Marine Pollution Bulletin 160 (2020) 111553

Contents lists available at ScienceDirect

Marine Pollution Bulletin

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

Baseline

Microplastics presence in cultured and wild-caught cuttlefish, *Sepia officinalis*

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ARTICLE INFO

Keywords:
Aquaculture
Cuttlefish
Food web
Human consumption
Ingestion
Microplastics

ABSTRACT

Amongst cephalopods microplastics have been reported only in jumbo squid gut. We investigated microplastics in the digestive system of wild cuttlefish (*Sepia officinalis*) as they are predators and prey and compared the stomach, caecum/intestine and digestive gland (DG) of wild and cultured animals, exposed to seawater from a comparable source. Fibers were the most common type (≈90% of total count) but were ≈2× higher in relation to body weight in wild vs. cultured animals. Fibers were transported to the DG where the count was ≈2× higher /g in wild (median 1.85 fibers/g) vs. cultured. In wild-caught animals the DG was the predominant location but in cultured animals the fibers were more evenly distributed in the digestive tract. The potential impact of microplastics on health of cuttlefish is discussed. Cuttlefish represent a previously unrecognized source of microplastic trophic transfer to fish and finding fibers in cultured animals has implications for aquaculture.

1. Introduction

Plastics, particularly in the form of microplastics, have been identified in the gastrointestinal tract of a diverse range of vertebrate and invertebrate species (Andrady, 2011; Lusher et al., 2013; Gall and Thompson, 2015; Lusher, 2015; Lusher et al., 2017; Nelms et al., 2018, 2019; Bucci et al., 2020; Kuhn and van Franeker, 2020). However, as far as we can ascertain there are only two published reports identifying plastics in the digestive tract of cephalopods. Both reports are from jumbo squid (*Doscidicus gigas*) as an invasive species in Pacific Canada (Braid et al., 2012) and as a commercial species in Ecuador (Rosas-Luis, 2016). In a study aimed at analysis of food and paralytic shellfish toxins plastic pellets (nurdles) were identified incidentally in the stomach of 8 out of 30 *D. gigas* (26%) from two locations, with 11 nurdles being the highest number found in a stomach (Braid et al., 2012). Fishing line was also identified but the incidence and quantity was not reported. Rosas-Luis (2016) specifically studied the presence of plastics in *D. gigas* finding them in 12 of 160 stomachs sampled (7.5%) making up < 1% by weight of total contents (food remains), but in 25% of the stomachs containing plastic it was the only item. Multifilament polyethylene fishing line was found in 11/12 stomachs and pieces of polyvinylchloride (PVC) fishing float found in one stomach. The lack of information on the presence of plastics in the digestive tract of cephalopods prompted us to investigate the common European cuttlefish, *Sepia officinalis*.

Cuttlefish are a widely exploited marine resource in the Mediterranean and the common European cuttlefish (*S. officinalis*) has been identified as a species potentially suitable for industrial aquaculture (Sykes et al., 2006, 2014), so it is relevant to investigate the occurrence of microplastics in the digestive tract of both wild-caught and cultured individuals. While wild-caught animals will primarily ingest MPs from the food web, cultured animals have a higher potential for MP ingestion from plastics used for aquaculture seawater systems. The project aims were: 1) Investigate the presence of microplastics in the lumen of the stomach or caecum/intestine (digestive tract) or in the digestive gland (also called the hepatopancreas or midgut gland) of wild-caught cuttlefish to extend the range of cephalopod species studied; 2) Investigate whether microplastics were present in the digestive system (digestive tract + digestive gland) of cultured cuttlefish; 3) Compare the findings from the two cuttlefish populations.

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https://doi.org/10.1016/j.marpolbul.2020.111553
Received 9 April 2020; Received in revised form 5 August 2020; Accepted 5 August 2020

2. Material and methods

2.1. Ethical statement

The study investigated *S. officinalis* that died naturally (presumed senescence) during culture at Ramalhete marine station (Faro, Portugal) and others bought from the nearby market of Olhão (South Portugal). Study approval was not required from the CCMAR Animal Welfare Committee (ORBEA CCMAR-CBMR) or from Direcção-Geral de Alimentação e Veterinária (DGAV) of the Portuguese Government.

2.2. Sample collection and preparation

Six cultured adult cuttlefish (body weight [mean ± SD] 433.7 ± 218.7 g; ♀ 284.7 ± 86.0 g (N = 3), ♂ 582.7 ± 213.4 g (N = 3) and ≈300 days post hatch) and 6 wild-caught cuttlefish (body weight ♀ 248.3 ± 44.3 g) were used for sampling the digestive system. The cultured cuttlefish were fed live grass shrimp (*Palaemon* sp. from the surrounding lagoons of Ramalhete) during the hatchling stage (≈ up to 30 days post hatch) and frozen grass shrimp ad libitum (remainder of the life cycle). Culture methods are detailed in Sykes et al. (2006, 2014). The location of the marine station where the culture was undertaken is inside the Ria Formosa lagoon system, drawing its water from the lagoon and the capture zone of the wild-caught cuttlefish is on the coast just outside the lagoon, providing a unique opportunity for a direct comparison The wild-caught cuttlefish were bought at Olhão market in November 2018, after being commercially captured nearby, outside the Ria Formosa lagoon system on the Algarve coast in an inshore zone with a sandy bottom (see Quintela and Andrade, 2002 for map). The grass shrimp were caught in saline ponds in the Sado estuary in Setubal, Portugal, an environment comparable to that in the vicinity of the Ramalhete marine station.

Cultured cuttlefish were thawed in a refrigerator. All animals were washed in filtered seawater (20 μm retention). Dorsal mantle length (cm), body wet weight (g) and the wet weight (g) of different regions of the digestive system were determined. The following samples were taken for analysis: digestive gland; oesophagus and stomach plus their content; caecum and entire intestine plus their content (see Tompsett, 1939 for a guide to the anatomy of the cuttlefish digestive system) were collected from each cuttlefish and placed in glass flasks. Procedural controls were used to screen for possible airborne contamination from microfibers at all stages of sampling and processing (Supplementary File). Samples were frozen at −20 °C until analysed.

2.3. Enzymatic digestion of tissues

Samples were subjected to an adapted enzymatic digestion protocol based on Lindeque and Smerdon (2003), Cole et al. (2011), Lusher et al. (2017), and Nelms et al. (2018). The thawed samples were homogenized with a glass rod and Milli-Q water (Millipore ultrapure water), before oven drying at 60 °C for 24 h. After cooling, the dry weight (DW) of each tissue was determined (Kern EW6000-1 M) and used to calculate the required volume of the homogenizing solution (400 mM Tris-HCl buffer [VWR Chemicals], 60 mM EDTA [ethylene diamine tetra acetic acid disodium salt dihydrate; VWR Chemicals], 150 mM NaCl [VWR Chemicals], 1% SDS [sodium dodecyl sulfate; Amresco] and Milli-Q water), previously filtered through Whatman grade 4 filters (25 μm retention). Fifteen milliliters per 0.2 g of dried tissue of the homogenizing solution was added to each sample, flasks were covered with aluminum foil and samples incubated at 50 °C for 30 min in a water bath. The flasks were stirred on a shaker (VWR International) for 30 min while cooling. Proteinase K (VWR Life Science) at 500 $\mu g.mL^{-1}$ per 0.2 g DW of sample tissue was added and samples incubated at 37 °C for 24 h in the water bath. Afterwards, 3 mL of 5 M $NaClO_4$ [VWR Chemicals] per gram DW was added and flasks shaken for 30 min at room temperature on the shaker at low speed (2/10). Again, samples were incubated at 60 °C overnight in the water bath. Samples were then filtered on a vacuum system through a Whatman grade 4 filter (25 μm retention). They were dried in closed glass Petri dishes in an oven overnight (at 37 °C) and later stored in a desiccator, wrapped in aluminum foil, until observation under a light microscope.

2.4. Microplastic quantification and identification

The filter papers (36 samples distributed on 50 filters because of sample size) were placed inside closed glass Petri dishes and observed under a microscope (Zeiss Stemi 2000-C) with a 25–50× magnification and photographed with a VWR Visicam 10.0 camera. A gridded transparent sheet was placed over each Petri dish and microplastics categorized, counted and measured in a row-by-row pattern to minimize errors. Microplastics were categorized as fibers, microfilm pieces, plastic fragments (irregular shaped and flat) or beads (spherical or ovoid). A set of samples (11% of total; N = 108) was selected randomly for independent "blind" counting to check the accuracy of analysis. Colour was not analysed.

2.5. Fourier transformed infra-red spectrometry (FT-IR) analysis

FT-IR analysis was carried out on a subset of the microplastics (≈10%) using an Agilent Cary 630 FT-IR with Attenuated Reflective Spectroscopy (ATR) accessory, set to take 16 background scans and 16 sample scans with a resolution of 4 at a range of 4000–650 cm^{-1} (Supplementary File). The difference between HDPE and LDPE was determined using Jung et al. (2018).

2.6. Statistics

Results are expressed as either mean ± Standard Deviation (SD) (N = number of observations) or median ± 95% Confidence intervals (CI) (N = number of observations) depending on the data set. Statistical comparisons were made using non-parametric tests (Mann-Whitney) or ANOVA (Kruskal-Wallis with Dunn's correction for multiple comparison) using GraphPad Prism 8 (GraphPad Software, La Jolla, CA, USA).

3. Results

Blank samples (Supplementary File) showed no signs of contamination during the collection and processing (concerning reagents, materials and airborne fibers).

3.1. Microplastics in the digestive system of cultured cuttlefish

Microplastics were identified in all six cultured cuttlefish with the total counts ranging from 11 to 45/animal (median 28.5/animal). Fibers were the most common type of microplastic accounting for 93.5% of the total counts over all animals compared to 5.9% for beads /plastic fragments and 0.6% for microfilm pieces (total N = 169). The beads/plastic fragments/microfilm pieces were only present in three animals (2♂; 1♀) and were distributed in both the digestive gland and digestive tract (Fig. 1). The total of 158 fibers in the six animals were distributed relatively evenly between the digestive gland (34.8%), stomach (35.4%) and the caecum/intestine (29.8%) but the number in each structure varied considerably between individuals (Fig. 2). However, the total number of fibers in the three ♀ animals was smaller than in the three ♂ (median 15 [95% CI 11–17] vs. 38 [95% CI 32–45]). As the ♀ had a body weight approximately half that of the ♂ we calculated the total number of fibers/g body weight for the two groups giving values of 0.06 fibers/g (95% CI 0.03–0.08) in ♀ compared with 0.06 fibers/g, (95% CI 0.06–0.09) in ♂. Confining analysis to fibers in the digestive gland gave values for ♀ of 0.8 fibers/g digestive gland (95% CI 0.5–1.4) and for ♂ of 0.9 fibers/g digestive gland (95% CI 0.5–1.0).

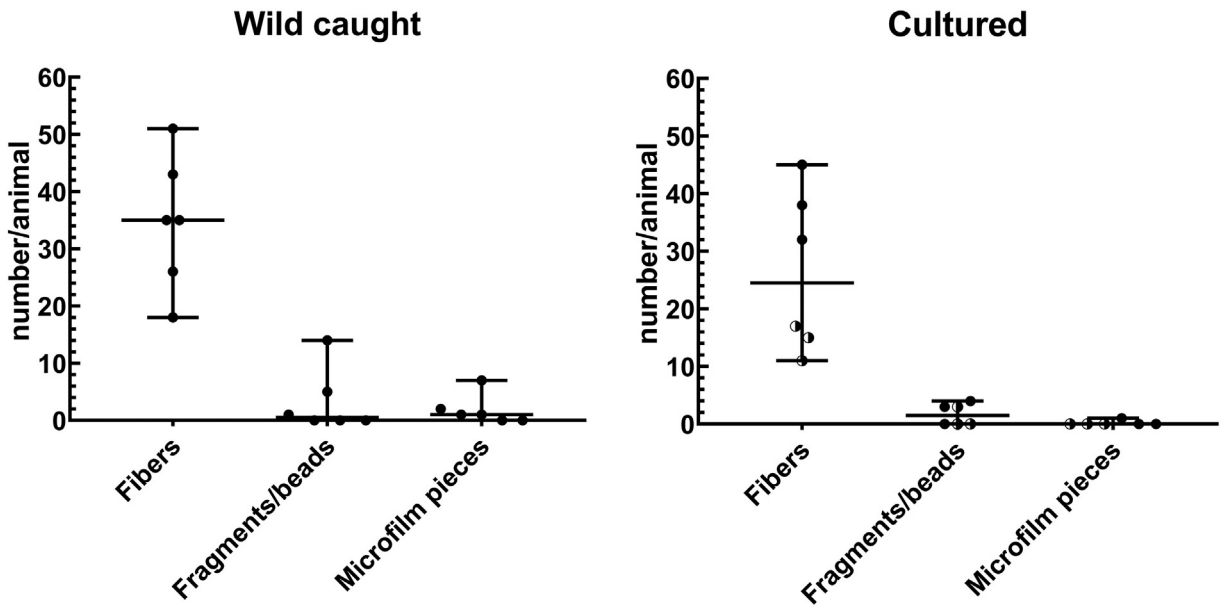

Fig. 1. Median (± 95%CI) total number of microplastics/animal (see text for definition) extracted from the digestive gland and digestive tract from either wild-caught (N = 6♀) or cultured (N = 6; 3♀ [white/black circle], 3♂♀ [black circle]) cuttlefish.

Combining the data for males and females gave an overall median fiber count in the digestive system (digestive tract + digestive gland) of 0.06 fibers/g body weight (95% CI 0.03–0.09) and 0.9 fibers/g digestive gland (95% CI 0.5–1.4).

3.2. Microplastics in the digestive system of wild-caught cuttlefish

Microplastics were identified in all six wild-caught ♀ cuttlefish with the total counts ranging from 27 to 52/animal (median 39/animal). Fibers were the most common type of microplastic accounting for 87% of the total counts over all animals compared to 8.4% for beads/plastic fragments and 4.6% for microfilm pieces (total N = 239) (Fig. 1). The beads/plastic fragments were present in three animals and microfilm pieces present in four animals. Plastic fragments/beads were found in the digestive gland of only one animal but microfilm pieces were found in the digestive gland of four animals. Both microfilm pieces and plastic fragments or beads were found in the digestive tract but were relatively uncommon (in total 18 plastic fragments/beads [14 in the stomach of one animal] and 11 microfilm pieces [5 in the caecum/intestine of one animal]). The total of 208 fibers in the six animals were distributed unevenly between the digestive gland (73.0%), stomach (7.2%) and the caecum/intestine (19.8%) but the number in each structure varied considerably between individuals (Fig. 2). Expressing the total fiber count in the digestive system (digestive tract + digestive gland) per gram body weight gave a median value of 0.12 fibers/g (95% CI 0.11–0.19; N = 6) and confining the analysis only to fibers found in the digestive gland the median count was 1.85 fibers/g digestive gland (95% CI 1.14–3.62; N = 6).

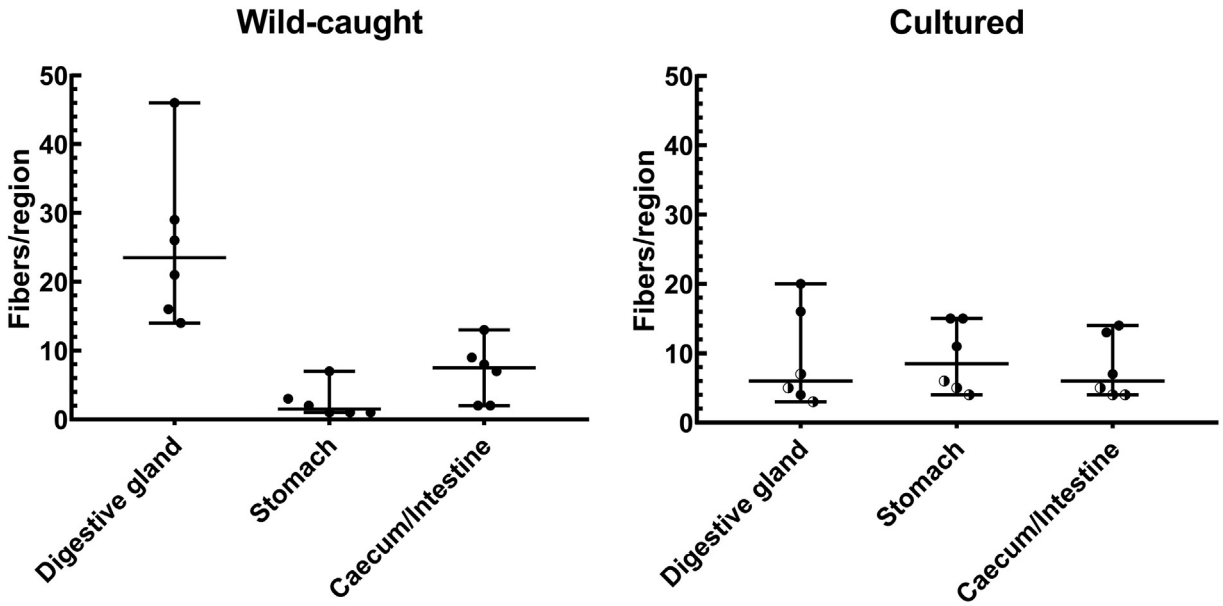

Fig. 2. Median (± 95%CI) total number of microplastic fibers (see text for definition)/region extracted from each of three regions of the digestive system from either wild-caught (left-hand panel N = 6♀; Digestive gland vs. stomach $p < .001$, Kruskal-Wallis with Dunn's correction) or cultured cuttlefish (right hand panel; N = 6: 3♀ [white/black circle] 3♂ [black/circle]) animals.

3.3. Comparison of microplastic distribution in cultured and wild-caught cuttlefish

Data comparison between wild-caught and cultured animals is limited because of the small number of animals sampled and the groups are not balanced for size, sex or age; the latter will also relate to exposure time to microplastics. Calculating the number of fibers/g body weight showed that in wild-caught animals there was approximately twice the number compared to cultured animals (median 0.12/g [95% CI 0.11–0.19] vs. 0.06/g [95% CI 0.03–0.09]). As fibers were the predominant microplastic type in both groups (wild-caught 93.5% vs. cultured 87%) detailed comparison is limited to this type with the following differences apparent between the two sources of cuttlefish:

a) wild-caught animals had a higher mean total fiber count/g body weight than cultured (0.14 ± 0.04 fibers/g vs. 0.06 ± 0.02 fibers/g, $p < .01$, Mann-Whitney);
b) wild-caught animals had a higher mean number of fibers/g of digestive gland compared to cultured animals (wild- caught 2.14 ± 1.10 fibers/g vs. cultured 0.86 ± 0.34 fibers/g, $p < .05$, Mann-Whitney);
c) in the cultured animals the distribution of fibers (assessed as number of fibers/region) was relatively even across the digestive gland, stomach and caecum/intestine with no obvious regional difference. However, in the wild-caught animals the number of fibers was higher in the digestive gland than in the stomach ($p < .001$, Kruskal-Wallis with Dunn's correction) and the stomach and caecum/intestine did not differ from each other (Fig. 2);
d) wild-caught females were of a similar body weight to cultured females (248.3 g ± 44.4 g [N = 6] vs. 284.7 g ± 86.0 g [N = 3]) facilitating direct comparison of microplastic load. The mean number of fibers/g body weight in the wild-caught animals (all females) was significantly higher than in the cultured female animals (0.14 ± 0.04/g body weight vs. 0.05 ± 0.02/g body weight; $p < .05$, Mann-Whitney) and although the mean number of fibers/g in the digestive gland was also higher this was not significantly different (2.14 ± 1.10/g digestive gland vs. 0.91 ± 0.45/g digestive gland).

3.4. Fourier transformed infra-red spectrometry (FT-IR) analysis

A subset of 10 microplastics (1–5 mm) were identified using FT-IR and three polymer types were determined (see Supplementary File): polypropylene (PP), low-density polyethylene (LDPE) and high-density polyethylene (HDPE). Overall, 3 out of 7 fragments were LDPE, 2 were HDPE, and 2 were PP. Two films were tested and determined to be PP and one fiber was tested and found to be PP.

4. Discussion

This study is the first to identify microplastics (as defined in Methods) in the digestive system (digestive tract + digestive gland) of both cultured and wild-caught cuttlefish and they are only the second cephalopod species in which microplastics have been identified in the digestive system (the other species is the jumbo squid, *D. gigas*, Braid et al., 2012; Rosas-Luis, 2016). Limitations of the study include: 1) Group sizes were relatively small (N = 6) and not balanced for sex, age, and weight making statistical comparisons limited, but the underlying major findings are unaltered; 2) It is not known if fibers are confined to the digestive system or can access tissues consumed by humans; 3) We were unable to test the smaller microfibers using FTIR and hence the count may have included natural anthropogenic fibers which are commonly ingested by marine species (Le Guen et al., 2020).

The data on microplastics in wild-caught and cultured animals will be discussed separately in relation to the anatomy and physiology of the digestive tract and potential implications.

4.1. Microplastics in wild-caught cuttlefish

The presence of microplastics and particularly fibers in the contents of the stomach and caecum/intestine in wild-caught cuttlefish was not surprising considering that microplastics had been identified in sediment of the type of habitat occupied by the cuttlefish (Frias et al., 2010; Martins and Sobral, 2011; Mizukawa et al., 2013; Antunes et al., 2013) and can be ingested by species upon which they prey such as crustaceans (Lusher et al., 2017; Ribeiro et al., 2017), and fish (Neves et al., 2015; Barboza et al., 2018).

The predominance of fibers in the digestive tract of cuttlefish is consistent with the previous study of jumbo squid (Rosas-Luis, 2016) and also with findings in a number of marine vertebrates (e.g. pelagic and demersal fish (Lusher et al., 2013); cetaceans and pinnipeds (Nelms et al., 2019)). Microplastics may also be ingested adherent to prey or in the small amount of sea water likely ingested with prey. Faecal samples were not taken but it is likely that some of the ingested microplastics transit the entire digestive tract as has been shown in both invertebrates and vertebrates (e.g., Antarctic krill, Dawson et al., 2018; grey seals Nelms et al., 2018). Larger pieces of microplastic were rare in the cuttlefish and this may be a consequence of the feeding habit using the beak and radula followed by swallowing through a relatively narrow oesophagus (Boyle et al., 1979). However, pieces of plastic could be voided from the digestive tract lumen by vomiting/regurgitation but it is not known with certainty if cephalopods possess this ability, although circumstantial evidence is supportive (Sykes et al., 2020).

Aggregated fibers could cause obstruction of the digestive tract lumen but the oro-anal peristaltic contractile activity would reduce this possibility (Andrews and Tansey, 1983), although if mechanisms controlling these movements were compromised by chemicals adsorbed on the microplastic this would facilitate obstruction. Smaller plastic fragments, microfilm pieces and fibers are likely to become entrapped in the leaflets lining the caecum (Budelmann et al., 1997) and individual fibers could breach the epithelium in areas not protected by chitin (e.g. intestine). However, the presence of microplastic fibers in the digestive gland of all animals, and occasionally larger pieces of microfilm or beads/plastic fragments, shows that microplastics are transported from the caecum to the digestive gland via the ducts. The duct lumen is < 1 mm even in large cuttlefish so there is a realistic possibility of the microplastics clumping and causing obstruction; maintenance of patency is essential for normal digestion and survival as experimental obstruction of the ducts in *Octopus vulgaris* resulted in death in 48 h (Wells and Wells, 1989).

We found a median number of ≈2 microfibers/g of digestive gland in wild-caught animals and as the digestive gland is ≈5% of the body weight, in a mature 500 g *S. officinalis* we estimate that there could be ≈50 microfibers in the digestive gland. The fate of the microplastic fibers after entering the digestive gland in cuttlefish is not known but there are at least three options which merit further investigation: i) Fibers accumulate and remain in the lumen of the duct system within the digestive gland; ii) Fibers are returned to the caecum; iii) Fibers traverse the epithelium of the digestive gland tubules and potentially enter the haemolymph. Further research is required to investigate the material type and the distribution of fibers and other microplastics within the digestive gland ducts and the interstitial layer between the epithelium and capillary-like vessels to better assess the potential biological impact.

Above we focus on potential physical effects of microplastics but chemicals used in their manufacture may leach from the plastics. Adsorbed chemicals could desorb to have toxic effects on the movements of the digestive tract and biochemical processes in the digestive gland. Additionally, microplastics may have adherent viruses, bacteria and parasites and so act as a vector for transport into the body. Information is beginning to emerge about pathogens in cephalopods (Gestal et al., 2019) and the gut microbiome (Lutz et al., 2019) which could be disrupted by pathogens and toxic chemicals.

4.2. Microplastics in cultured cuttlefish

Microplastics in all three categories were found in the digestive system of the cultured animals with fibers representing the predominant group as in the wild-caught animals. The number of fibers in the cultured group was approximately 50% of that in the wild-caught animals expressed either as *per* g. body weight or as *per* g. digestive gland. The major sources of the fibers were most likely the seawater supply (filtered, but drawn from the Ria Formosa lagoon) and the food (grass shrimp captured from saline ponds). Although the cultured males and females differed in body weight they were of comparable ages at the time of analysis, but as the number of fibers/g body weight is comparable it suggests that fiber load is related to food intake/unit time rather than time in the tank. Production of "plastic free" cuttlefish for human consumption will be a major challenge but in the present study we did not analyse the parts of the cuttlefish usually eaten (mantle, tentacles, nidamental glands) so we do not know if the microplastics (or smaller breakdown products) are confined to the digestive system. Culture of "plastic free" animals will require the use of prepared/synthetic foods, where the microplastic contamination can be strictly regulated but this is currently one of the industrial aquaculture production bottlenecks for cephalopods (Villanueva et al., 2014). On the other hand, the seawater systems used for such production will have to use either artificial or micro-filtered seawater to prevent microplastic contamination; this will also be an issue in Recirculating Aquaculture Systems finfish production in the coming years.

The overall microplastic exposure in cultured animals was lower than their wild counterparts and this is perhaps not surprising because of the multiple filtration systems used to deliver seawater to the culture tanks. Although we do not know the age of the wild-caught animals in comparison to the cultured animals (≈300 days post hatching) we consider it likely that they were younger as they were captured rather than died naturally. The aquatic environment for the cultured animals probably exposed them to a lower level of microplastics/unit time than the wild environment. However, this requires further study, but as the females in both groups were fortuitously of similar weight a comparison of fibers/g revealed a trend for a higher level in the wild-caught animals. In both groups fibers were numerically predominant. However, they were more evenly distributed between the digestive gland, stomach and caecum/intestine in the cultured animals and the density of fibers in the digestive gland was lower. We can only speculate on the reasons for this difference but access to the digestive gland requires fibers to bypass the main digestive tract lumen, enter the single narrow opening to the bifurcated digestive gland ducts, and then to be transported in the flow of chyme to the digestive gland. The probability of these events occurring will be related to the density of fibers ingested, their size and the time over which there is exposure; in both cases we suspect that this was higher in the wild-caught compared to the cultured animals but confirmation awaits formal investigation.

The potential physical and chemical effects of MPs are the same in both cultured and wild-caught cuttlefish. However, the lower overall exposure in cultured animals would place them at a lower overall risk of an impact on health.

In conclusion, we have provided preliminary evidence for the presence of microplastics in the digestive tract of both wild-caught and cultured cuttlefish and briefly discussed anatomical features of the digestive system which may make it prone to obstruction or damage. The present study needs to be replicated using more samples of cuttlefish and other cephalopods to fully assess the extent of microplastic ingestion by this class representing ecologically and commercially important species inhabiting the seas from the Polar regions to the tropics and at all depths. The demonstration of the presence of microplastics in wild-caught cuttlefish has potential implications for consumption by predators (e.g., conspecifics, sea bass and seals) leading to biomagnification. Although we do not know if microplastics entering the digestive tract of wild-caught cuttlefish can reach the tissues consumed by humans, in cultured cuttlefish reared using existing aquaculture methodology (Sykes et al., 2014) the presence of microplastics in the digestive system illustrates the challenges in commercial rearing of aquatic species for human consumption.

Funding

AVS was supported by Fundação para a Ciência e a Tecncologia (Programa Investigador FCT 2014 (IF/00576/2014) and Plurennial funding to CCMAR (UID/Multi/04326/2019)) and Programa Operacional Mar2020/Portugal2020/FEAMP (Project SEPIACUL 16-02-01-FMP-53).

CRediT authorship contribution statement

GMC, AVS, DG, and PLRA participated in the conception and design of research; ARO and ASS performed the sample collection, microplastics extraction, quantification and identification; all authors were involved in the data analysis. ARO, PLRA and AVS wrote the initial draft of the manuscript and all authors edited and approved the final version. All authors agree to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

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.

Acknowledgments

PLRA wishes to acknowledge the receipt of an honorary Research Fellowship at Stazione Zoologica Anton Dohrn, Naples and would like to thank the President of the Stazione Zoologica, Professor R. Danovaro, Dr. G. Fiorito and the Head of the Department of Biology and Evolution of Marine Organisms (Dr I. Arnone).

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.marpolbul.2020.111553.

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