Ingestion of microplastic debris by green sea turtles (*Chelonia mydas*) in the Great Barrier Reef: validation of a sequential extraction protocol.

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Abstract

Ocean contamination by plastics is a global issue. Although ingestion of plastic debris by sea turtles has been widely documented, contamination by microplastics (< 5 mm) is poorly known and likely to be under-reported. We developed a microplastic extraction protocol for examining green turtle (*Chelonia mydas*) chyme, which is multifarious in nature, by modifying and combining pre-established methods used to separate microplastics from organic matter and sediments. This protocol consists of visual inspection, nitric acid digestion, emulsification of residual fat, density separation, and chemical identification by Fourier transform infrared spectroscopy. This protocol enables the extraction of polyethylene, high-density polyethylene, (aminoethyl) polystyrene, polypropylene, and polyvinyl chloride microplastics >100 µm. Two macroplastics and seven microplastics (two plastic paint chips and five synthetic fabric particles) were isolated from subsamples of two green turtles. Our

results highlight the need for more research towards understanding the impact of microplastics on these threatened marine reptiles.

Keywords: marine turtle, plastic ingestion, plastic contamination, extraction technique, chemical digestion, Fourier transformed infrared spectroscopy.

Highlights:

- We combined and validated pre-established methods for microplastic extraction.
- This protocol is suitable for samples comprising both organic and mineral material.
- Macro- and microplastics were detected in sea turtles from the Great Barrier Reef.
- This protocol improves method harmonisation in marine debris ingestion research.

1. Introduction

Plastics are one of the most common and persistent pollutants in coastal and marine environments worldwide (Gall and Thompson, 2015; Moore, 2008). Anthropogenic marine debris was first identified as an issue in the Great Barrier Reef two decades ago (Haynes, 1997). Recent estimates suggest that more than 5 trillion pieces of plastic debris, weighing 298 tons, may be floating in the world's oceans (Eriksen et al., 2014). These estimates of plastic pollution are higher if particles in beach sand and those deposited onto seafloors are also included (Galgani et al., 2015).

Plastic pollutants are broadly divided into two categories; macroplastics (> 5 mm) and microplastics (< 5 mm, Barnes et al., 2009; Moore, 2008). Both macro- and microplastics are ubiquitous and widespread in the marine environment; polluting the ocean surface, water column, and benthos (Cole et al., 2011; Galgani et al., 2015; Woodall et al., 2014). Microplastic pollutants are broadly classified as either primary or secondary microplastics (Cole et al., 2011). Primary microplastics are deliberately manufactured in the sub-visible size range, such as pelletised raw materials for manufacture of plastic products (Ashton et al., 2010) and plastic beads destined for use

in processes and applications such as air-blasting, medicinal vectors and cosmetic exfoliants (Cole et al., 2011; Fendall and Sewell, 2009). Secondary microplastics are created by the physical, chemical, and biological degradation of plastic debris in the environment (Cole et al., 2011; Duis and Coors, 2016; Moore, 2008).

Marine life is mainly impacted by plastic debris through the processes of entanglement and ingestion (Derraik, 2002). Ingested macroplastics can either pass through the intestinal tract, or accumulate there for several months, effectively blocking the tract and/or reducing the feeding stimulus with lethal or sub-lethal effects (Laist, 1987; Lutz, 1990; Nelms et al., 2016; Santos et al., 2015). Ingestion of macroplastics has been implicated in the mortality of a wide range of organisms including sea birds (Provencher et al., 2014), cetaceans (Jacobsen et al., 2010; Laist, 1987), sirenians (Beck and Barros, 1991; Ceccarelli, 2009; Laist, 1987) and sea turtles (Santos et al., 2015).

Similarly, ingestion of microplastics has also been reported for a wide range of marine wildlife including fishes (Foekema et al., 2013), cetaceans (Lusher et al., 2015), zooplankton (Sun et al., 2017) and sea turtles (Santos et al., 2015). Like macroplastics, ingested microplastics can impact organisms physically (Wright et al., 2013) and increasing concern has been expressed regarding their capacity to act as a vector for toxic chemicals (Besseling et al., 2013; Derraik, 2002; Moore, 2008; Von Moos et al., 2012). Once ingested, chemical effects can occur via three processes: 1) leaching: plasticisers, UV stabilisers, and other chemicals added to polymers during production leach into the organism post-ingestion; 2) sorption: pollutants such as polychlorinated biphenyls (PCBs), polycyclic aromatic hydrocarbons (PAHs), metals, and pesticides adsorbed onto microplastics from the surrounding environment are released internally post-ingestion; and 3) trophic flow: accumulated toxins are bioaccumulated through the food chain (Bejgarn et al., 2015; Hamlin et al., 2015; Koelmans et al., 2014).

One iconic animal impacted by marine debris is the sea turtle. All seven turtle species are known to be affected by plastic debris globally (Clukey et al., 2017; Gall and Thompson, 2015; Nelms et al., 2016). Two factors that likely increase the risk of plastic ingestion by sea turtles relative to other marine species are: 1) visual feeding strategies which select for structures analogous to jellyfish and soft floating plastics, and 2) backward-facing oesophageal papillae which inhibit

regurgitation and facilitate particle accumulation in the gut (Schuyler et al., 2014; Vegter et al., 2014; Wyneken, 2001).

Of all sea turtle species, the green turtle (*Chelonia mydas*, Linnaeus 1758) and leatherback turtle (*Dermochelys coriacea*, Vandelli 1761) are the most susceptible to marine debris because of their respective herbivorous and gelatinous diets (Di Beneditto and Awabdi, 2014; Schuyler et al., 2013). Plastic debris can also become entangled among green turtle food sources such as seagrass leaves and macroalgae (Awabdi et al., 2013). Microplastics have been found in sea turtle stomach content in Brazil and the North Atlantic (Mascarenhas et al., 2004; Pham et al., 2017), raising concerns about potential cumulative impacts of microplastics on these slow-growing animals, including dietary dilution and malnutrition (Nelms et al., 2016).

Methods for extracting microplastics have been developed for a range of sample matrices. Visual assessment using microscopy is routinely used to extract microplastics from waste water, sea water, sediments, ice, plant matter, biological tissues, and whole organisms. Density separation is commonly used to extract microplastics from water or sediments (Claessens et al., 2013; Hidalgo-Ruz et al., 2012; Reisser et al., 2013; Thompson et al., 2004). Chemical digestion is used to extract microplastics from whole organisms (Claessens et al., 2013) and from ingested material, for example, from pelagic fish or cetacean chyme (i.e. ingested material and digestive tract fluid) (Foekema et al., 2013; Lusher et al., 2015). Many of these methods are suitable and efficient for either homogenously organic or homogenously inorganic sample matrices; however, each of them alone are unlikely to be suitable for microplastic extraction from green turtle chyme. Green turtle chyme can have a diverse organic composition and can also contain sediments. In fact, when green turtles shift from their pelagic stage to coastal benthic habitats, their diet broadens from mainly animal matter such as jellyfish and sponges to include herbivorous components, particularly seagrass, algae, and associated sediments and epibionts (Bjorndal, 1997). Due to the diverse diet of coastal turtle populations, chyme from non-pelagic green turtles is expected to be relatively complex, comprising a range of organic (plant and animal material) and inorganic (mineral and sediment) matrices. Therefore, a protocol capable of efficiently extracting microplastics from all matrices is required in order to accurately establish contamination levels. The objective of this study is to develop and validate a microplastic

extraction protocol suitable for investigating green turtle chyme samples, thereby improving method harmonisation in marine debris research (Tate et al., 2012).

2. Materials and methods

2.1 Sample collection and preparation

Two unsuccessfully rescued green turtles (Turtle A: StrandNet #55364 and Turtle B: StrandNet #53584), collected near Cairns (central Great Barrier Reef, Australia) were used for this study. Turtle A was a juvenile with a curved carapace length of 45.4 cm. Turtle B was an adult female with a curved carapace length of 103 cm. Foreguts (including oesophagus, stomach, and small intestine) of both turtles were necropsied, the rest of the digestive tract being required for a different study. The foregut content was visually inspected and any visible macroplastics were removed for subsequent analysis. For each turtle, chyme was transferred to a metal bucket and homogenised by manual stirring using a metal spoon.

2.2 Sequential extraction protocol

A preliminary pilot test using chemical digestion (HNO_3, 69.5 %) of green turtle chyme was unsuccessful, as some fat and sediments remained. A sequential extraction protocol (Figure 1) was therefore developed by combining existing separation methods (both physical and chemical) as well as emulsification of fats to simplify the complex mixture through deconstruction of the mostly plant-derived biomass. The suitability of the protocol to separate microplastics was assessed by measuring physical (i.e. change in size) and/or chemical (i.e. alterations to polymer type identified via Fourier transform-infrared spectroscopy, FTIR) degradation of known polymer types. The efficiency of the protocol was then determined by spiking homogenised green turtle chyme samples with plastic micro-beads and quantifying recovery rates. The protocol was then applied directly to green turtle chyme to quantify plastic ingestion. Because of the challenges in confirming the polymer composition of particles smaller than 100 µm using FTIR spectroscopy, this size was established as the lower limit of analysis. To prevent procedural contamination all lab equipment was rinsed with reverse osmosis

(RO) water before use and filters were always kept covered.

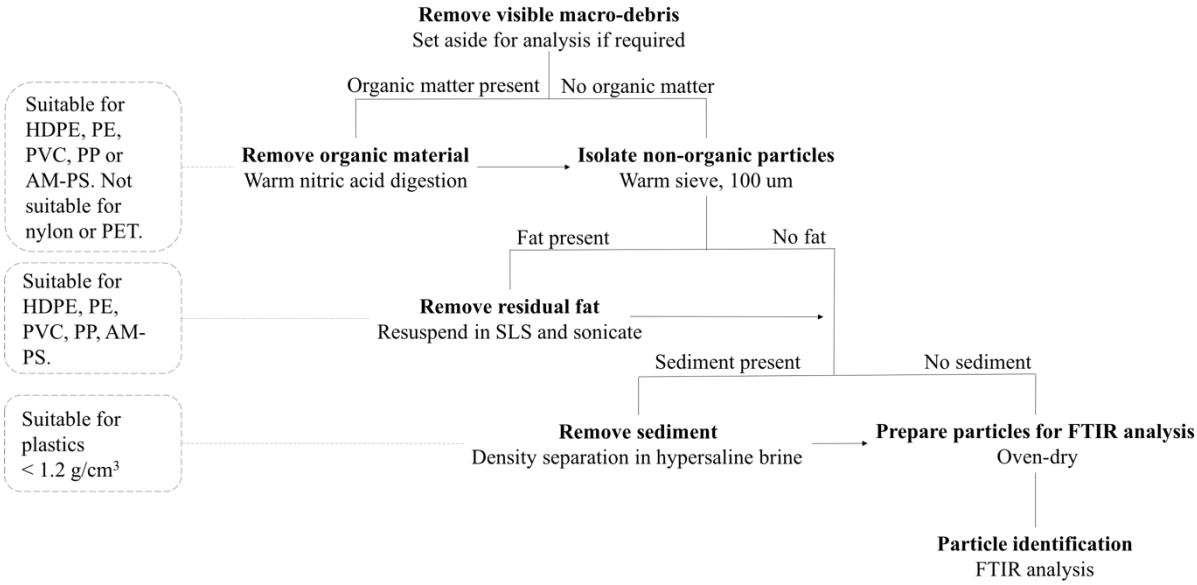


Figure 1: Sequential extraction protocol for the extraction of microplastics from green turtle chyme showing polymer suitability.

Acid digestion of organic materials

All chyme samples were processed in 50 mL glass test tubes (~ 6 g wet weight (w/w) per test tube) using a method modified from Claessens et al. (2013). Nitric acid (HNO_3, 69.5 %, Scharlau) was added to each sample (3:1, HNO_3 mL: chyme g w/w), followed by an overnight digestion at room temperature (~ 20 °C) and two hours of heating in an 80 °C block heater. Warm samples were then sieved over a 100 µm steel mesh.

Emulsification of fats

If an intractable fatty residue remained after acid digestion of samples, all remaining materials were re-suspended in 200 mL of a warm sodium lauryl sulfate (SLS, Acros Organics) solution (1.0 g SLS/L; ~ 50 °C) followed by sonication for 30 seconds (47 kHz ± 6 %, Branson® 2200 Ultrasonic Clearer). The solution was kept warm and sieved through a 100 µm steel mesh.

Density separation from sediments

If sediments remained after filtration (i.e. >100 µm), all remaining materials were re-suspended in 200 mL of a hypersaline brine solution (NaCl, 1.2 g/cm^3, Sigma Aldrich) prepared by saturation of RO water with NaCl (water solubility at 20 °C = 357 g/L), modified from Hidalgo-Ruz et al. (2012). The solution was manually stirred for 30 seconds using a glass stirring rod and allowed to rest for 1 hour. Supernatant was collected using a Pasteur pipette and transferred to a glass beaker before further filtration. The pipette was rinsed three times with RO water into the glass beaker to collect any particles that may have adhered to the internal wall of the pipette.

Filtration

Collected supernatant was vacuum-filtered (Millipore HA cellulose nitrate/acetate 0.45 µm pore membrane filters) (Claessens et al., 2013; Hidalgo-Ruz et al., 2012). Each filter was then put in a covered glass dish and oven-dried at 60 °C for 4 hours.

2.2 Validation of the sequential extraction protocol

Polymer identification

Polymer composition was determined using Fourier transform-infrared spectroscopy (Kroon et al 2017; Foekema et al., 2013; Hall et al., 2015; Lusher et al., 2013; Thompson et al., 2004). Briefly, FTIR spectra were acquired in transmission mode on a PerkinElmer Spectrum 100 FTIR Spectrometer using an attenuated total reflectance (ATR) accessory as per Kroon et al. (2017). Individual items were placed on the ATR diamond using forceps. A pressure clamp was used to ensure good contact with the sample. The Data Tune-up command to smooth and perform a baseline correction using default PerkinElmer parameters was applied to all spectra. Spectra were searched (4000-600 cm^{-1}) using Euclidian distance against commercially available NICODOM IR libraries (Polymers and Additives, Coatings, Fibres, Dyes and Pigments, Petrochemicals Full Version; NICODOM Ltd., Czech Republic) and a percent match between the reference spectra and the sample obtained. As per Kroon et al. (2017) samples with a percent match of < 60 % were considered a low

match, 60 - < 70 % and intermediate match and ≥ 70 – 100 % a high match. All spectra were further inspected and any unexplained bands investigated by reviewing the lower percent matches and the literature. This technique was used throughout the study to confirm each target polymer type, to determine whether any degradation was evident as a result of treatment of target polymer types and to identify the polymer composition of extracted particles from both turtles.

Protocol suitability

Seven target polymers from daily-use items were used to test the suitability of the sequential extraction protocol (Table 1). Firstly, commercially manufactured high-density polyethylene (HDPE) micro-beads were isolated from the soap matrix of a commercially available facial cleanser (Garnier®, Pure and Active Daily Pore Scrub Wash). The size range of the micro-beads (192 ± 48 μm) was measured as the largest length of randomly-selected micro-beads (n = 20) under a dissecting microscope using the software ImageJ® (Rasband, 2012). Also, poly(ethylene terephthalate) (PET) from a single-use water bottle, polyethylene (PE) from a soft drink bottle lid, nylon (polyamide, PA) from a fishing line, vinyl chloride/vinyl acetate/vinyl terpolymer (PVC) from a conduit pipe, (aminoethyl) polystyrene (AM-PS) from a styrofoam box and polypropylene (PP) from a multiple-use shopping bag were used. Ten pieces approximately 1 cm in length were cut from each plastic item, with the exception of the facial cleanser micro-beads, which were only available as particles. Each plastic item was also grated into particles < 1 mm^2 (Table 1) with a metal kitchen grater. During this study, the term *pieces* refers to plastic items cut to ~ 1 cm (in length) and the term *particles* refers to plastic items grated to < 1 mm^2.

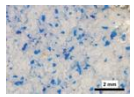
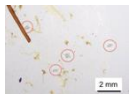
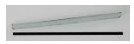
Acid digestion and emulsification are harsh chemical treatments with the potential to degrade plastics. To determine if the sequential extraction protocol affected the physical structure of the seven target polymers, the area of each of the ten pieces per plastic item was measured using the software Image J® before and after acid digestion and emulsification. A Student's T-test (two-tailed, paired samples) was run with a null hypothesis of no change in the area of the pieces before and after the digestion and emulsification steps.

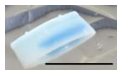
In order to establish whether the sequential extraction protocol affected smaller particles, the experiment was repeated exposing smaller particles (< 1 mm^2) of each of the seven plastic polymers (n = 20 per polymer) to the acid digestion and emulsification methods. Particles were manually sorted and counted.

The seven target polymers were also subjected to density separation (NaCl, 1.2 g/cm^3) after treatment with the acid digestion and emulsification methods. The capacity of each treated polymer to float or sink was recorded.

ATR-FTIR spectroscopy was used to determine whether the sequential extraction protocol affected polymer identification. The polymer composition of each plastic item was measured prior to treatment. Pieces were then treated with the acid digestion and emulsification methods. Pieces that could be recovered after these two methods were rinsed with RO water and dried before repeating the chemical analysis. A percent similarity, calculated by comparing the FTIR spectra of the treated polymers against those of the untreated polymers (PerkinElmer COMPARE algorithm), was used to assess whether there was any chemical degradation.

Table 1: The physical (shape, colour, and density) and chemical (degradation and composition) characteristics of polymers exposed to the sequential extraction protocol and their recovery rates. One example of each of the source plastic material, the simulated microplastic (~ 1 cm in length) and the microplastic after treatment with nitric acid and sodium lauryl sulfate is provided. Scale bars on pieces represent 1 cm.

Plastic items	Source plastic item	Particle size (length in mm ±SD) x width[a]	Piece before treatment	% particles recovery[b]	T-test *P*-value[c]	Piece after treatment	Floatation after density separation	Density (g/cm^3)[d, e]	Match score (%)[f]	Match description[g]
Facial cleanser micro-beads		0.16 ± 0.13		100 %	-		Yes	0.917-0.965	NT: 98 T: 98	High-density polyethylene
Fishing line		0.46 ± 0.04		0 %	Degraded	-	-	-	NT: 97 T: Degraded	82% nylon, 18% lamé
Styrofoam box		1.03 ± 0.34		100 %	0.44		Yes	1.04-1.1	NT: 91 T: 91	(Aminomethyl)polystyrene
Pipe		1.02 ± 0.12		100 %	0.80		No	1.16-1.58	NT: 95 T: 78	Vinyl chloride/vinyl acetate/vinyl terpolymer
Multiple-use shopping bag		(2.12 ± 0.39) x 0.094		100 %	0.84		Yes	0.9-0.91	NT: 98 T: 98	Polypropylene

Single-use water bottle		0.33 ± 0.15		0 %	Degraded	-	-	-	NT: 99 T: Degraded	Poly(ethylene terephthalate)
Soft drink bottle lid		0.41 ± 0.15		100 %	0.89		Yes	0.917-0.965	NT: 97 T: 97	Polyethylene

Notes:

[a] Width only mentioned when particles were fibres from synthetic fabrics (i.e. multiple-use shopping bag); [b] from 20 particles treated with nitric acid (HNO_3) and sodium lauryl sulfate (SLS); [c] comparison of the size of plastic item pieces (~ 1 cm in length) before and after treatment by HNO_3 and SLS, ($p > 0.05$); [d] as reported by Hidalgo-Ruz et al. 2012 (Table 7); [e] low density polyethylene (LDPE) ρ = 0.910-0.925 g/cm^3, high density polyethylene (HDPE) ρ = 0.941-0.965 g/cm^3; [f] NT: not treated, T: treated with HNO_3 and SLS; [g] chemical composition of all polymers remained unchanged after treatment with HNO_3 and SLS.

1

Extraction efficiency

Three replicates, each containing 6 g (w/w) of homogenised chyme from Turtle B, were used for method validation. Each replicate was spiked with five micro-beads and then processed with the sequential extraction protocol (described above). Since both fats and sediments were present in these samples, all three extraction methods; acid digestion, emulsification of fats, and density separation were used.

Procedural contamination

Three procedural contamination blanks comprising 6 mL RO water were processed in accordance with the sequential extraction protocol. Filters were visually inspected under a dissecting microscope. Any particle or fibre present on the filters was treated as procedural contamination.

Extraction and quantification of plastics from turtle chyme

Due to the challenges faced by this study (time limitations, complexity of samples), only a subsample of the homogenised turtle foregut content was analysed to quantify microplastic contamination. For each turtle, 48 g of homogenised foregut content was transferred into a glass beaker; this represented approximately half of Turtle A foregut content (weighing 98 g) and approximately 1 % of Turtle B foregut content (weighing 4.70 kg). Four replicates of 12 g (w/w) were prepared for Turtle A. For Turtle B, replicates had to be reduced to 6 g (w/w) to manage the high reactivity of these samples to nitric acid which otherwise caused an unmanageable amount of foam. The sequential extraction protocol outlined above was applied to these samples and potential microplastics were identified.

3. Results

Protocol Suitability

HNO_3 completely dissolved pieces and particles of PET and PA; no pieces or particles of these two target polymers were recovered after acid digestion (Table 1). The acid digestion and emulsification

methods did not affect pieces or particles of: PE, HDPE, PVC, AM-PS and PP; there was 100 % recovery of these pieces and particles after both treatments (Table 1).

T-test results for all recovered (i.e. incompletely dissolved) pieces (n = 10) indicated that acid digestion followed by emulsification resulted in no significant change in the area of non-digested target polymers pieces ($p > 0.05$) (Table 1). However, pieces that were coloured before treatment (i.e. from bottle lid and multiple-use shopping bag) suffered some discolouration (Table 1).

As expected, all recovered treated pieces (i.e. those not digested by the acid or emulsification treatment) floated in hypersaline NaCl ($\rho < 1.2$ g/cm^3) indicating their densities were not substantially altered (Table 1). The exceptions were the PVC particles, which did not float; pieces of PVC (1 cm in length) were observed at the bottom of the hypersaline brine solution. The density of PVC pieces remained higher than the hypersaline solution (PVC $\rho = 1.16$ to 1.58 g/cm^3, hypersaline NaCl $\rho =$ 1.2 g/cm^3) (Table 1).

Polymer identification using FTIR showed that matches of target polymer types to the spectral reference library were similar before and after acid digestion and emulsification (Table 1).

Extraction efficiency

Extraction efficiency of the sequential extraction protocol was 100 %; all spiked micro-beads were recovered from samples of turtle chyme.

Procedural contamination

Procedural contamination blanks revealed the presence of hair-like fibres and very fine dark particles < 100 µm on each filter. Because these particles were smaller than 100 µm, they didn't interfere with the extraction of microplastics sized > 100 µm conducted in this study. Procedural contamination particles were too small to be analysed by FTIR spectroscopy and their composition could not be confirmed.

Plastic quantification in turtle samples

After acid digestion, visual inspection of the residual chyme samples from Turtle A confirmed that no sediments were present, eliminating the need for density separation. However, samples from Turtle B proved multifarious and were therefore processed using all the methods in the extraction protocol (i.e. digestion, emulsification, and density separation methods). Macro- and microplastics were extracted from both turtles (Table 2). Three microplastic particles ranging between 0.45 mm - 2.51 mm were extracted from Turtle A. FTIR analysis identified these as two plastic paints; a transparent particle of polyethylene acrylic acid copolymer (EAA; match score 94 %), a dark green particle of polyvinyl acrylic paint (PVA; match score 78 %), and one mixed yarn synthetic fabric; a transparent round-shaped particle composed of cotton: olefin: polyester (match score 79 %). Two items of macroplastic debris were found in the foregut content of Turtle B: a 4.5 metre-long line matching to PA (nylon: wool: lamé; match score 95 %; Figure 2A) and a 21.5 cm^2 piece of soft plastic debris matching to HDPE (match score 97 %; Figure 2B). Four particles ranging from 0.76 – 2.95 mm were extracted from Turtle B. One white and one black particle each matched to a mixed-yarn synthetic fabric of cotton: wool: nylon (match score 86 % and 85 %, respectively), a piece of transparent film matched to cotton (match score 94 %) and a transparent particle matched to cotton: rayon: acrylic (match score 93 %). Based on the visual assessment, the cotton component of these particles is likely to be reconstituted cellulose, such as that used to produce biodegradable plastics, synthetic fabrics, and flexible films. After visual inspection of the spectra against their matching spectral library references and based on their match scores being > 70 %, all particles were confirmed as plastic paints and synthetic fabrics, as per Kroon et al., 2017.

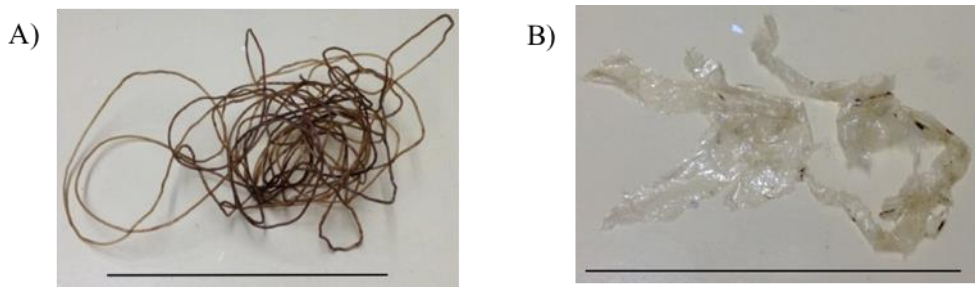


Figure 2: Macroplastic debris recovered from Turtle B; A) nylon line, B) high-density polyethylene. Scale bar represents 10 cm.

Table 2: Macro- and microplastics extracted from subsamples of chyme from two green turtles.

Size	Physical characteristics [a]	Match (%)	Match description
Turtle A			
0.45 mm	Transparent particle	94	Polyethylene acrylic acid copolymer
1.13 mm	Transparent, rounded particle	79	69 % cotton, 19 % olefin, 12 % polyester
2.51 mm	Dark green particle	78	Polyvinyl acrylic paint
Turtle B			
4.52 m	Line	95	60 % Nylon, 37 % wool, 3 % lamé
21.5 cm^2	Soft debris	97	High-density polyethylene
0.76 mm	White particle	86	45 % cotton, 40 % wool, 15 % nylon
0.84 mm	Black particle	85	45 % cotton, 40 % wool, 15 % nylon
1.52 mm	Transparent film	94	Cotton 90 %
2.95 mm	Transparent particle	93	Cotton, rayon, acrylic

[a] For microplastics (>100 um and < 5 mm), the description of the particles is given after processing with the sequential extraction protocol.

4. Discussion

In this study we developed and validated a sequential extraction protocol modified from Claessens et al. (2013) and Hidalgo-Ruz et al. (2012) for quantification of microplastics recovered from green turtle chyme. Because of the green turtle's diverse diet (herbivorous with opportunistic feeding on animals and incidental ingestion of sediment), their chyme can comprise of a range of biological and mineral matrices, such as seagrass, sediments, and potentially also fat (e.g. from cephalophods, jellyfish, and sponges). Consequently, the extraction of microplastics from chyme required a combination of different extraction methods, including acid digestion, fat emulsification, and density separation. Our protocol allowed for microplastic extraction from green turtle chyme with 100 % extraction efficiency for microplastics sized between 100 µm and 5 mm for five polymer types: PE, HDPE, AM-PS, PP and PVC. No physical (i.e. change in size) or chemical (i.e. polymer identification

using FTIR spectroscopy) degradation to these five polymer types was observed after exposure to the protocol. Four of the five target polymer types were readily recovered after each method. PVC was the exception; because the spiked PVC particles (ρ = 1.16 to 1.58 g/cm^3) are more dense than the hypersaline brine solution they did not float and were therefore not separated from the sediments in one of the chyme samples (Turtle B). They could only be detected in this sample after a visual assessment of the sediments was conducted (i.e. they were large enough in size to readily identify). This suggests that smaller and visually less-distinguishable PVC-derived microplastic contaminants present in turtle chyme containing sediments may not be detected, therefore resulting in an underestimation of the microplastic numbers present. With this in mind, and given that most common plastic polymers have a density lower than 1.2 g/cm^3 (Hidalgo-Ruz et al., 2012), this density separation method is suitable for the extraction of most microplastics. To recover a wider range of polymers including PVC, solutions with a density > 1.2 g/cm^3, such as sodium polytungstate (1.4 g/cm^3; Corcoran et al., 2009), or sodium iodide (1.6 g/m^3; Roch and Brinker, 2017) could be used. Four of the target polymer types deemed suitable for this extraction protocol represent 70 % of the plastics produced globally in 2007: HDPE = 21 %, PP = 24 %, PS = 6 %, PVC = 19 % (Andrady, 2011). This extraction protocol is therefore considered suitable for the majority of plastic pollutants that turtles are likely to ingest. In fact, PE and PP account for 98.5 % of the plastic detected in waters around Australia (Reisser et al., 2013), including where the turtles for the present study were collected. Microplastics made of PE and PP have been found in loggerhead turtles, fishes, and in True's beaked whales from the North Atlantic Ocean (Foekema et al., 2013; Lusher et al., 2015; Pham et al., 2017).

HNO_3 digestion degraded PA, consistent with that reported by Claessens et al. (2013). PA is used in the fabrication of fishing gear such as netting and traps (Andrady, 2011; Jones, 1995). These items have been reported to be the main source of entanglement for marine fauna (Gall and Thompson, 2015; Nelms et al., 2016), and lines are commonly ingested by sea turtles (Clukey et al., 2017; Schuyler et al., 2013). PET was also affected by HNO_3. PET is used in the fabrication of packaging such as single-use plastic bottles and food containers (Andrady, 2011; Barnes et al., 2009). Plastic bottles, such as the one used for validation of the methods, are a ubiquitous marine pollutant

(Eriksen et al., 2014) and although not found in this study, plastic bottle fragments have previously been found in sea turtle gut content (Wedemeyer-Strombel et al., 2015).

Another limitation of using HNO_3 digestion as a separation method is a notable discoloration of the target polymers tested. Most particles extracted from green turtles (i.e. after being processed with the sequential extraction protocol) were transparent or white, but two particles were still highly coloured (dark green and black). These results suggest that the colour agents in these plastic particles may be of different chemical composition to those in the test polymers (i.e. pigments rather than dyes), or incorporated using different manufacturing techniques (i.e. dispersion vs. dissolution) and therefore display some resistance to HNO_3. The potential discoloration of microplastics by the extraction protocol makes comparison with other studies more difficult, as most studies on microplastic ingestion by marine biota rely on visual inspection using microscopy as the primary identification technique (Cole et al., 2011). However some have identified microplastics using Raman spectroscopy (Remy et al., 2015) or FTIR spectroscopy (Güven et al., 2017; Hidalgo-Ruz et al., 2012; Lusher et al., 2013), a technique that is gaining traction as it becomes less expensive and more sensitive.

The limitation of our sequential extraction protocol in not being able to recover PA and PET, as a result of chemical digestion, or to not separate polymers with densities > 1.2 g/cm^3 (i.e. PVC) from sediments, must be taken into consideration when applied to complex biological samples possibly contaminated with a variety of microplastic polymer types. Blind use of this protocol and the individual methods therein will potentially result in an underestimation of microplastics in instances when these polymer types are present in pre-digested samples. We recommend further testing of the suitability of this protocol on more polymer types.

Hair-like fibres recovered on blank filters were likely to be airborne contamination, potentially from clothing (Foekema et al., 2013) even though care was taken during processing to avoid contamination. Since these contamination particles were < 100 µm in size they did not interfere with the extraction of particles from turtle samples in this study. Nevertheless, if interested in microplastics < 100 µm, further care must be taken to avoid this contamination; FTIR spectra should be acquired and added to a contaminant spectral library against which samples can be searched and

contaminants eliminated (Kroon et al., 2017). Further reductions in procedural contamination could be achieved through wearing synthetic-free natural fibre clothing as done by in Roch and Brinker (2017), or working in a sealed damp-wiped room (Taylor et al., 2016).

The rising concerns of microplastic pollution on marine wildlife drives the need for a reliable and comparable detection protocol (Nelms et al., 2016; Roch and Brinker, 2017). A protocol similar to the one validated in this study was recently published by Roch and Brinker (2017), whereby a combination of digestion of organic materials (from fish) and a density separation from mineral residue was used. Karlsson et al. (2017) used an enzymatic digestion method for the extraction of microplastics from biota (marine invertebrates and fish). The advantage of this method was that it did not alter the physical integrity of PA, PS, PP, and PE. The disadvantage of this and similar methods is that the microplastics are contaminated with a proteinaceous residue that is not easily removed, complicating chemical analysis (i.e. FTIR or Raman; Miller et al., 2017; Courtene-Jones et al., 2017). For future investigations of microplastics in green turtle chyme, alternate, less harsh digestion methods (i.e. sulfuric acid or enzymatic digestion) combined with a density separation and, if necessary, emulsification of fats should be tested to confirm extraction of PA-type polymers. Regardless, a combination of extraction methods like ours appears to be the most appropriate and consistent approach to extract microplastics from marine organisms and multifarious biological samples such as green turtle chyme.

Although a limited number of specimens were available, microplastics were recovered from both turtles analysed. A total of two macroplastics and seven microplastics; two plastic paint chips and five mixed-yarn synthetic fabric particles, were found in the foregut of the two turtles. EAA, the main component of one of the paint particles, is commonly used as a coating and in food packaging (Dupont, 2017). Paints, such as antifouling paint, and leachates from plastic products are known to pollute the marine environment. They can transport and/or leach toxic chemicals such as metals or tributyltins, and have the potential to impact the health of marine organisms by decreasing fecundity and fertilization success and inhibiting the development of eggs (Lithner et al., 2009; Negri and Heyward, 2001; Ozretić et al., 1998; Soroldoni et al., 2017; Wilson et al., 1993). Paint chips have also been found in sediment samples and sea birds (Fischer et al., 2015; Laist, 1997). Synthetic fabrics are

a large source of pollution to the marine environment via waste water (Browne et al., 2011; Law and Thompson, 2014; Salvador Cesa et al., 2017). During textile manufacture, extensive amounts of chemicals such as pesticides, monomers, additives, solvents, and dyes are used (Browne et al., 2011; Bruce, 2016; Luongo, 2015; Salvador Cesa et al., 2017). Once in the environment, these chemicals can become bioavailable and toxic to organisms that ingest them (Avagyan et al., 2015; Luongo, 2015; Salvador Cesa et al., 2017). Green turtles are likely to ingest paint and fabric particles entrapped in seagrass or macroalgae, or through trophic flow if the micro-particles were ingested by seagrass epibionts in the first instance (Remy et al. 2015). These results are cause for alarm because only a fraction of the foregut content was assessed, representing 1 % and 50 % of foregut content for Turtle B and Turtle A, respectively. Measurements of green turtle gastro-intestinal tracts by Magalhães et al., 2012 showed that the oesophagus, stomach, and small intestine together represent less than half of the total length of the digestive tract. In addition, Clukey et al. (2017) found that 70 % of ingested plastics were located in the large intestine compared with the stomach and small intestine for juvenile green turtles. The results presented here could therefore be just "the tip of the iceberg" as a much higher quantity of plastics could have been present in the large intestine of the two turtles analysed.

Marine plastic pollution affects sea turtles worldwide (Clukey et al., 2017; Nelms et al., 2016; Schuyler et al., 2014). In Brazil, 70 % of juvenile turtles analysed for plastic ingestion along the coast had ingested debris, with a mean number of 47.5 items per turtle (Santos et al., 2015). In the North Pacific Ocean, Wedemeyer-Strombel et al. (2015) reported that 83 % of the sea turtles studied had ingested anthropogenic debris. Marine debris ingestion by sea turtles is a global issue of increasing magnitude (Schuyler et al., 2014). Our results are consistent with these and other studies showing that green turtles inhabiting the Pacific Ocean are directly impacted by plastic pollution through ingestion (Boyle and Limpus, 2008; Clukey et al., 2017). Furthermore, they are supported by the fact that there is a high prevalence of secondary microplastics specifically made of PE and PP in Australian waters (Reisser et al., 2013). Macro-debris similar to the nylon line and soft, transparent debris found in Turtle B have been found in sea turtles around the world (Angelo Abreo et al., 2016; Clukey et al., 2017; Schuyler et al., 2014; Wedemeyer-Strombel et al., 2015). While awareness of the

issue is increasing, the lethal and sub-lethal impact of anthropogenic debris ingestion on sea turtles remains poorly known and warrants further investigation (Clukey et al., 2017; Nelms et al., 2016; Vegter et al., 2014).

Depth-integrated plastic concentration in Australian waters was estimated to be 8966.3 ± 1330.75 pieces (mostly < 5 mm) per km^2 (Reisser et al., 2013). Australian beaches are littered with pollution (44 % from the sea vs. 56 % from land) that is almost exclusively composed of plastic; 1,449,091 items were collected on Australian beaches in 2016 (Tangaroablue, 2016). Recent research indicates that tourism can be among the most significant sources of marine debris in the Great Barrier Reef World Heritage Area (Wilson and Verlis, 2017). These alarming numbers are the result of local and global mismanagement of plastic waste (i.e. available to enter the environment). In 2010, the amount of plastic waste entering the ocean worldwide (for 192 coastal countries) was estimated to 4.8 to 12.7 million tons, with Australia's contribution estimated at 0.01 to 0.25 million tons (Jambeck et al., 2015). Plastics floating on the surface of the open ocean accumulate in the convergence zone of each of the five subtropical gyres (Cózar et al., 2014) where sea turtles nest, migrate, or forage, depending on species and life stage (Clukey et al., 2017; Nelms et al., 2016). Plastic pollution also reaches even remote, non-industrialised places such as Antarctica (Isobe et al., 2017) and the Torres Strait (Ceccarelli, 2009) raising environmental concern. Plastics, and especially microplastics, are extremely difficult to remove from marine environments, thus the most effective mitigation strategy is to reduce inputs (Jambeck et al., 2015). Reducing inputs of plastics in the marine environment could be achieved by national and international measures towards improving waste management, enforcing legislation, decreasing use and production of single-use plastics, and enhancing ecological consciousness through education (Derraik, 2002; Haynes, 1997; Jambeck et al., 2015).

5. Conclusion

Our validated sequential extraction protocol can be used for efficient extraction of microplastics from green turtle chyme samples, which may comprise seagrass, sediment, and animal matter. The

detection of microplastics from a small portion of chyme from two green turtles highlights the need for analysis of an increased sample size (i.e. through opportunistic necropsies) in order to improve our knowledge of the microplastic loads of sea turtles from the Great Barrier Reef World Heritage Area. Despite being iconic animals, sea turtles are categorized as vulnerable to critically endangered with decreasing population trends (IUCN, 2017). Analysing chyme samples from a greater number of turtles will not only provide a greater understanding of the exposure of turtles to plastic pollution and ingestion, but will also increase our knowledge on the role plastic pollution plays in declining turtle health, in particular the sub-lethal and lethal effects of ingested anthropogenic debris (Clukey et al., 2017; Vegter et al., 2014). Finally, these findings highlight the need for increased organized efforts for plastic pollution mitigation and reduction into the marine environment.

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