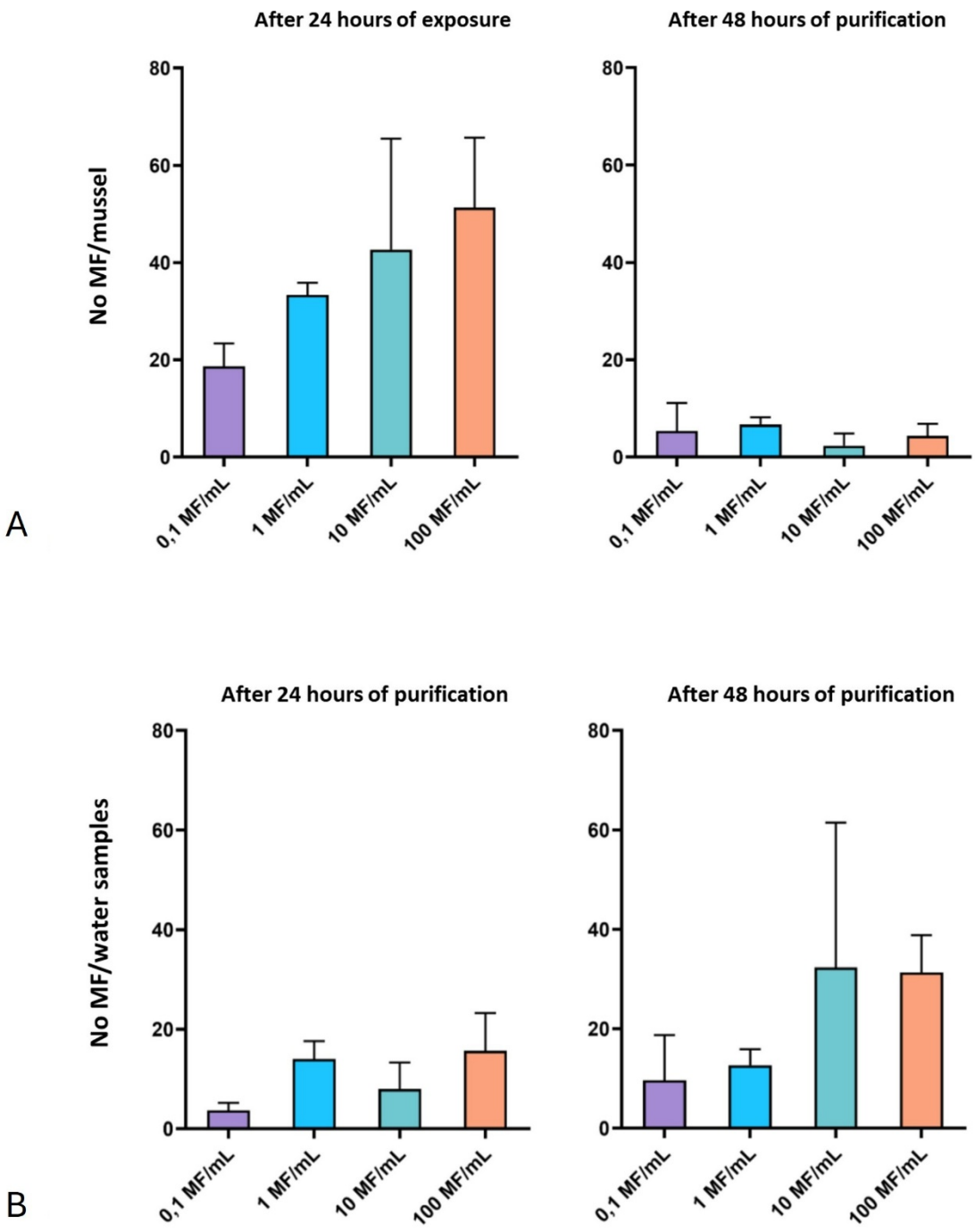

Figure 2. A comparison of the number of MFs remaining after 24 hours of exposure and 48 hours of purification (A) and a comparison of the number of MFs remaining on water filters after 24 and 48 hours of purification at different mussel concentrations (B).

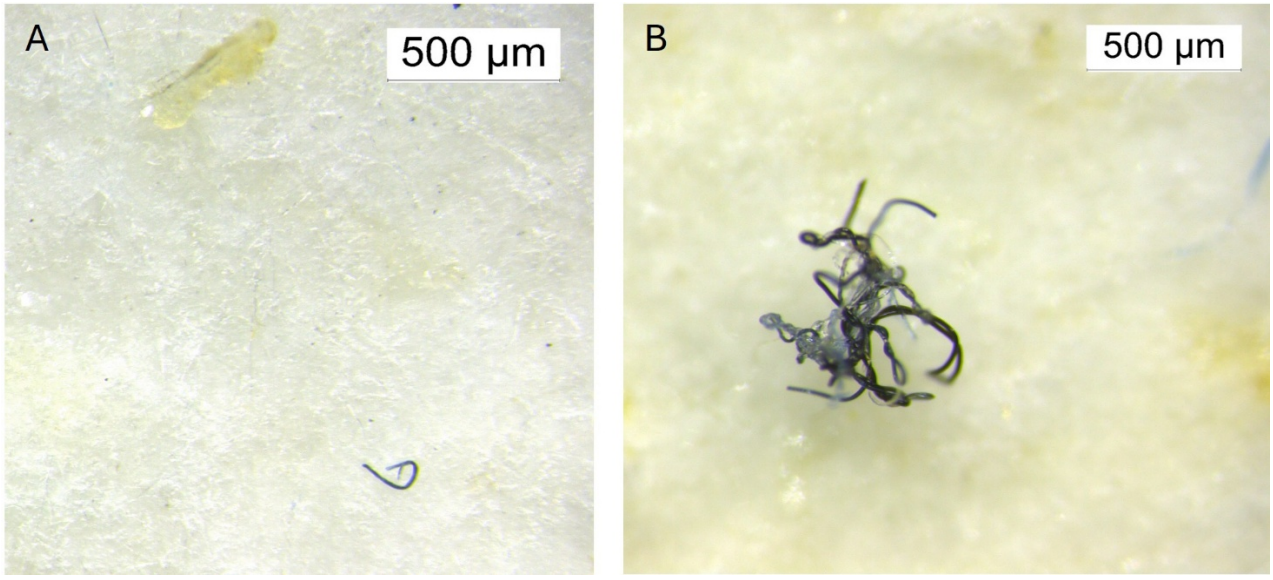

Figure 3. Stereomicroscope aspect of MFs on digestate filters (A). In some cases, fibres may become entangled, forming aggregates (B).

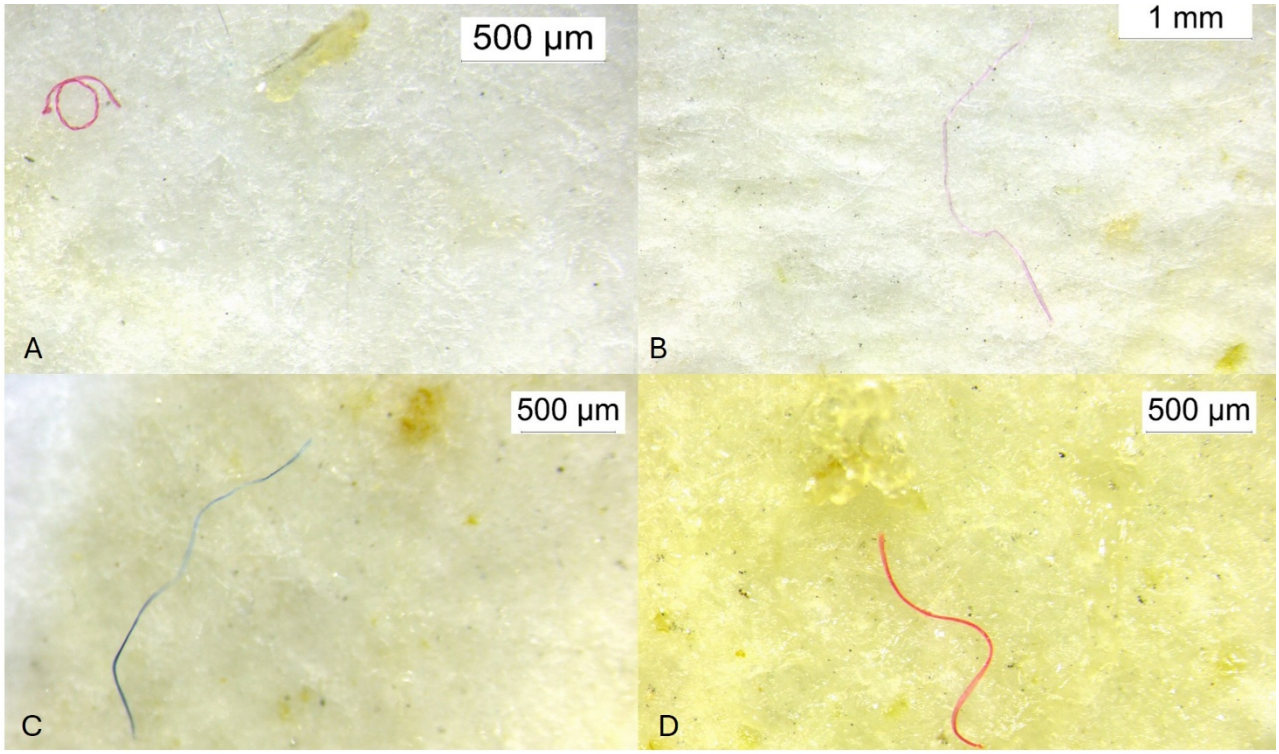

Figure 4. Representative stereomicroscopic images of MFs detected in water and digestate filters, not attributable to experimental contamination, showing different morphologies and colouration (A-D).