Detection of ultrafine plastics ingested by seabirds using tissue digestion

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Highlights

- Potassium hydroxide digested shearwater tissue ~~in a rapid and affordable manner~~rapidly and affordably
- Ultrafine plastics (1 mm – 1 µm) were observed in 7.0% of shearwater gizzards
- Ultrafine plastics accounted for 3.6% of ~~all items~~ debris recovered from shearwaters

Abstract

Plastic debris is a major global threat to marine ecosystems and species. However, our knowledge of this issue may be incomplete due to a lack of a standardized method for quantifying ingested ultrafine particles (1 mm – 1 µm) in wildlife. This study provides the first quantification of ultrafine plastic in seabirds using chemical and biological digestion treatments to extract plastic items from ~~the~~ seabird gizzards. The ~~digestive~~ alkaline agent, potassium hydroxide, outperformed the enzyme corolase, based on cost and efficiency (e.g., digestion time). Ultrafine plastics were observed in 7.0% of Flesh-footed Shearwater (*Ardenna carneipes*) gizzards collected from Lord Howe Island, Australia and accounted for 3.6% of all plastic items

recovered (13 out of 359 items). Existing methods for extracting ingested plastic from seabirds do not account for ultrafine ~~or nano-sized (<1 µm)~~ particles, therefore our results indicate current seabird plastic loads, and the associated physical and biological impacts, are underestimated.

Keywords: extraction methods; marine debris; microplastic; plastic pollution

1. Introduction

Plastics have become a revolutionary alternative ~~for global~~ industr~~y~~ies~~,~~ and society ~~in general~~, ~~by~~ transforming the delivery of health care and ~~how products are~~ packag~~ing~~ed~~,~~ stor~~asgeed~~, and ~~shipped~~ transportation of goods . Over the past century, global plastic production has grown exponentially to over 345 million tonnes per annum, the majority of which is designed to be thrown away after single use . A combination of high disposability and gaps in waste management systems has led to plastic debris becoming recognized as a major environmental issue, causing widespread contamination of aquatic and terrestrial environments, and serious economic and ecological harm .

~~Huge~~ Vast areas of the ocean now contain up to 890,000 plastic items km^{-2} with 1.1-2.4 million tonnes of new plastic entering the ocean from global riverine systems every year~~an estimated 20 million new items entering the ocean each day~~ . This growing mass of floating debris creates a ‘plastic soup’ of large and small items distributed throughout the water column , entangling or being ingested by >2240 marine species . Once ingested, plastic debris ~~has been linked to~~ expos~~es~~ure ~~of~~ wildlife to toxic substances and associated adverse effects, including endocrine disruption and reduced body condition .

Our understanding of this complex issue is increasing rapidly, but information on the quantity of very small particles (e.g., ultrafine plastics 1 µm - 1 mm) ~~plastic~~ ingested by marine wildlife ~~in the ultrafine (1 mm – 1 µm) and nano-size (<1 µm) range~~ has been a conspicuous gap . ~~These tiny particles are abundant in the environment; once in the ocean, plastic material breaks up into smaller pieces through photodegradation and wave action, becoming ultrafine and eventually nanoparticles .~~ While such particles may appear harmless due to their small size,

they pose a significant threat since tiny particles can penetrate tissues and accumulate in organs, leading to changes in behaviour and metabolism . Particle size also plays a pivotal role in the exposure of wildlife to chemicals, as smaller particles are generally more toxic than the corresponding bulk material at the same mass concentration .

Globally, seabirds are considered reliable sentinels of ocean health, with long-term monitoring programs providing valuable information on trends in plastic and chemical contamination in the world's oceans . Exposure of seabirds to small plastics (e.g., ultrafine ~~and nano~~ particles) is poorly documented , but likely occurs through direct ingestion, secondary ingestion via prey, or through the breakup of larger items during the digestion process. Many birds (especially seabirds) have a stomach consisting of two well-defined chambers: the proventriculus and muscular gizzard . ~~Similar to~~Like pumice and some prey hard parts (e.g., squid beaks), the movement of ingested plastic through seabird gastrointestinal tracts appears to be influenced by particle size, with fragments that are too large to pass into the gizzard being retained in the proventriculus. Importantly, items that are too large to pass directly into the intestine are temporarily retained in the gizzard until they are sufficiently broken up, presumably into ultrafine ~~or nano-sized~~ particles, before they are excreted .

The lack of data on ultrafine ~~and nano~~ particles in seabirds is due, in part, to the difficulty in quantifying these tiny particles and, until recently, a lack of standardised methodology which provided a framework and motivation to collect such data . To date, the focus of seabird plastic studies has been on lavage (stomach flushing) of live animals which underestimates ingested debris , or necropsy of deceased animals using a 1 mm mesh size to separate debris items, thus providing no data on the presence of very small items . Ultrafine ~~and nano~~ plastics can also be overlooked due to concealment by biological tissue, and visual separation is both difficult and time consuming. However, without data on ultrafine ~~and nano~~particles, plastic loads in individual birds are underestimated, and information on this emerging threat is therefore lacking.

Recently, chemical digestion techniques have been used to enhance the quantification of plastic by removing biological tissues. While the majority of research has used tissues from fish, two studies obtained rapid, complete digestion of soft tissues from avian intestines using

chemicals such as potassium hydroxide (KOH) over a period of 3-14 days . However, results of some studies suggest digestive treatments, including KOH, hydrogen peroxide (H□O□), and hydrochloric acid (HCl) are inconsistent or can cause alterations to plastic polymers . Enzymatic digestion has been proposed as a safer alternative to chemical digestion and has been recently used to extract ingested plastics from shellfish with no evidence of damage to the plastics .

In eastern Australia, the ingestion of macro- (>5 mm) and micro-plastic particles (1 – 5 mm) by Flesh-footed Shearwaters (*Ardenna carneipes*) has been linked to perforations of the digestive tract, and a range of sublethal effects which likely contribute to the decline of this species . No data exist on the ingestion of ultrafine ~~or nano-sized~~ particles in Flesh-footed Shearwaters, or seabirds more broadly, and few methods are available for obtaining this type of data, especially from muscular/fibrous tissues, such as the gizzard where such items are likely to accumulate. Therefore, the aims of this study were to ~~1) identify digestive treatments for the extraction and quantification of marine plastics from seabird gizzards, and 2~~1) quantify ingested ultrafine plastics in Flesh-footed Shearwater ~~gizzard~~s, and 2) identify digestive treatments for the extraction and quantification of marine plastics from seabird gizzards.~~–~~

3. Materials and methods

3.1. Sample collection and preparation

~~The ingestion of plastic by Flesh-footed Shearwaters breeding on Lord Howe Island, New South Wales (31.5191°S, 159.0649°E) has been documented annually since 2011 as part of a long-term monitoring program.~~ During 4-9 May 2017 and 1-9 May 2018, freshly deceased Flesh-footed Shearwater fledglings (80-90 days old; e.g., road kill) were collected on Lord Howe Island, New South Wales (31.5191°S, 159.0649°E). ~~and t~~The gizzard from each bird was extracted intact, placed in a numbered bag, and stored at -20°C. Samples were then transported to the University of Tasmania where they were thawed for 1-2 hours, weighed using an electronic balance to 0.00001 g, and the length and width recorded with digital Vernier calipers to 0.01 mm. Care was taken to prevent contamination of samples with ultrafine plastic particles from outside sources: all samples were processed in the presence of a fume hood, and work

benches and tools were wiped clean with sterile paper and 70% ethanol between samples, all lab consumables were made of glass ~~(not plastic)~~, and researchers wore clothing made of non-synthetic materials whenever possible.

3.2. Digestion treatments

Two digestion agents, 1 M KOH and 2.5 ml corolase 7089 , were selected for this study, based on cost, accessibility, treatment time, known or predicted effect on plastic polymers, and ease of transport (Table 1).

When digesting samples using KOH, we ~~modified~~ followed the procedures similar to those outlined by . However, as this protocol was developed for fish tissue (sprat *Sprattus sprattus*), we completed initial tests to determine an optimal digestion protocol for seabird gizzards. In brief, a sub-set of gizzards was randomly assigned to two test groups: room temperature (18-21°C) and heat bath (60°C). This temperature (60°C) was chosen as it is below the melting point of most polymers. The room temperature samples achieved near complete digestion (i.e., only oily fatty deposits remained) after 15 days of exposure, while small remnants of the gizzards remained after only 10 hours of exposure to the heat bath. To ensure complete digestion, for our final protocol each gizzard was placed in a glass flask containing 20 ml of 1 M KOH solution, then in a 60°C water bath for 12 hours. The residual solution was then passed through nested mesh sieves in descending size order (4.75, 1.00, and 0.33 mm) to capture the liberated plastics. KOH was chosen because it is inexpensive (AU$0.16 per gizzard), readily available, can be safely transported, and the treatment time was relatively short.

For the enzymatic digestion, we used 2.5 ml of corolase (activity 840 UHb) in 25 ml of filtered H□O following ~~modifications to~~ methods similar to ~~used by~~ Catarino et al. (2017~~; Appendix 1~~). As this protocol was developed for mollusk tissue (blue mussel *Mytilus edulis*), we again completed tests to determine an appropriate strategy for seabird gizzards. A sub-set was randomly assigned to three test groups: 0.5 ml of corolase in 50 ml filtered H□O, 1.5 ml of corolase in 50 ml filtered H□O, and 2.5 ml corolase in 25 ml filtered H□O with the gizzard placed in a fresh solution every 3 hours over a 9 hour period. Samples in the first two groups did not

exhibit any evidence of digestion after 20 hours, while the gizzards treated with higher enzymatic concentration were completely digested within 9 hours. Based on these results, we used this combination as our final protocol.

Once the digestions are complete, ~~T~~the residual solution is ~~then~~ passed through nested mesh sieves as described above. When required (i.e., for very small particles), plastic items were confirmed through visual identification using a dissecting microscope and floatation in a weak salt solution. Results are reported as frequency of occurrence (FO), number, and mass of plastics, with summary data as the mean ± standard deviation with range~~, and plastic type and colour categories following~~ .

4. Results

A total of 57 Flesh-footed Shearwaters gizzards were examined. Mean mass, length, and width was 2.05 ± 0.66 g, 22.48 ± 4.27 mm, and 17.52 ± 2.60 mm, respectively. A total of 359 pieces of plastic were recovered, with 91.5% of gizzards containing plastic with an average of 0.05 ± 0.06 g and 6.02 ± 5.50 pieces of plastic (Table S1). The majority (60.4%; n = 217) of recovered plastic items were in the micro-plastic (1.00-4.74 mm) size range, with mean length 3.02 ± 1.00 mm and width 2.11 ± 0.86 mm. Macro-plastics (≥4.75 mm) were also abundant, accounting for 35.9% (n = 129) of items recovered (length 8.38 ± 7.03 mm, width 3.24 ± 1.95 mm). Ultrafine debris (i.e., items extracted from the 0.99-0.33 mm sieve) accounted for 3.6% of all items (13/359 items). These 13 particles were recovered from six of the 57 gizzards (FO: 7.01%). Mean length, width, and mass of the 13 ultrafine particles were 0.95 ± 0.21 mm, 0.58 ± 0.22 mm, and 0.0005 ± 0.0009 g, respectively. All ~~of~~ the ultrafine particles were classified as hard fragments (Fig. 1~~2~~).

The cost per gizzard using 20 ml of 1 M KOH was $0.16/sample (2017 AUD). Corolase was inherently more expensive and needed to be replenished at 3-hourly intervals due to the activity potency, increasing the cost per gizzard to $9.30 (2017 AUD). Digestion using the KOH solution was straightforward, requiring little or no ongoing adjustment during the digestion process while the corolase digestion required replacing the corolase with a fresh batch every

three hours to maintain the subculture *Bacillus subtilis* activity potency (total volume used over 10 hours is 7.5 ml corolase in 75 ml filtered H□O). KOH is also readily available from a variety of commercial suppliers whereas corolase 7089 could only be purchased in bulk (~25 kg) from a limited number of overseas suppliers (Table 1).

5. Discussion

While our understanding of plastic pollution is increasing rapidly, there are key areas where a lack of information limits our ability to accurately describe the pressures plastic imposes on species and ecosystems. Quantitative data on the presence of ultrafine~~ or nano-sized~~ particles is one such area. Recent findings from a handful of studies of fish and invertebrates suggest that particles in this size range are abundant and have the potential to cause significant harm . To date, there is no information on the hazard ultrafine~~ or nano-sized~~ particles may pose for birds. This is due, in part, to the difficulty of identifying and working with tiny particles, particularly in the field (e.g., remote areas where lab facilities are limited or unavailable). However, the recent development of a standardized method for describing ingested plastic in birds enables reliable data to be collected on small particles under most scenarios .

Plastic items ingested by seabirds have been traditionally collected in one of two ways: necropsy of dead birds, or lavage (stomach flushing) of live birds. The latter is a non-lethal method of collecting samples from a random sub-set of the population . However, lavage suffers from imperfect detection as only the proventriculus is emptied, and a small proportion of plastic items are missed due to tissue obstruction . Successful digestion of soft tissues and recovery of plastics in marine invertebrates suggested chemical or enzymatic digestion may provide an opportunity to separate ingested plastics from seabird tissues in a rapid and reliable manner. We found that digestion of seabird gizzards using KOH was both time and cost effective, with all soft tissue digested, leaving only plastic debris and hard biological fragments (e.g., squid beaks). The addition of a 60°C water bath reduced previous published treatment times by ~75% (from 2 days to 12 hours; Table 1) with no apparently consequences. In contrast, digestion of seabird

gizzards using corolase was more challenging due to the high cost and limited accessibility of this reagent.

Current protocols for examining ingested plastic in seabirds typically use a 1 mm mesh sieve to capture and sort liberated plastics into size classes after necropsy or lavage . Sieves with smaller dimensions are uncommon as they can become blocked with biological material, and items <1 mm have been considered rare or of low interest in studies of seabirds as they are assumed to pass through the digestive tract (Provencher et al. 2017). Our results demonstrate that discounting ingested plastics <1 mm under-estimates plastic load in 7% of Flesh-footed Shearwaters. While the mass of ultrafine particles in each of these birds was low (0.0005 ± 0.0009 g), additional plastic particles passing through seabirds' digestive tracts increase the risk of harm from chemical pollutants and infectious agents present on the plastics' surface . It is possible some, or all, of the ultrafine particles arose through the breakup of larger items in either the proventriculus or through the mechanical grinding of the gizzard. As noted by , plastic fragments in two seabird species decreased in size from the proventriculus and gizzard, with the smallest items being detected in the intestines.

While our findings have improved our understanding of the occurrence of ultrafine particles in seabirds, there remains no data on nano-sized plastics for any seabird species. Both size classes play an important role in facilitating the translocation of toxins and microbes, with nano-plastics able to pass through the gut epithelial membrane and circulatory system of organisms . Further development of extraction and quantification methods for ultrafine and nano-sized particles will enable research on a wider range of species, and for larger numbers of samples to be processed quickly in both laboratory and field settings.

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Table 1. Review of digestive treatments for the extraction of marine plastics from biological tissue samples (adapted from .

Species	Tissue	Treatment (including agent)	Digestion time	Polymer type	Agent accessibility	Cost per sample (AU$)[a*]	Source
Copepod (*Temora longicornis*)	Whole animal	500 µg mL^{-1} proteinase-K, 15 ml of sample, incubated for 2 hr at 50˚C	2 hr	Polyester & nylon fibres, PET, PVC~~fibre~~	Uncommon	$22.40	
Blue mussel *(Mytilus edulis)*	Whole animal	25 ml trypsin 0.3125%, heated magnetic stirrer @ ~38ºC for 30 min	30 min	PET, PA, HDPE, PVC, PP	Uncommon	$3.29	
Blue mussel *(Mytilus edulis)*	Whole animal	1.5 ml corolase 7089 (activity 966/UHb/ml) in 100 ml MilliQ. Heated magnetic stirrer 1 hr @ 60˚C	12 hr	Nylon fibre	Uncommon	$1.86	
Blue mussel (*Mytilus edulis*)	Whole animal	20 mL 22.5 M 69% HNO_3, 3 mussels per sample. Room temp. overnight	12 hr	Nylon fibre, PS	Common	$28.40	
12 × terrestrial bird sp.	Oesophagus, intestine	10% KOH (volume not reported), digest at room temp	2-3 wk	Not reported	Common	$0.29	
Mussel/crab/fish	Whole animal	20 ml 10% KOH, incubated @ 60˚C	24 hr	LDPE, HDPE, PP, PA, PS~~PET, CA~~	Common	$0.29	
North Sea fish sp.	Stomach, oesophagus, intestine	3x volume of biological sample of 10% KOH solution added, stored @ room temp.	2-3 wk	Not reported~~N/A~~	Common	$0.89	
Beaked whale (*Mesoplodon mirus*)	Oesophagus, stomach, intestine	3x volume of biological sample of 10% KOH solution added, stored @ room temp.	3 wk	PP and rayon fibers~~N/A~~	Common	$0.89	
Sprat (*Sprattus*	Whole animal	20 ml 1 M KOH, digest at room temp.	2 days	Various	Common	$0.16	

sprattus)				plastics[b]~~CA, LDPE, PLA~~			
Flesh-footed Shearwater (*Ardenna carneipes*)	Gizzard	20 ml 1 M KOH. Digest in water bath @ 60˚C	12 hr	Not recorded	Common	$0.16	This study
Flesh-footed Shearwater (*Ardenna carneipes*)	Gizzard	2.5 ml corolase 7089 (activity 840/UHb/ml) in 25 ml filtered water, replaced every 3 hr. Digest in water bath @ 60˚C	9 hr	Not recorded	Uncommon	$9.30	This study

[a]~~*~~based on prices available from Sigma Aldrich Australia and AB Enzymes in 2017

[b]Various plastic types broadly categorized into synthetics, rubbers and biodegradables

Cellulose acetate (CA), high-density polyethylene (HDPE), low-density polyethylene (LDPE), polylactic acid (PLA), polyamide (PA), polypropylene (PP), polystyrene (PS), polyvinyl chloride (PVC), polyethylene terephthalate (PET)~~Polyethylene terephthalate (PET), cellulose acetate (CA), low-density polyethylene (LDPE), polylactic acid (PLA), polyamide (PA)~~

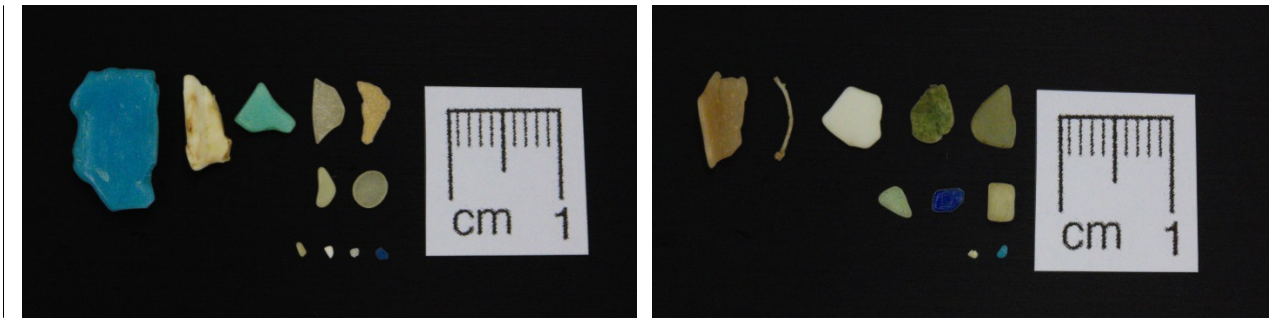

Fig. 1. Plastic items extracted from two Flesh-footed Shearwater gizzards after chemical and enzymatic digestion. In each panel, macro-plastics (>4.75 mm) are shown in the top row, micro-plastics (>1.00-4.74 mm) in the middle row, and ultrafine particles (1 µm – 1 mm) along the bottom row.