Marine Environmental Research 111 (2015) 18–26

Contents lists available at ScienceDirect

Marine Environmental Research

journal homepage: www.elsevier.com/locate/marenvrev

Experimental development of a new protocol for extraction and characterization of microplastics in fish tissues: First observations in commercial species from Adriatic Sea

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ARTICLE INFO

Article history:
Received 28 February 2015
Received in revised form
15 June 2015
Accepted 23 June 2015
Available online 2 July 2015

Keywords:
Microplastics
Marine organisms
Extraction
Polymers
FT-IR
Commercial fish

ABSTRACT

The presence of microplastics in the marine environment has raised scientific interest during the last decade. Several organisms can ingest microplastics with potentially adverse effects on the digestive tract, respiratory system and locomotory appendages. However, a clear evidence of tissue accumulation and transfer of such microparticles in wild organisms is still lacking, partially hampered by technical difficulties in isolation and characterization protocols from biological samples. In this work, we compared the efficacy of some existing approaches and we optimized a new protocol allowing an extraction yield of microplastics from fish tissues ranging between 78% and 98%, depending on the polymer size. FT-IR analyses confirmed that the extraction procedure did not affect the particles characteristics. The method was further validated on the fish mullet, *Mugil cephalus*, exposed under laboratory conditions to polystyrene and polyethylene; the particles were isolated and quantified in stomach and liver, and their presence in the hepatic tissue was confirmed also by histological analyses. A preliminary characterization revealed the presence and distribution of microplastics in various fish species collected along the Adriatic Sea. FT-IR analyses indicated polyethylene as the predominant polymer (65%) in the stomach of fish. The overall results confirmed the newly developed method as a reliable approach to detect and quantify microplastics in the marine biota.

1. Introduction

Plastic pollution in the oceans has been recognized as a world phenomenon with nearly 300 million tons of debris floating at sea surface, or accumulating on seafloor and shorelines from polar regions to the equator (Boerger et al., 2010; Browne et al., 2011; Eriksen et al., 2014; Suaria and Aliani, 2014).

In the recent years, scientific interest has been directed also toward microplastics, i.e. plastic fragments with a grain size lower than 5 mm, which are manufactured *ex novo* for their use in cosmetics, industrial or medical applications, or derived from chemical, physical and biological degradation of larger plastic debris (Barnes et al., 2009; Wright et al., 2013).

Laboratory experiments have shown that microplastics can be ingested by different marine organisms, including polychaetes, crustacean, bivalves and echinoderms (Browne et al., 2008; Gregory, 2009; Graham and Thompson, 2009; Kach and Ward, 2008; Thompson et al., 2004; Von Moos et al., 2012; Van Cauwenberghe et al., 2015b). Due to their hydrophobic properties, microplastics can also adsorb several classes of organic pollutants (Teuten et al., 2007), which may be transferred to organisms and enter the marine food-webs (Teuten et al., 2009; Farrell and Nelson, 2013; Setala et al., 2014); experimental evidence has been recently obtained for the transfer of pyrene from microplastics to mussels (Avio et al., 2015). The consequences of microplastics ingestion may affect the feeding activity, respiratory functions, reproductive output, and also modulate several molecular and cellular pathways (Gregory, 2009; Cole et al., 2015; Avio et al., 2015).

The multiple risks that microplastics pose to marine life prompted their inclusion in some international legislation and marine protection projects, like the European Marine Strategy Framework Directive (MSFD) and the Marine Debris Program of the US National Oceanographic and Atmospheric Administration (NOAA). In this scenario, a better knowledge on the presence and

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http://dx.doi.org/10.1016/j.marenvres.2015.06.014
0141-1136/

characterization of microplastics in marine food webs has become a research priority, and a fundamental step toward a more integrated ecological risk assessment.

Environmental studies on microplastics are often hampered by the limited availability of standardized protocols and technical difficulties in the extraction and characterization of these particles from marine samples. Suitable methodologies have been recently reviewed for sediments and seawater indicating the density separation as the most common approach (Hidalgo-Ruz et al., 2012; Van Cauwenberghe et al., 2015b). More complex is the extraction and quantification of microplastics from organisms, being fragments easily masked within biological material and tissues. In addition, microplastics comprise a very heterogeneous assemblage of pieces that vary in size, shape, color, specific density, chemical composition and other characteristics which should be considered during the development of appropriate methods for their extraction and characterization.

Some recent protocols tested the extraction of microplastics from marine invertebrates after a pre-digestion of organic matter (Claessens et al., 2013); the comparison of various acid, basic or oxidizing treatments revealed that pH-sensitive polymers can be dissolved or partially degraded by certain acids, thus affecting both the estimation and the characterization of the polymers by FT-IR (Claessens et al., 2013). The enzymatic digestion of organic matter with proteinase k resulted as a reliable method to extract microplastics from zooplanktons samples (Cole et al., 2014), but not a cost-effective approach for larger organisms.

The methodological difficulties in isolation protocols can partly explain why, to date, only a few studies specifically addressed the occurrence of microplastics in wild fish populations. Larger plastic fragments (1–5 mm) have been detected in various Pacific and Atlantic species (Choy and Drazen, 2013; Davison and Asch, 2011; Boerger et al., 2010; Pinnegard, 2009; Laist, 1997), while plastic particles <1 mm have been observed for the first time in demersal and pelagic fish from the English channel (Lusher et al., 2013), and in wild gobids, *Gobio gobio*, from French rivers (Sanchez et al., 2014). All these studies were based on a direct visual sorting of the fish stomach contents, without testing the efficiency of the separation methodology. The destruction of organic material with 10% KOH has been recently applied on intestines of fish from the North Sea, following a 2–3 weeks period of alkaline digestion (Foekema et al., 2013): plastic particles were found in 2.6% of examined fish, with typically one item per fish, ranging in size from 0.04 to 4.8 mm (Foekema et al., 2013).

The aim of the present work was to propose a reliable technique for the isolation of microplastics from marine organisms, preventing any aggressive procedure, and thus allowing the further characterization of the polymers by FT-IR or Raman spectroscopy analysis. In this respect, isolated gastrointestinal tracts of fish were spiked with known amounts of previously characterized microplastics polymers: the extraction yields were then assessed for different methods, including some published protocols and a new one that combined various steps of the available techniques. Tested protocols allowed to extract particles generally within 24 h; in this respect, we did not include the protocol of Foekema et al. (2013) requiring up to 3 weeks of alkaline digestion.

Possible dimensional limits of the newly developed procedure were tested toward microplastics of different size classes, from 5 mm to less than 0.1 mm, while FT-IR analyses were used to evaluate the integrity of polymers structure. The method has been further validated to analyze accumulation and transfer of microplastics in the gastrointestinal tract and liver of mullets, *Mugil cephalus*, exposed in laboratory conditions to polyethylene and polystyrene polymers. This species was selected as an experimental model due to its commercial importance and wide distribution in the Mediterranean Sea; being an omnivorous fish, it is also potentially exposed to pollutants and microplastics ingestion, and previous studies demonstrated its utility for ecotoxicological studies (Gorbi et al., 2005; Whitfield et al., 2012). Finally, a preliminary field assessment study was carried out to investigate the occurrence, typology and characteristics of microplastics in wild fish species from the Adriatic Sea, with different trophic guilds and ecological characteristics.

The results of this study were expected to provide a practical contribution toward the standardization of appropriate procedures for isolating microplastics from marine organisms, providing new insights on the presence, distribution and typology of these particles in commercial fish species from the Adriatic.

2. Material and methods

2.1. Polyethylene and polystyrene particles preparation

A stock of polyethylene and polystyrene powder was obtained from a private plastic company and sorted in four different grain size classes (5–1 mm; 1–0.5 mm; 0.5–0.1 mm; 0.1–0.01 mm). The particles were used to test various extraction protocols and for the laboratory exposure of the mullets *M. cephalus*.

2.2. Maintenance and acclimation of fish in laboratory conditions

Mullets, *M. cephalus*, were obtained from a local aquaculture and maintained in laboratory conditions with filtered and aerated seawater, at 18 ± 1 °C, salinity 37 for at least two weeks before exposures. Fish were daily fed with a specific grower feed (by Aller-Aqua, Aller Thalassa 2 mm, crude protein 50%, crude fat 15%); this commercial pellet was analyzed and confirmed to be microplastics free (data not shown). All the maintenance and experimental procedures were in accordance with requirements of the Ethical Committee of the Polytechnic University of Marche for scientific activities with marine organisms.

2.3. Protocols for plastic extraction

The efficacy to extract microplastics from fish tissues was evaluated for six different protocols, including five already published and a newly developed one; these procedures were compared in terms of percentage recovery of known amounts of previously characterized particles “spiked” in gastrointestinal tract of laboratory-acclimatized fish.

To this aim, 60 acclimatized mullets (length 24.6 ± 2.7 cm) were euthanized by submerging the fish in ice before cervical transection; gastrointestinal tracts (from the esophagus to the anal sphincter) were removed, homogenized and spiked with microplastics (35 g of tissues were added and accurately mixed with 5 polyethylene and 5 polystyrene particles, 1–0.5 mm grain size). Samples were processed as outlined below, using 10 gastrointestinal tracts for each protocol.

Protocol 1, consisted in the direct visual sorting of the stomach content according to Choy and Drazen (2013). Particles were counted, photographed and measured with a stereomicroscope (Optika SZM-D, equipped with a DinoEye Camera AM-423X and Dino Capture 2.0 software).

Protocol 2, previously proposed for microplastics determination in sediments (Thompson et al., 2004) was slightly modified for fish samples. Briefly, after the addition of plastic particles, the gastrointestinal tracts were homogenized in ultraturrax and 1 L of hypersaline NaCl solution (1.2 g/cm^3) was added to each samples: quite surprisingly this treatment did not result in the expected density gradient separation, at least within 24 h, thus preventing