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Application of an enzyme digestion method reveals microlitter in *Mytilus trossulus* at a wastewater discharge area

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ABSTRACT

The ingestion of microlitter by blue mussels (450) was studied at a wastewater recipient area in the Baltic Sea. The mussel soft tissues were digested using enzymatic detergents and the detected litter particles characterized with FT-IR imaging spectroscopy. Microlitter concentration in seawater and WWTP effluent were also measured. Microlitter was found in 66% of the mussels. Mussels from the WWTP recipient had higher microlitter content compared to those collected at the reference site. Plastics made up 8% of all the analysed microlitter particles. The dominating litter types were fibres (~90% of all microlitter), 42% of which were cotton, 17% linen, 17% viscose and 4% polyester. The risk of airborne contamination during laboratory work was lowered when mussels were digested with their shells on instead of dissecting them first. The approach was found applicable and gentle to both non-synthetic and synthetic materials including fragile fibres.

1. Introduction

Microlitter (ML) is an ever-increasing form of marine litter commonly defined as particles < 5 mm in diameter (Arthur et al., 2009). These particles include e.g. fibres, fragments, flakes and spheres that typically originate as fragmentation of larger litter items (secondary microlitter) or are intentionally manufactured to their specific size (primary microlitter, Kershaw, 2015). Microlitter consists of various anthropogenic particles, from either organic or synthetic origin, but microplastics (MP) have gained most of the attention in research. The globally increasing production of mismanaged plastic waste (Jambeck et al., 2015) combined with the longevity of plastics in marine environment has raised serious concern of the harm they may pose to the marine environment (Thompson et al., 2004).

Marine microlitter derives from a variety of sources. Like larger litter items, most of the smallest litter fractions are land-based (Andrady, 2011). Terrestrial sources and pathways of microlitter include e.g. industrial outfalls, traffic, municipal wastewaters and sludge (Barnes et al., 2009; Andrady, 2011; Magnusson et al., 2016a; Talvitie et al., 2017). Once in the environment, the sources of marine litter are difficult to identify, and the smaller and more fragmented the particles are, the more difficult the identification becomes. However, some sources and pathways can be monitored, and the actual load of microlitter entering marine environment be assessed, like in the case of municipal wastewater treatment plants (WWTPs) (Murphy et al., 2016; Talvitie et al., 2017; Mintenig et al., 2017; Ziajahromi et al., 2017).

WWTPs receive water from households, industries, commercial establishments and sometimes as urban rainwater runoff (e.g. Magnusson and Noren, 2014). Households generate large quantities of microlitter of which the most commonly known types are textile fibres from washing of clothes (Browne et al., 2011; Sillanpää and Sainio, 2017) and plastic microbeads from personal care products (Fendall and Sewell, 2009). During conventional wastewater treatment processes, the majority of microlitter (98–99%) can be removed from the influent (Magnusson et al., 2016a; Murphy et al., 2016). Despite the seemingly high removal rate, wastewater effluent can still be a significant source of microlitter as large volumes of wastewaters are discharged into the aquatic environments constantly (Magnusson and Noren, 2014; Murphy et al., 2016; Talvitie et al., 2017). However, the actual microlitter load deriving from WWTPs depends on the population equivalent load served (PE) and the treatment level. A study conducted in a relatively small secondary WWTP with PE of 14,000, estimated that 40,000 microplastic particles were released daily with the effluents (Magnusson and Noren, 2014). On the other hand, only primary treated effluent from large (PE > 1 million) WWTPs can release hundreds of millions MPs into the environment per day (Ziajahromi et al., 2017).

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Microplastics are of special concern due to their small size (< 5 mm). With decreasing size, their availability and potential to accumulate throughout the marine food web increases, where they may cause severe harm at different trophic levels (e.g. Laist, 1987; Mato et al., 2001; Sussarellu et al., 2016). The ingestion of microplastics has been demonstrated in a variety of invertebrate taxa and has been connected to harmful effects such as immune system stress responses in mussels (Von Moos et al., 2012; Avio et al., 2015), reduced activity levels e.g. burrowing activity in polychaete worms (Wright et al., 2013) as well as lowered reproductive levels in oysters (Sussarellu et al., 2016). Microplastics may expose organisms to various hazardous substances including plasticizers (Fries et al., 2013), persistent organic pollutants (POPs) (Rios et al., 2007; Chua and Pumera, 2014) and toxic metals (Rochman et al., 2014). These substances are either added upon manufacturing or adsorbed from the surrounding water (Teuten et al., 2009). Microplastics have also been found in seafood cultivated for human consumption (Van Cauwenberghe and Janssen, 2014).

One of the challenges in microlitter studies is to verify the presence of anthropogenic particles in environmental samples. Various digestion methods, using strong acids (e.g. De Witte et al., 2014; Van Cauwenberghe and Janssen, 2014), bases (Claessens et al., 2013, Dehaut et al., 2016), oxidizing chemicals (Li et al., 2015, Dehaut et al., 2016) and enzymes (Cole et al., 2014, Catarino et al., 2017) have been developed. Some of these methods can damage or destroy examined litter particles, leading to underestimations of microlitter content (Dehaut et al., 2016). Previous studies from WWTPs have shown that the proportion of non-synthetic materials in the municipal wastewaters is high (e.g. Talvitie et al., 2017). Therefore the mussel tissue digestion method selected for this study had to be applicable to non-synthetic fibres as well. The effect of various digestion chemicals on different microlitter materials was tested and based on the results a gentle enzyme-based mussel tissue digestion method was developed.

To study the ingestion of microlitter originating from a potential point source, mussels were incubated in cages at close vicinity to a discharge pipe of a municipal WWTP in the city of Hanko (thereafter called recipient area), SW coast of Finland. In this study, we investigated the role of wastewater as microlitter gateway into the Baltic Sea, indicated by the litter contents of a local blue mussel (*Mytilus trossulus*) community and their environment. We also examined options for reducing airborne contamination during mussel treatment in the laboratory.

2. Materials and methods

2.1. Mussel caging experiment

Approximately 450 adult mussels of similar size (shell length 2–3 cm) were collected by scuba diving 1500 m off the southern coast of Hanko (59°48′42.2″N, 23°01′20.1″E) at a depth of 6 m (Fig. 1). 50 mussels were selected to represent the original mussel community (source population), while the remaining 400 individuals were distributed to two stainless steel cages (cage size 350 × 350 mm, mesh size 5 mm) holding five metal mesh trays in five storeys layers (200 individuals/cage). Cages were deployed on the seafloor (hard bottom) ca 5 and 10 m depth. Cage 1 was left at the site of the original mussel collection and cage 2 placed 700 m apart from Cage 1, at 30 m from the mouths of the wastewater discharge pipe. Reference mussels were collected onboard R/V Aranda in May from the Sea of Åland (Fig. 1), at an area not subjected to any known direct anthropogenic stress sources.

The cages were set up on the same day in May 2016 and left in place for the following 4 weeks. 50 mussels were collected from the cages every 7 days during a 4-week period. For the duration of transport to laboratory, mussels were placed in coolers with ice. This approach kept the mussels' environment cool and moist, but did not allow the specimens to filter water and egest the possible microlitter contents during transfer.

Each mussel was rinsed with tap water and the shell length measured with a calliper (average shell length 2.03 ± 0.39 cm) and weighted in a clean laboratory environment (all surfaces were wiped clean, no other laboratory users at the same time, laboratory coats were used). Each mussel was then rinsed again and transferred to a rinsed glass vial (57.5 × 27.3 mm, Fiolax, Germany), each to one vial and the caps were finally rinsed and screwed on. Samples were frozen and stored until further processing. To control contamination, procedural blanks (empty vials) were set up upon bottling the mussels (one for every 10 mussels).

2.2. Mussel digestion method

Frozen mussel samples were placed in a newly wiped fume hood and either left to defrost in room temperature (1 − 2h) or placed in a warm bath to aid the defrosting process. To examine the potential contamination induced by shell removal, half of the samples in each batch (cage 1, cage 2, source population, reference) went through shell removal (non-shelled) and half were left untreated (shelled). The wet weight of the soft tissues of non-shelled mussels from each corresponding study site was measured and the average soft tissue wet weight (cage 1: 0.26 ± 0.01 g, cage 2: 0.28 ± 0.02 g, source population: 0.33 ± 0.01 g, reference: 0.15 ± 0.01 g) was used to calculate microlitter and microplastic content (MP/ML ww g^{-1}) for each individual mussel.

To make sure even the finest ML particles such as non-synthetic fibres would not be damaged during the mussel digestion process, a digestion method adjusted from Löder et al. (2017) was utilised and its applicability tested and compared with other digestion protocols (see Table 1 for further method development details). Pre-filtered Sodium Dodecyl Sulphate (SDS, 5 g/ L, Sigma-Aldrich) and detergent enzymes (Biozym F and Biozym SE, Spinnard, Bad Segeberg, Germany) were used to digest the organic matter. A stock solution was prepared containing 25% of Biozym F (lipase) and 25% of Biozym SE (protease and amylase) and 50% of SDS. This solution was added in each vial (ca 6 mL). Due to the varying shell sizes, the quantity was not absolute. Most importantly, the solution covered each mussel completely.

Once the solution was added, vials were capped with aluminium foil lids and samples placed in an oven at 37.5 °C for 48 h (Fig. 2). The samples were gently swirled in their vials once or twice a day to aid the disintegration of tissue. After the incubation, samples were rinsed with Milli-Q water on 20 μm plankton net filters one by one using a vacuum filtration device using 40 kPa suction. No clogging of the filters was observed. Due to the viscosity of the pure SDS/enzyme mixture being relatively high in the first place, there was no significant difference in the viscosity once the mussel had dissolved in it. The filters were transferred to individual petri dishes and covered at all times possible to prevent airborne contamination.

2.3. Wastewater and seawater sampling

The municipal WWTP which is in the focus of this study treats the wastewaters of ca. 10,000 inhabitants in the Hanko area and 2 million cubic meters (2,077,980 m^3 in 2016) of secondary treated effluent is discharged from the WWTP annually. The treatment process of the plant is based on a conventional activated sludge method including mechanical, chemical and biological treatments and is a representative of the majority of Finnish WWTPs. The effluent is discharged into a relatively open sea area at a depth of ~10 m. Recipient seabed consists of mainly rock, sand and gravel. To study the abundance and distribution of wastewater derived microlitter in the aquatic environment, both the effluent water and the discharge area seawater were investigated.

All equipment including filters, hoses, tubes and glassware were thoroughly rinsed with tap water before use to prevent contamination. Recipient water samples (both caging locations) and effluent samples