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Are Nitric Acid (HNO₃) Digestions Efficient in Isolating Microplastics from Juvenile Fish?

Trishan Naidoo · Kimerra Goordiyal · David Glassom

Abstract A standard method for the detection and isolation of microplastics is required to adequately investigate plastic ingestion by juvenile fish. Dissections of juvenile fish guts require precise handling, which can affect the processing time if sample numbers are high. To investigate the efficacy of nitric acid (HNO₃) in aiding the isolation of microplastics using whole fish, we digested juvenile glassfish, *Ambassis dussumieri* (Cuvier, 1828), at room temperature and at 80 °C. For a complete digestion, overnight incubation in 10 mL of 55% analytical-reagent (AR) HNO₃ was sufficient for a whole fish of 1 g at room temperature. When coupled with elevated temperature, the digestion time is shortened to a few minutes and larger fish of 3 g can be digested in 30 min. Four of the five types of plastic survived the process, with nylon being the exception. This is a shortfall to the method; however, until a better method replaces it, we still value the use of HNO₃ for its simple, inexpensive, swift and complete digestions of whole fish. Four fish species from two feeding guilds were digested using this method to validate its use. The number of plastic particles ingested did not differ between benthic and pelagic species and microplastic fibres comprised the majority of the plastic types found.

Highlights • HNO₃ digestions on juvenile fish are simple, inexpensive and swift.
• Overnight incubation was sufficient for whole fish of 1 g at room temperature.
• With elevated temperature, fish of 3 g digested in 30 min.
• Four of the five types of plastic survived the digestion, nylon being the exception.

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1 Introduction

Microplastic ingestion by larval and early juvenile fish (hereby termed early stages) has not been as well-documented as their adult counterparts. Research has mainly been limited to experimental studies, such as de Sá et al. (2015). This is concerning as these early stages are vulnerable to environmental perturbations (Whitfield 1990) and may suffer a more pronounced effect of microplastic ingestion due to the size of juvenile fish relative to microplastic particles. Juveniles may encounter plastics more often due to their sheltering and feeding under plastic debris within current lines in the ocean and using polluted estuaries, which may also have as high organic pollutant loads, associated with plastic particles, as nurseries (de Sá et al. 2015).

Efficiently isolating microplastics from these early life stages of fish is a key step toward identifying how microplastic ingestion ranks among the many other mortality risks to fish populations and may thus guide future stock management (Markic and Nicol 2014).

Information on the size or stage at which a fish would be at risk of ingestion of a specific suite or size of microplastics is vital, as smaller organisms may not be able to consume all particles within the general microplastic size range (Cole and Galloway 2015). In part, the reason for the lack of such information is the difficulty in isolating plastics from these small fish and the lack of a standard method to do so, making comparisons difficult (Avio et al. 2015). Unlike adult fish, examining the gut contents of juveniles may require high-precision dissections, primarily due to their small size, making it difficult to process large sample numbers under time constraints (Khan et al. 2015). In addition, organic matter and other foreign particles consumed by fish may mask microplastics, complicating the isolation process (Lusher et al. 2017).

The use of nitric acid (HNO_3) to digest tissue for microplastic quantification, by Claessens et al. (2013), could aid the isolation process. The method has been considered as one of the more destructive plastic isolation techniques, since it may degrade plastics (Lusher et al. 2017), yet derivatives of it have been prescribed by the Convention for the Protection of the Marine Environment of the North-East Atlantic (OSPAR) as a monitoring protocol (Dehaut et al. 2016). It has also been used to isolate microplastics from clams (Davidson and Dudas 2016), shrimp (Devriese et al. 2015) and lugworms (van Cauwenberghe et al. 2015). Desforges et al. (2015) suggested that more polymers should be tested using nitric acid isolation techniques to determine if they survive the digestion protocol. Despite the drawbacks, nitric acid may be the simplest, most inexpensive way of processing large sample numbers quickly, which is useful for routine bio-monitoring projects (Desforges et al. (2015).

Existing isolation methods include using other acids, strong bases, other oxidising agents and enzymes to digest biological tissue (see Lusher et al. (2017) for a review). However, these have limitations. For example, Collard et al. (2015) found that using sodium hypochlorite did not degrade polyamides but reduced the mass of PVC, while other studies suffer from shortcomings such as long incubation times (Foekema et al. 2013), discolouration of plastics (Dehaut et al. 2016), using expensive digestion agents (Cole et al. 2014), staining tissues, rendering particles the same colour as the stain less visible (Davison and Asch 2011), using many components and digestion steps making the process slower and at the end still using HNO_3 to clear up membrane

filters further (Collard et al. 2015) or ground gut samples before extraction which could potentially invalidate counts if plastics are also broken down (Avio et al. 2015).

In our case, we aimed to adapt the methodology of Claessens et al. (2013) for use on juvenile fish. The proposed method would need to be quick, use realistic acid volumes and concentrations and be applicable to a range of plastic types. It would also need to have some degree of sample storage practicality to avoid plastic deterioration if samples are to be stored for a few days after the digestion preceding filtration. We used juvenile glassfish *Ambassis dussumieri* (Cuvier, 1828) as a sentinel species to investigate the method. This species can spend its entire life cycle in estuaries (van der Elst 1993) and may therefore be influenced by the high particle concentrations generally found in estuaries, especially near urban areas (Naidoo et al. 2015). We aimed to expand on previous studies investigating the efficiency of the HNO_3 digestion method by examining the acid volume use, the time taken for digestions and the plastic types that survive the HNO_3 digestions. The objectives were to (1) digest juvenile fish of similar sizes in different volumes of acid to check for acid use efficiency, (2) digest fish at an elevated temperature to examine if this increases efficiency and (3) immerse different plastics in acid, either directly or within fish spiked with plastics, to assess if any plastic degradation occurs. Juvenile fish of four species from two feeding guilds were digested using the protocol to validate it and to assess the frequency of microplastic ingestion by juvenile fish in the harbour. We predicted that (1) a low acid volume would be sufficient to digest whole juvenile fish, without clogging up membrane filters; (2) higher temperatures would speed up the reaction; (3) all plastic types would be resistant to acid digestion for at least 24 h and (4) microplastic concentrations and types from field collected fish will differ between species that belong to different feeding guilds.

2 Materials and Methods

2.1 General Procedures

Glassfish were collected from the Durban harbour, KwaZulu-Natal, South Africa (29° 52′ S, 31° 04′ E) using seine nets. Fish were rinsed with distilled water, and fish mass (g) and length (mm) were recorded. All

acid digestions were performed within a fume cupboard, using 55% AR HNO$_3$. Glass honey jars (350 mL) set in water baths and covered with watch glasses were used to contain the reactions and prevent contamination. All glassware and handling equipment were rinsed in distilled water and checked under the microscope for microplastic contamination before use. At the end of each reaction, the products were added to 100 mL of distilled water to dilute the digested products before vacuum filtration, for both handling and equipment safety. In pilot digestions, 2-, 5- and 10-μm filters clogged up easily and therefore, 20-μm filters were used in this study. After filtration, filters were placed under a microscope and microplastics were counted and photographed. Forceps were then used to carefully transfer plastics to a mass balance. All statistical analyses were done on R 3.3.2 (R_Development_Core_Team 2014).

2.2 Acid Volume

This was the primary variable to determine since using too little acid may leave incomplete digestions that clog up membrane filters, making them difficult to examine and using too much would be wasteful. Twelve juvenile fish of similar size, 1.290 ± 0.120 g (std. dev.), were placed into individual 350-mL glass honey jars containing three replicates of either 5, 10, 20 or 30 mL of acid and digested overnight at room temperature. The digested tissue was vacuum-filtered, and filters were examined visually to determine the optimal volume for tissue degradation.

2.3 Tissue Digestion

Fish of masses ranging from 0.124 to 3.027 g were digested in 10 mL HNO$_3$ at either room temperature or 80 °C in a water bath. For each fish, the length (mm) and mass (g) were recorded before acid addition. Once completely disintegrated, the time elapsed was recorded (min). An analysis of covariance (ANCOVA) was first run to determine if there were significant differences between the temperatures while controlling for fish mass. However, the assumption of homogeneity of regression slopes was violated (significant interaction between the independent variable and the covariate). Therefore, mass, which is the covariate, was converted into categories of small (≤ 1.000 g), medium (> 1.000 g ≤ 1.900 g) or larger fish (> 1.900 g) and a

factorial analysis of variance (ANOVA) was then used to analyse the data. The elapsed time was log-transformed to satisfy the assumptions of normality and homoscedasticity of the residuals. These assumptions were tested using a Lilliefors Kolmogorov-Smirnov normality test ($D = 0.078$, $p = 0.845$) and Levene's test for homogeneity of variance ($F = 2.460$, $df = 5$, $p = 0.056$), respectively. Multiple comparisons of the mass categories were performed with a Tukey's HSD test.

2.4 Plastics Immersed Directly in Acid and Spiked in Fish

Plastic materials used for the digestions were obtained from previous sampling work and clean-ups done in Durban harbour. Five polymers (plastic types) were used in the experiment. Polyethylene was duplicated to include thin film and microscrubbers with a high surface area-to-volume ratio (Table 2). Digestions were run by immersing three replicates of each of the plastic types directly in HNO$_3$ for 1 month at room temperature. In similar experiments, plastics were also spiked into *Ambassis dussumieri* by opening up the gastrointestinal cavity and using a large syringe to insert plastics within. For larger plastic types, fine forceps were used to insert plastics into the cavity. These were then digested at 80 °C. Experiments were run according to the general procedures described above, with 10 mL HNO$_3$ placed in pre-washed glass honey jars. The different types of plastics that were used were in accordance with the common types recorded in field surveys (Naidoo et al. 2015) and those that had been ingested by fish in an urban harbour (Naidoo et al. 2016). Samples of all plastic types were run on Fourier transform infrared spectrometry (FTIR) to determine the polymers that were used. The fixed mass of plastics in each replicate was recorded before being used. The mass of each plastic type immersed directly in acid was measured at various times during the 1-month trial period. Foam material was resuspended in distilled water after the digestion and dried to constant mass to get more accurate mass readings. During these measurements, a metal sieve was used to capture the plastics for mass measurements before they were placed back into the acid. Larger plastic types were counted before being placed back into the acid, and particles were measured at random to check for any difference in size or degradation. All fish

used for the spiked digestions were of similar weight (1.091 ± 0.118 g) to keep mass standard.

Single paired-sample *t* tests were performed on each of the plastic types immersed directly in acid wherever possible to determine if the mass after 1 month had changed. Before paired *t* tests were run, the assumption of normality was tested using a Shapiro-Wilk's test with $W = 0.964$ and $p = 0.637$, $W = 0.964$ and $p = 0.637$, $W = 0.964$ and $p = 0.637$ and $W = 1.000$ and $p = 1.000$ for film, polyester, PVC and scrubbers, respectively. The assumption of normality was not satisfied for foam data, and therefore, a Wilcoxon signed-rank test was performed. For experiments digesting fish spiked with plastics, a one-way ANOVA was used to compare the loss of plastic mass during the digestion for the various plastic types. Data were log-transformed. The residuals of the ANOVA were plotted to test if they resembled that of a normal distribution, and a Levene's test was used to test for homogeneity of variance ($p = 1.028$). Finally, a Tukey HSD test was used for comparison among plastic types.

2.5 Ingestion by Juvenile Fish

Microplastic abundance in four fish species, collected from the Durban harbour, was investigated (Table 1). Nine fish of each species were placed into individual 350-mL glass vials containing 1 mL of acid per 1 g of fish and digested overnight at room temperature. The digested tissue was then filtered and examined visually using a Zeis™ DV4 dissecting microscope.

A one-way ANOVA was used to compare the mean abundance of ingested microplastics among the four fish species. Data were $\log_{10}$-transformed, and the assumption of normality was satisfied using a Shapiro-Wilk's test. A Tukey HSD test was used to compare the number of ingested plastics among the fish.

3 Results

3.1 Acid Volume

A volume of 10 mL of HNO_3 filtered through a 20-μm membrane filter was found to be the ideal volume to use for the rest of the experiments. Filters with a smaller mesh size and a lower acid volume clogged up the membrane when digesting a fish (Fig. 1), while using a higher volume than 10 mL of acid was wasteful.
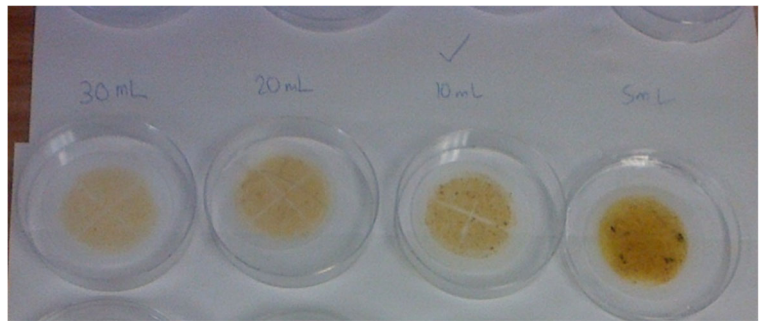
3.2 Tissue Digestion

Fish digested more rapidly at the elevated temperature than those at room temperature (Fig. 2). The largest fish from the 80 °C treatment digested 26 times faster than the largest fish from the room-temp treatment (Fig. 2). Although the main effects of temperature and mass categories were significant ($F = 1334.332$, $df = 1$, $p = < 0.0005$ and $F = 65.143$, $df = 2$, $p = < 0.0005$ respectively), there was a significant interaction between them ($F = 7.394$, $df = 2$, $p = 0.002$). Comparisons between combinations of temperature settings and mass categories were all significantly different at the 0.05 level, with the exception of the medium- and large-size categories digested at 80 °C ($p = 0.996$).

3.3 Plastic Digestion and Fish Spiking

3.3.1 Plastics Immersed Directly in Acid

Overall, plastics used in each replicate had a mean of 0.050 ± 0.002 g ($n = 42$). The number of particles in each replicate ranged from just two pellet particles to numerous microscrubbers. Most plastic types tested survived the exposure to HNO_3 for the experimental period of 1 month, with the exception of nylon (Table 2). During the first 24 h of exposure, all of the nylon particles were disintegrated completely by the HNO_3

Table 1 Juvenile fish from two feeding guilds collected from Durban harbour

Feeding mode	Species name	Common name	Mass (g)
Zooplanktivorous	*Ambassis dussumieri* (Cuvier, 1828)	Glassfish	0.43–1.31
	Hilsa kelee (Cuvier, 1829)	Razorbelly	0.23–1.82
Benthivorous	*Sillago sihama* (Forsskål, 1775)	Silver sillago	0.66–1.92
	Gerres filamentosus (Cuvier, 1829)	Pursemouth	0.29–0.58

Fig. 1 Filter membranes after *A. dussumieri* digestions in 5, 10, 20 and 30 mL HNO_3

(Table 2). No significant change in mass could be detected after film, foam, PVC and scrubber particles were immersed in acid for 1 month (Table 2). There was not enough variability to test the difference for pellets; however, there was very little mass change after 1 month (Table 2). There was a small change in the mass of polyester after 1 month; however, this was not significant (Table 2).

3.3.2 Plastics Spiked in Fish

There was an overall difference among plastic types for the change in mass during the fish-spiked digestions ($F = 57.650$, $df = 6$, $p = <0.0005$). However, this difference was found only between nylon and all other plastic types (Table 2). The nylon completely disintegrated during the digestion, while other plastic types survived the digestion with no major loss of mass (Fig. 3). Apart from nylon, the most mass change was observed in foam material (0.010 ± 0.010 g) and the least change was observed in pellets and PVC, neither of which showed any change in mass. Other plastic types showed very

Fig. 2 The total digestion time (min) of fish placed in size categories based on their initial mass. HNO_3 digestions occurred either at room temperature or at an elevated temperature of 80 °C. Bars represent the standard deviation

limited change in mass; for example foam, film and polyester had slightly positive changes in mass of 0.010 ± 0.010, 0.002 ± 0.000 and 0.003 ± 0.000 g, respectively, owing to the digestion debris that adhered to their surfaces (Fig. 3c, i, l). Mass of scrubbers had a slightly negative change of -0.002 ± 0.000, but none of these was significantly different from each other. Partial whitening occurred in the PVC particles immersed in acid which did not occur as much when spiked in fish (Fig. 3n, o).

3.4 Ingestion by Juvenile Fish

Microplastic particles were ingested by all juvenile fish from both feeding guilds, and the mean abundance of these particles was not significantly different among the four species ($F = 2.442$, $df = 35$, $p = 0.082$) (Fig. 4). Microplastic fibres contributed the highest proportion for all species (*A. dussumieri*, 98%; *Hilsa kelee*, 94%; *Sillago sihama*, 98% and *Gerres filamentosus*, 94%). The colour composition of fibres varied considerably but were predominantly clear, black and red (Fig. 5).
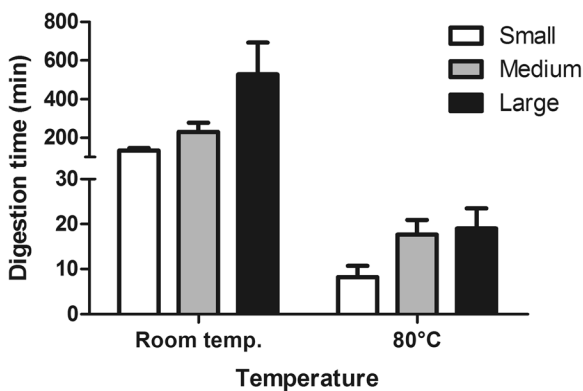
4 Discussion

We aimed to test if the use of an HNO_3 method would be suitable for isolating plastics from juvenile fish. For the digestion of a fish of 1 g, 10 mL of 55% AR HNO_3 was found to be the optimal acid volume. This was half of the volume used by Claessens et al. (2013) who used 69% HNO_3 to digest mussels. This difference could be due to the fish being much smaller with softer tissue than mussels or due to the higher fat content of mussels that may require a higher volume of acid to dissolve. Initially, whole juvenile fish of around 1 g digested in 5 mL of acid-clogged membranes making material inspection difficult. Increasing the volume to 10 mL

Table 2 Plastic polymers and size measurements (mm) used in the digestion experiments. Mean mass measurements (g) ± std. dev. are shown for plastics immersed directly HNO_3 for 1 month and for those that were spiked in fish. Paired-sample t test results are given for plastics immersed directly in acid, and for spiked experiments, the different letters in lowercase denote a significantly different change in mass among plastic types

	Nylon	Pellet	Film	Foam	Polyester	PVC	Scrubber
Polymer	Polyhexamethylene nonanediamide	Polyethylene, high-density	Polyethylene, high-density	Polystyrene	Poly 1,4-butylene terephthalate	Polyvinyl chloride	Polyethylene, high-density
Shape	Cylindrical	Round	Square	Round	Square	Irregular	Round
	5 mm	4 mm²	5 mm²	5 mm²	5 mm²	L 1.940 ± 0.610	D 0.267 ± 0.050
	D 0.434 ± 0.030		H 0.128			W 0.608 ± 0.139	
	$n = 5$					$n = 5$	$n = 5$
Directly immersed in acid							
Before	0.050 ± 0.000	0.050 ± 0.002	0.050 ± 0.001	0.052 ± 0.002	0.049 ± 0.001	0.050 ± 0.001	0.050 ± 0.000
After 1 month	0	0.049 ± 0.003	0.048 ± 0.001	0.052 ± 0.002	0.044 ± 0.003	0.049 ± 0.002	0.049 ± 0.001
	All digested	*	$t = 2.6458$	$V = 0$	$t = 3.0237$	$t = 0.75593$	$t = 1.7321$
			$df = 2$		$df = 2$	$df = 2$	$df = 2$
			$p = 0.118$	$p = 0.180$	$p = 0.094$	$p = 0.529$	$p = 0.225$
Spiked in fish then digested in acid							
Before	0.050 ± 0.000	0.045 ± 0.001	0.049 ± 0.001	0.050 ± 0.000	0.050 ± 0.002	0.050 ± 0.000	0.050 ± 0.000
After	0	0.045 ± 0.002	0.051 ± 0.002	0.060 ± 0.012	0.053 ± 0.002	0.050 ± 0.001	0.048 ± 0.002
Δmass	− 0.050 ± 0.000	0.000 ± 0.001	0.002 ± 0.001	0.010 ± 0.012	0.003 ± 0.003	0.000 ± 0.001	− 0.002 ± 0.002
Tukey's group	a	b	b	b	b	b	b

D diameter, W width, L length, H height
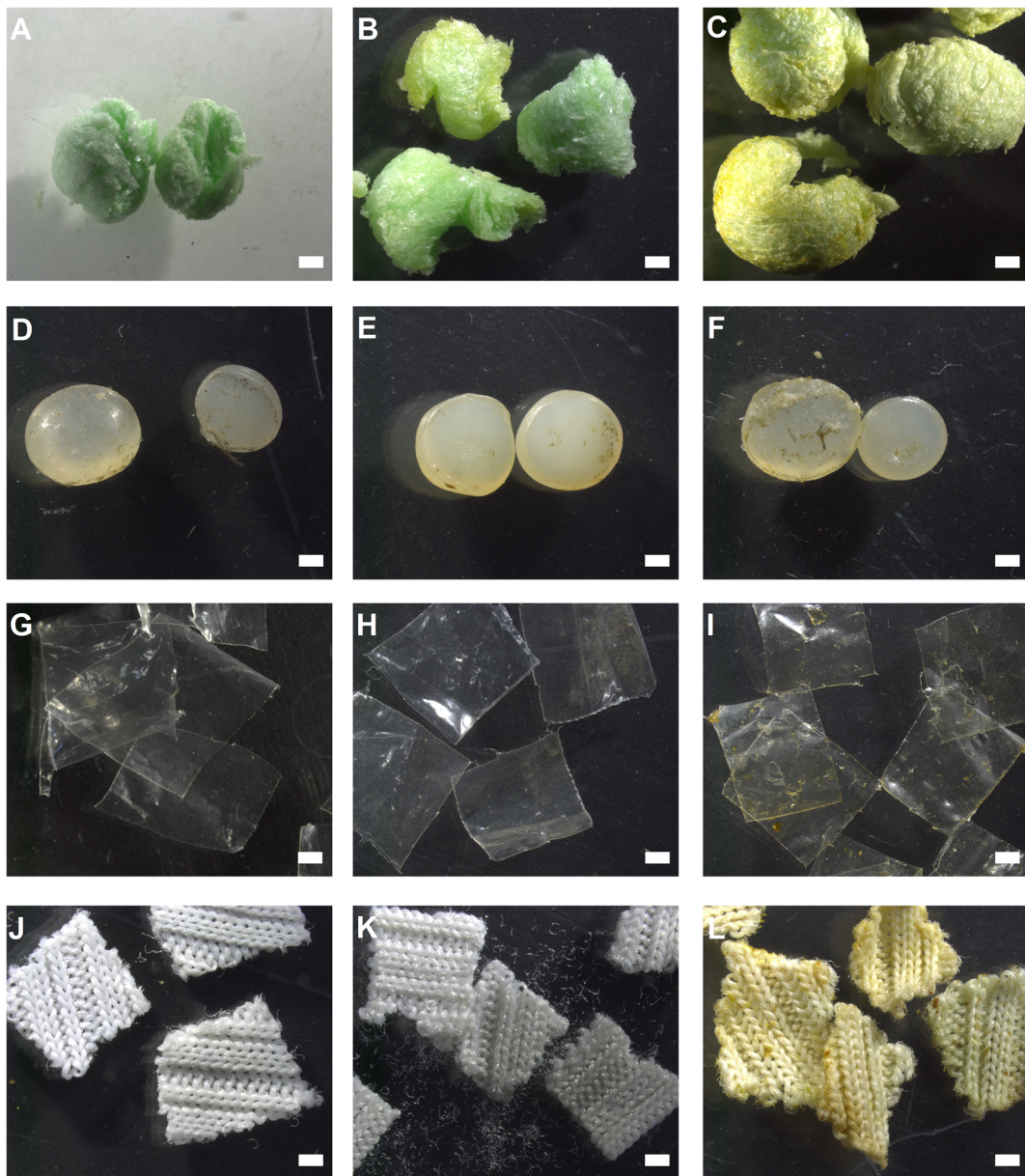
* - Test was not performed

Fig. 3 Raw plastics on the left, plastics immersed in acid in the centre and plastics spiked in fish before digestion on the right. **a–c** Foam. **d–f** Pellets. **g–i** Film. **j–l** Polyester. **m–o** PVC. **p–r** Microscrubbers. **s** Nylon. **t, u** Closer view of microscrubbers. All scale bars represent 1 mm, except those in **t** and **u** which are 0.2 mm

solved this. A similar fish mass-to-acid volume ratio may therefore prove useful for future digestion experiments on juvenile fish using the same acid concentration.

At elevated temperatures, the digestion times decreased dramatically, lending clear support for the initial hypothesis that the reaction would be sped up. The juvenile fish digested in a matter of minutes when heated, and this is beneficial for processing large sample numbers. Smaller fish digested faster. Additionally, this method eliminates the need for prior dissection of the fish, in contrast to studies that have generally removed gastrointestinal tracts first, rather than using whole animals in their isolation protocols (Lusher et al. 2017). These dissections are difficult to perform on fish in their early stages, and some protocols do a separate digestion
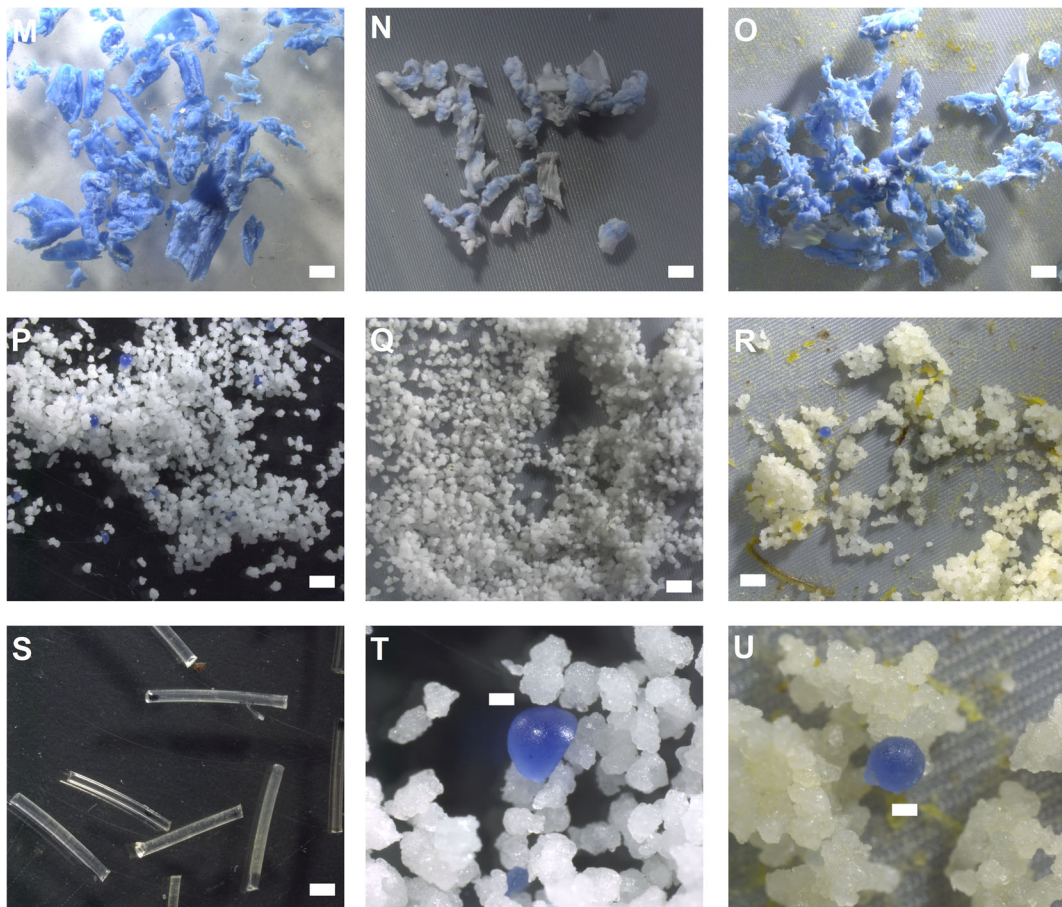
Fig. 3 (continued)

for fish tissue to assess the whole-body burden of plastics afterward (Lusher et al. 2017), which is more relevant for human consumption monitoring (Dehaut et al. 2016).

Most plastics showed miniscule to no mass change for either the direct immersion or spiked experiments;
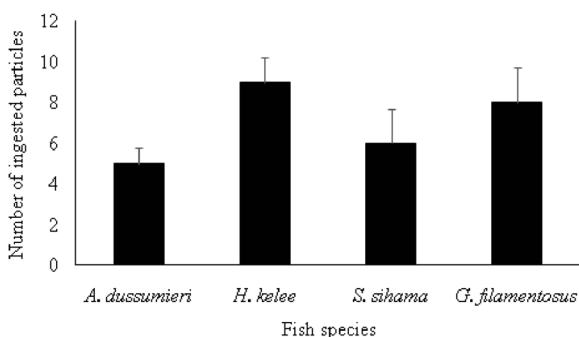

Fig. 4 Number of microplastics ingested by four species of juvenile fish (mean + SE)

however, nylon immersed in acid digested away within the first 24 h. Nylon was also completely disintegrated during digestions following the fish spiking. This was in contrast to Claessens et al. (2013) who found that 98% of nylon fishing fibres (100 × 400 μm), which were much smaller than those used here, could be recovered when using HNO_3. Therefore, studies using this method for counts should state that these counts are conservative (Desforges et al. 2015). Modulating the concentration of acid does not seem to work, since a decrease in concentration could result in lower digestion efficiency that could block up filter membranes (Karami et al. 2017). It may be the case that one has to determine the common plastics in the environment and visually in a small sample of fish guts first before acid use. The proportion of nylon relative to other microplastics can then be factored in after the digestion. If there is a high concentration of nylon material, as could be the case for estuaries that are heavily impacted by fishing gear (e.g.

Fig. 5 The proportion of fibre colours found in four species of juvenile fish
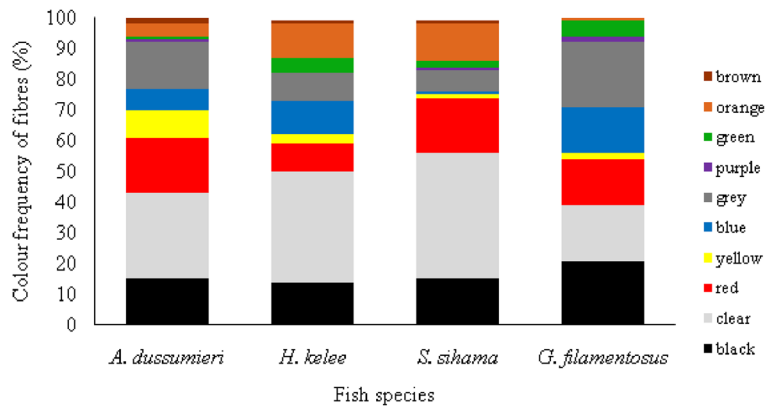
Possatto et al. 2011), then this method would not be suitable as the plastics would be severely under-sampled. However, in Europe for example, less than 1 million t per annum of nylon including other polyamides is produced, compared to an overall plastic production of 49 million t per annum (PlasticsEurope 2015); therefore, nylon is unlikely to comprise the largest proportion of plastics in many areas.

Although the nylon disintegrated, all other polymers tested seemed to show little degradation when exposed to the protocol and this is considering that the plastics were immersed in acid for 1 month. Desforges et al. (2015) noted that using HNO_3 at 80 °C caused a yellowing of the filters after the digestion process. There was some degree of yellow material present on the filters here from the digested tissue, but this did not hamper visual inspection (Fig. 3o, r, u). This could mean that samples can be stored a little while after digestion, if the filtration step cannot be done all in a single day.

Using this protocol on field-collected juvenile fish extracted similar quantities and types of microplastic particles from two zooplanktivorous and two benthivorous fish. This is supported by previous work showing that microplastics can be ingested by fish from different feeding guilds (Lusher et al. 2013; Neves et al. 2015; Campbell et al. 2017). There was no significant difference in the average number of ingested microplastics between the fish from the two feeding modes (Fig. 4). Therefore, the hypothesis that the average number of ingested microplastics will differ between fish species with different modes of feeding was rejected. A similar pattern of microplastic ingestion between pelagic and benthic fish was reported by Lusher et al. (2013), who sampled five fish per habitat. This trend was also reported by Neves et al. (2015), who

assessed the presence of microplastics in four pelagic and five benthic fish.

The high percentage of fibres over other types of microplastic detected in this study (94–98%) was consistent with the findings of Lusher et al. (2013) (68%), Neves et al. (2015) (66%) and Mizraji et al. (2017) (99%). A similar colour range of ingested fibres was identified across all four sampled fish species, suggesting that both the zooplanktivorous and benthivorous fish were feeding indiscriminately (Fig. 5). The high prevalence of fibres was expected because the fish were collected from the Durban harbour, where there is a lot of activity such as fishing, boat and ship mooring and discharge of industrial and wastewater effluents (Rathbone et al. 1998; Forbes and Demetriades 2008; Browne et al. 2011; Naidoo et al. 2015; Nel et al. 2017). The excessive consumption of fibres by fish, especially juveniles, maybe potentially hazardous because of the additives and pollutants associated with plastics that have been implicated in a variety of harmful effects (reviewed by Wright et al. (2013) and Jovanović (2017)).

In conclusion, we recommend the nitric acid digestion for use on juvenile fish, particularly for researchers working with high sample numbers, on a tight deadline and provided that nylon is not largely prevalent in the environment that is being considered. The lengthy, expensive and partially complete digestions of some of the other methods will inevitably impact sampling efficiency during large monitoring programs, while the advantages of using nitric acid are that it is readily available, cheap and quick under the right conditions. The method was successfully used to document the presence of microplastics in two pelagic (i.e. *A. dussumieri* and *H. kelee*) and benthic (i.e. *G. filamentosus* and *S. sihama*) fish species from the Durban harbour.

Compliance with Ethical Standards

Conflict of Interest   The authors declare that they have no conflict of interest.

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