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What goes in, must come out: Combining scat-based molecular diet analysis and quantification of ingested microplastics in a marine top predator

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Abstract

1. Microplastics (plastic particles <5 mm in size) are highly available for ingestion by a wide range of organisms, either through direct consumption or indirectly, via trophic transfer, from prey to predator. The latter is a poorly understood, but potentially major, route of microplastic ingestion for marine top predators.
2. We developed a novel and effective methodology pipeline to investigate dietary exposure of wild top predators (grey seals; *Halichoerus grypus*) to microplastics, by combining scat-based molecular techniques with a microplastic isolation method. We employed DNA metabarcoding, a rapid method of biodiversity assessment, to garner detailed information on prey composition from scats, and investigated the potential relationship between diet and microplastic burden.
3. Outcomes of the method development process and results of both diet composition from metabarcoding analysis and detection of microplastics are presented. Importantly, the pipeline performed well and initial results suggest the frequency of microplastics detected in seal scats may be related to the type of prey consumed.
4. Our non-invasive, data-rich approach maximizes time and resource–efficiency, while minimizing costs and sample volumes required for analysis. This pipeline could be used to underpin a much-needed increase in understanding of the relationship between diet composition and rates of microplastic ingestion in high trophic level species.

KEYWORDS
diet analysis, DNA, marine mammals, metabarcoding, microplastics, next-generation sequencing, pinnipeds, prey composition

1 | INTRODUCTION

An estimated 9.6 to 25.4 million tonnes of plastic are projected to enter the global ocean annually by 2025 (Jambeck et al., 2015). As a result, improving our understanding of the relationship between plastic pollution and impacts on marine species is a widely acknowledged global priority (UNEP, 2016). Microplastics (plastic particles <5 mm in size) are ubiquitous in many aquatic environments and, due

to their small size, are highly bioavailable to a wide-range of species, from low-trophic level organisms to top predators (Desforges, Galbraith, & Ross, 2015; Nelms et al., 2019; Steer, Cole, Thompson, & Lindeque, 2017).

Marine microplastics present in seawater, sediment or on vegetation, may be consumed as a result of being mistaken for food or due to indiscriminate feeding strategies (e.g. filter feeding; Besseling et al., 2015; Hall, Berry, Rintoul, & Hoogenboom, 2015). Additionally, they may be ingested indirectly as a result of trophic transfer, whereby prey-containing microplastics are consumed (Farrell & Nelson, 2013; Lourenço, Serra-Gonçalves, Ferreira, Catry, & Granadeiro, 2017; Nelms, Galloway, Godley, Jarvis, & Lindeque, 2018). Ingestion of microplastics has been found to cause detrimental effects, such as intestinal damage, oxidative stress, energetic depletion and reduced reproductive output in some low trophic-level organisms (Cole, Lindeque, Fileman, Halsband, & Galloway, 2015; Lei et al., 2018). Furthermore, hydrophobic chemical contaminants present in seawater, such as heavy metals and polychlorinated biphenyls, can adhere to the surface of microplastics and, if ingested, may be released into the organism and exert toxic effects (Teuten et al., 2009).

Understanding predator diets is crucial for examining disruptions to trophic interactions and potential threats to species and habitats that may be caused by anthropogenic factors (Jeanniard-du-Dot, Thomas, Cherel, Trites, & Guinet, 2017), such as plastic pollution. Marine mammals, in particular, are often considered sentinels for marine ecosystem health due to their high trophic level, extensive foraging ranges, sampling of the full water column and longevity (Bossart, 2011; Fossi et al., 2014; Moore, 2008). Although they ingest microplastics, the route of uptake and resulting biological effects remain unclear (Lusher, Hernandez-Milian, Berrow, Rogan, & O'Connor, 2018; Lusher et al., 2015; Nelms et al., 2019). For this method development, we chose to focus on a single species (grey seals; *Halichoerus grypus*) as a case study but the pipeline developed here could be applied to any predatory species for which the question of microplastic ingestion is relevant.

Grey seals are top predators in United Kingdom (UK) waters, consuming a range of demersal fish species, such as sand eel, cod and other gadoid fish (Brown, Bearhop, Harrod, & McDonald, 2012; Gosch, Hernandez-Milian, Rogan, Jessopp, & Cronin, 2014; Hammond & Wilson, 2016). While it has been shown they can ingest microplastics via trophic transfer from contaminated fish in a captive environment (Nelms et al., 2018), little is known about the extent to which seals ingest microplastics in the wild and whether the risk of doing so relates to their prey composition.

Obtaining dietary information can be difficult for many marine mammal species because they are logistically challenging to access and sample. Stranded animals, from which gut content may be extracted for dietary analysis, are investigated when accessible (Fernández et al., 2014; Mintzer, Gannon, Barros, & Read, 2008; Nelms et al., 2019). However, animals that died from infectious disease, starvation or other non-trauma-related causes of mortality, may introduce bias due to probable abnormal feeding behaviour prior to death (Fernández et al., 2014; Mintzer et al., 2008; Nelms et al., 2019). Grey seals offer the opportunity for relatively easy and representative sample collection because they routinely haul-out on land to rest, breed and moult, during which time they defecate. Although scats (faeces) only provide a snapshot of what the animal has recently consumed (previous *c*. 48 hr), and may be biased towards species present within the immediate proximity of the haul-out site (Grellier & Hammond, 2006; Jeanniard-du-Dot et al., 2017), scat-based methods are non-invasive and have traditionally been utilized to effectively examine diet composition of typical, living animals, using hard parts from undigested prey remains present in the scat (Grellier & Hammond, 2006; Jeanniard-du-Dot et al., 2017). These methods are, however, labour-intensive, time-consuming and often miss gelatinous, rare or less robust organisms (Deagle, Kirkwood, & Jarman, 2009). In addition, different prey species digest at varying rates so their importance in the diet may be under- or over-represented (Grellier & Hammond, 2006; Jeanniard-du-Dot et al., 2017). In recent years, molecular techniques, which can overcome these issues, have been developed using amplification, by Polymerase Chain Reaction (PCR), and sequencing, of a chosen species-specific gene fragment or barcode to better understand diet composition (Deagle et al., 2005). Such a technique, which provides presence/absence information for each potential prey species, can be performed on small quantities of faecal matter, but traditional cloning and subsequent sequencing of the amplicons is time-consuming and therefore limits the number of scats and sequences that can be processed. Quantitative PCR (qPCR) methods have also been developed to quantitatively assess the presence of a particular species in faecal matter (Matejusová et al., 2008), but this can also be time-consuming to develop and uses much smaller amplicons, such that primer design for distinguishing closely related species can be challenging. Both standard and qPCR require some knowledge of the likely prey encountered and the building of an appropriate primer and sequence library to cover all probable prey species (Deagle et al., 2005). Both may also underestimate contribution of species from which the DNA has degraded. More recent tools, such as next generation sequencing, offer a quick and reliable method of assessing diet composition from small sample volumes (McInnes et al., 2017). Metabarcoding is a rapid method of biodiversity assessment that combines two technologies: DNA-based identification (barcoding) and high-throughput sequencing (HTS) allowing the mass-amplification (using universal primers) of DNA barcodes from collections of organisms or environmental DNA (Deagle et al., 2019). Such a method yields a greater number of sequences and therefore a greater diversity of prey species without predefining the screening panel (Jeanniard-du-Dot et al., 2017; Thomas, Nelson, Lance, Deagle, & Trites, 2016), and in addition can provide an estimation of relative abundances in each sample (Albaina, Aguirre, Abad, Santos, & Estonba, 2016; Bucklin & Lindeque, 2016). The use of universal primers designed to amplify a short, highly variable region of DNA enables a large amount of information to be gleaned from degraded DNA, as would be present in faeces (McInnes et al., 2017). In recent years, the expense of HTS has decreased dramatically and metabarcoding is now seen as

FIGURE 1 (a) Scats were collected from haul-out sites on Skomer Island (represented by star), Wales; (b) Tissue samples from a dead weaned grey seal pup were collected from the Isle of May (represented by star), Scotland

a powerful and cost-effective tool for assessing diet composition (Berry et al., 2017; Bucklin & Lindeque, 2016).

To date, no studies have examined the direct relationship between diet composition and microplastic ingestion in wild marine mammals. This is important because prey type may be a crucial factor that determines the extent to which plastic is ingested, particularly for top predators for which trophic transfer is potentially the main route of entry (Nelms et al., 2018). Although both metabarcoding and microplastic extraction from faeces/gut content have been applied separately to a variety of marine and terrestrial taxa, including zooplankton, fish, turtles, birds and marine mammals (metabarcoding; Bucklin & Lindeque, 2016; Berry et al., 2017; McInnes et al., 2017, microplastics; Cole et al., 2014; Zhao, Zhu, & Li, 2016; Huerta Lwanga et al., 2017; Duncan et al., 2019; Nelms et al., 2019), they usually require different sample processing methods and have not been used concurrently. Here, for the first time, we combine existing DNA extraction techniques for determination of diet composition using molecular scatology methods, with specialist methods designed to isolate microplastics in the same protocol, providing a stream-lined methodology pipeline to assess diet and microplastic abundance simultaneously.

We performed a spiked trial to assess the recovery rate of purpose-made microplastics from seal scats when subjected to two DNA extraction treatments. Using the most appropriate treatment, we extended the full pipeline to 15 wild seal scats from Wales and used metabarcoding to identify the prey composition and relate it to microplastic content. We outline and discuss techniques for overcoming challenges that arise from performing these processes concurrently, such as DNA preservation during microplastic extraction and control of both biological and microplastic contamination. Our aims were to (a) develop a technique to combine diet analysis and microplastic quantification; (b) provide insights on the diet of a relatively understudied population of grey seals and (c) provide recommendations to improve future work linking diet and microplastic burden in marine top predators using scat samples, which may also be applicable to other species and ecosystems.

2 | MATERIALS AND METHODS

2.1 | Sample collection

Grey seal scats (n = 15) were collected from a number of haul-out sites (used by unknown individual females and pups) on Skomer Island, Wales (Figure 1a) in November 2013 (n = 9) and October 2014 (n = 6), and frozen at −20°C. Analysis was carried out at Plymouth Marine Laboratory, England.

2.2 | Spiked trial

Two scat sub-samples were spiked with purpose-made microplastics (see below for details) and subjected to different procedures, to develop the optimal protocol for extracting both DNA and microplastics, as outlined below.

2.2.1 | Sample processing

A scat was thawed and two ×2 g sub-samples were placed into separate sterile centrifuge tubes using a sterile metal spatula. Ten purpose-made microplastics of various types – to represent the diversity found in the marine environment and those which are likely to be encountered by seals and fish (two each of polypropylene, nylon fishing line, fishing rope, low-density polyethylene (LDPE) and expanded polystyrene) were added to each of the two tubes.

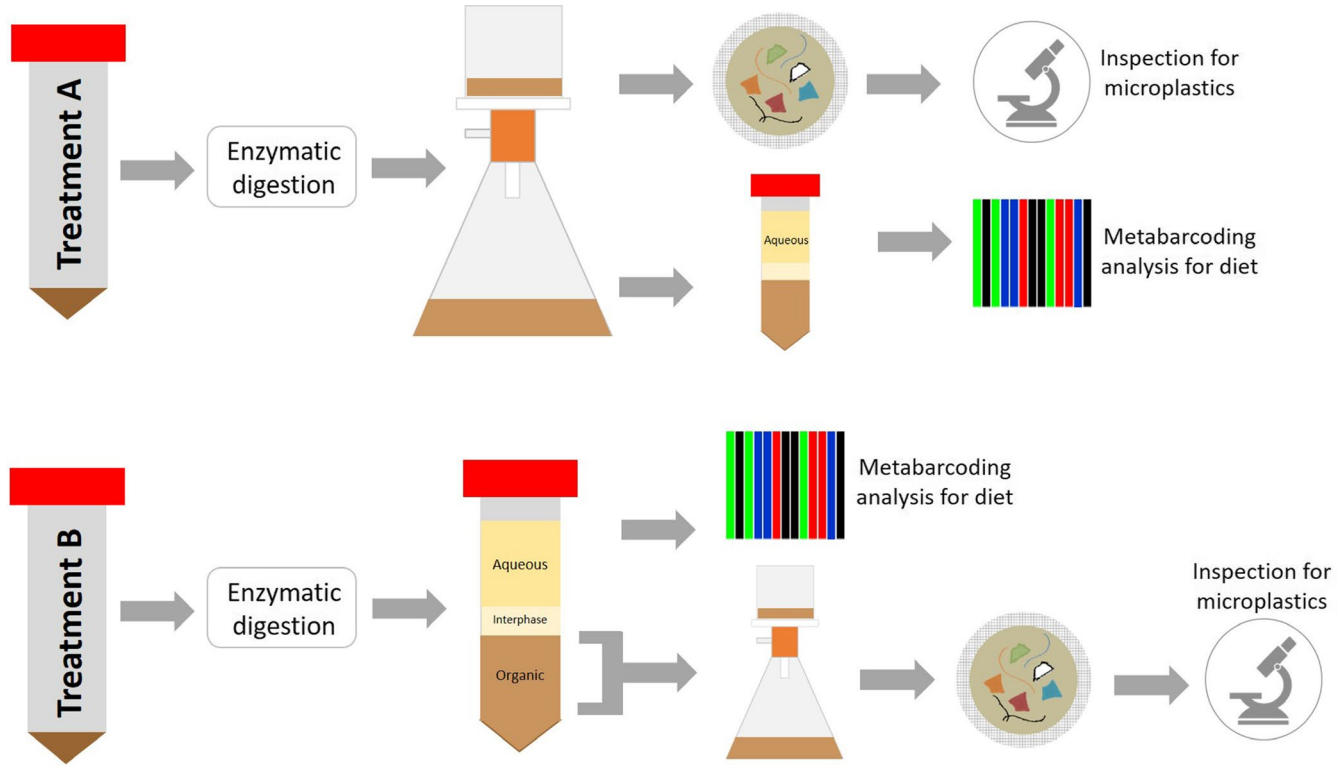

FIGURE 2 Schematic showing processes applied to Treatments A and B to extract DNA and isolate microplastics

2.2.2 | Enzymatic digestion

To each tube, 15 ml of homogenizing solution (400 mmol/L Tris-HCl pH 8, 60 mmol/L EDTA, 150 mmol/L NaCl, 1% SDS) and 500 µl of RNase (10 mg/ml) were added and the samples incubated at 37°C for 30 min. Molecular biology grade Proteinase K (14 µl at 250 µg/ml) was added and samples were incubated for a further 30 min at 37°C. Sodium perchlorate (4.28 ml of 5 mol/L $NaClO_4$) was added and the samples shaken at room temperature for 20 min and incubated at 65°C for a further 20 min.

2.2.3 | Combined DNA and microplastic extraction procedure comparison

Two different treatments were applied to the scat sub-samples, hereafter *Treatment A* and *Treatment B* (see Figure 2), each aimed at combining DNA and microplastic extraction into one procedure;

Treatment A

1. *Step 1 - Microplastic removal*: Following enzymatic digestion, the entire sample was filtered through a 35 µm mesh disc using a vacuum pump and collected in a sterilized (autoclaved) glass flask. The resulting solution was retained (at room temperature for a minimum amount of time to prevent DNA degradation) for subsequent DNA extraction. The mesh disc containing the scat residue and microplastics was stored in a Petri dish for later microscopic inspection.
2. *Step 2 - DNA extraction*: An equal volume of phenol/chloroform: isoamyl alcohol (24:1) was added to 15 ml of the scat solution obtained during filtering (Step 1), which was gently mixed by inversion and centrifuged for 5 min (G = 11,600). The aqueous phase was removed and an equal volume of chilled (–20°C) chloroform: isoamyl alcohol (24:1) added to the aqueous phase, which was further separated by centrifugation for another 5 min (*g* = 11,600). The DNA solution (aqueous phase) was removed and precipitated once with 2.5 volumes 100% ethanol overnight (–20°C) and washed with 70% ethanol, pelleted using centrifugation, air dried for *c.* 3 hr, then resuspended in 1 ml TE (10 mmol/L Tris, pH 8.0 and 1 mmol/L EDTA) buffer overnight.

Treatment B

1. *DNA extraction and microplastic removal:* Following enzymatic digestion, DNA was extracted using the methods outlined by Step 2 above. However, following separation by phenol/chloroform:isoamyl alcohol (24:1), the aqueous phase was retained for DNA extraction and only the interphase and organic phase were filtered through a 35 µm mesh using vacuum pump as in Step 1 for microplastic removal above.

2.2.4 | Molecular analysis for diet

Metabarcoding of DNA in the seal scats, to assess seal diet, was performed by amplification of a region of the 18S nuclear small subunit

(nSSU) ribosomal RNA (rRNA) gene and subsequent High Throughput Sequencing (HTS). This method was used because there is at least one variable position in the 18S V9 region, such that metabarcoding of this region can discriminate between species in a reliable way, providing a reference sequence is available in the sequence database (Albaina et al., 2016). First, the quality and quantity of extracted DNA were assessed using a Nanadrop 1000 Spectrophotometer (ThermoScientific, Delaware, USA). Universal primers (Euk_1391f, EukBr; Amaral-Zettler, McCliment, Ducklow, & Huse, 2009) were chosen to target the V9 hypervariable region of the 18S rRNA gene. PCR amplification was performed in triplicate, to reduce PCR bias and increase the likelihood of amplifying rare DNA, 25 μl reactions containing 2.5 μl of each primer (10 μmol/L), 2.5 μl dNTPs (2 mmol/L), 2.5 units of TaqDNA polymerase (5 units/μl; Qiagen), 2.5 μl $MgCl_2$ (25 mmol/L), 2.5 μl 10× buffer, 11 μl molecular grade water and 1 μl DNA extract (range = 0.9–42.7 ng/μl). Reactions were amplified through denaturation at 95°C for 2 min then 27 cycles of (30 s at 95°C, 45 s at 57°C and 45 s at 72°C) followed by a final extension step of 7 min at 72°C and then stored at 4°C. The PCR products were checked by gel electrophoresis before being pooled and cleaned up using QIAquick PCR purification kit (Qiagen). Illumina HiSeq high-throughput sequencing was performed by MR DNA (Molecular Research).

2.2.5 | Microplastic quantification

The dried mesh discs were examined and microplastic particles counted to determine the recovery rate of microplastics used to spike the samples.

2.3 | Optimized protocol

2.3.1 | DNA and microplastic extraction

Treatment A was used as the pipeline to obtain both diet information and microplastic burden for the 15 wild seal scats.

2.3.2 | DNA sequencing

Sequencing of the amplified 18S rRNA gene fragments from seal scat was performed at MR DNA (www.mrdnalab.com, Shallowater, TX, USA) on a MiSeq following the manufacturer's guidelines (MiSeq, Illumina). Sequence data were processed using the MR DNA analysis pipeline (MR DNA, Shallowater, TX, USA).

In summary, paired end sequences were joined and depleted of barcodes, chimeras and sequences with ambiguous base calls were removed before Operational Taxonomic Units (OTUs) were generated. OTUs were defined by clustering at 3% divergence (97% similarity) and any OTUs containing a single sequence were removed. The OTUs were assigned taxonomy using UCLUST (Edgar, 2010), a de novo picker within QIIME™ (Quantitative Insights Into Microbial Ecology). A representative set of sequences was then generated and these sequences were assigned taxonomy (at the level of 95% homology) using the BLASTn search of the NCBI non-redundant dataset. Only OTUs with >95% homology were retained for further analysis and OTUs assigned as predator DNA (as detailed above), fungi and bacteria were removed.

2.3.3 | Microplastic identification and characterization

Following the filtering step, the mesh discs were visually inspected for microplastics using a microscope (Olympus SZX16) and the particles were counted, photographed (microscope mounted Canon EOS 550D DSLR camera), measured and characterized by type, colour and size. Each potential microplastic was subjected to further analysis to confirm polymer type using attenuated total reflection-Fourier transform infra-red spectroscopy (ATR-FTIR; PerkinElmer Spotlight 400 FT-IR Imaging System). Each potential microplastic was scanned at a resolution of 8 per cm (wavelength range = 4000–650 per cm) and pixel size of 6.25 μm using SpectrumIMAGE™ software. Spectra were compared to a number of polymer libraries using Spectrum™ (PerkinElmer). Only those considered to have reliable spectra matches (after visual inspection) and a search score confidence of 0.70 or greater (Lusher, McHugh, & Thompson, 2013) were accepted when interpreting output.

2.3.4 | Contamination control

Strict contamination control measures are essential for studies aimed at assessing microplastic abundance. Though the aims of this study were to develop a methodology rather than produce abundance estimates, best practice contamination control measure were implemented during the handling of samples within the laboratory. Briefly these were; cotton laboratory coats worn at all times, surfaces and equipment thoroughly cleaned with 70% ethanol and/or rinsed with Milli-Q water. The sub-sample of scat for analysis was taken from the centre to avoid any possible contamination of the external surfaces. Microplastics detected in the samples were compared by characteristics (polymer, colour, type) with any plastic equipment used during sample collection, preparation and processing, such as nitrile gloves, polyethylene sample collection bags and Nylon mesh discs. For more details, see (Nelms et al., 2018). For the molecular aspect of this study all equipment was autoclaved following the Milli-Q water rinse to prevent any false positive amplification of DNA.

2.3.5 | Statistical analysis

The relationship between each of the top three most prevalent prey families (by proportion of sequences) and microplastic abundance was investigated using separate Generalized Linear Models (GLMs). Analyses were undertaken in the statistical computing software, R (GLM; R Core Team, 2018). The distribution of the data was checked for normality using a Q–Q plot and deemed not normal (zero-bounded, asymmetrical). Model selection was performed based on AIC scores for models with poisson and negative binomial error families and various link function combinations (identity, log and sqrt). Statistical significance was set at a probability level (α) of 0.05.

2.4 | Grey seal DNA

Using HTS methods, prior knowledge of diet composition is not required (as is the case when primers are selected for specific clades) because universal 18S primers allow for the detection of any eukaryote present within scat. It is essential, however, to have a robust reference sequence for the predator species to enable exclusion of these sequences in subsequent analysis. Grey seal 18S was not publicly available for comparison, so we generated the sequence information as follows;

2.4.1 | Sample collection

Tissue samples (liver, kidney and muscle) were taken from a freshly dead, weaned grey seal pup that had died of natural causes, on the Isle of May, Scotland (Figure 1b) in December 2017.

2.4.2 | DNA extraction (adapted from Berntson et al., 1999)

Small sub-samples (5 mm) of tissue were removed and 300 µl of cetyl trimethyl ammonium bromide (CTAB) buffer [2 ml Cetyl trimethyl ammonium bromide 10% in dH_20, 2.8 ml 5 mol/L NaCl, 0.4 ml 0.5 mol/L EDTA (pH 8), 1 ml 1 mol/L Tris-Cl (pH 8.0), 0.02 ml B-mercaptoethanol, 3.78 ml H_2O] was added. The samples were homogenized using a pestle and mortar and a further 300 µl CTAB buffer was added. Molecular biology grade sProteinase K (1 µl at 20 mg/ml) was added and the samples were further homogenized followed by incubation at 55°C with periodic agitation for 24 hr. An equal volume of cold (–20°C; 24:1) chloroform: isoamyl alcohol was added, followed by centrifugation at 7,700*g* for 10 min. Two volumes of cold (–20°C) 95% ethanol were added to the aqueous phase and DNA was precipitated for 1 hr at –80°C. The samples were centrifuged at 10,000*g* for 30 min before being washed with cold (–20°C) 70% ethanol and centrifuged again at 7,000*g* for 15 min. The ethanol was then poured off and air-dried for 45 min, after which the pellets were resuspended in 50 µl TE and stored at 4°C overnight. The quality and quantity of extracted DNA were assessed by visualization using gel electrophoresis (1% agarose) and with a Nanadrop 1,000 Spectrophotometer (ThermoScientific, Delaware, USA).

2.4.3 | Sequencing and data processing

PCR amplification was performed for each tissue type (liver, kidney and muscle; concentration of DNA range = 2,823.7–5,028.9 ng/µl) using the methods and universal primers as described above for seal scat. Following visualization of the amplification products using gel electrophoresis (2% agarose gel), DNA extracted from muscle was deemed the most appropriate and reliable for sequencing. Six replicates of the 18S V9 PCR products from grey seal muscle DNA (concentration of DNA range = 0.01–0.36 ng/µl) were sequenced in both directions by LGC Genomics, Berlin (Germany). Sequence data from the six replicates were aligned and a consensus sequence generated using MEGA 7 (https://www.megasoftware.net/). The resulting GenBank accession number for grey seal 18S V9 nucleotide sequence is BankIt2148050 seq MH845620.

3 | RESULTS

3.1 | Spiked trial

3.1.1 | Observations and microplastic recovery rate

During the spiked trial, phenol dissolved the purpose-made microplastics and affected the equipment used for filtering, as such Treatment B was not continued. Conversely, Treatment A resulted in a 100% recovery rate of microplastics used to spike the scat and was employed for full analysis of 15 scats.

3.2 | Optimized protocol

3.2.1 | Microplastics

Microplastics (a total of 17) were found in eight of the 15 subsampled scats (53%), ranging between 1–5 microplastics per scat, as confirmed by FT-IR. Fibres were most commonly detected (76.5%; *n* = 13) while fragments made up 23.5% (*n* = 4). The former ranged from 5.5 mm to 300 µm in length while the latter ranged from 400 µm to 150 µm along the longest edge. The majority were blue (52.9%) followed by red (17.6%), black (11.8%), clear, orange and purple (Figure 3). The most common polymer type was Nylon (47.1%; *n* = 8) followed by low-density polyethylene (LDPE), polyethylene terephthalate (PET) and polyethylene (all 17.6%; *n* = 3).

3.2.2 | Seal diet

In total, 1,449,416 sequences were returned and 9,683 OTUs were formed from the 15 scats. Following the removal of singletons 1,436,089 sequences and 6,993 OTUs remained, of which 353 OTUs were unknowns (<95% homologous) leaving 6,640 OTUs and 1,432,569 sequences of >95% homology (Table 1). Of these 386,968 (27%) sequences were assigned as predator (seal) DNA.

Biological rationale was employed to determine which taxa were subjected to further analysis, based on their likelihood to contain seal prey species, in a stepwise process of taxonomic elimination (Figure 4). For example, within the Kingdoms listed above, prey are most likely to belong to Metazoans within Eukaryota. Chordata was the most common phylum in this taxon at 71% of sequences, followed by Nematoda (23%) and Cnidaria (5%; Figure 4a). The high proportion of nematodes is likely due to the presence of parasitic worms in the seals' digestive tract, and perhaps other nematode species in the substrata from which the seal scat was collected. Seals are not known to eat Cnidaria and it is likely that their presence reflects the diet of the fish species consumed by the seals. Of the Chordata, mammalian DNA (predator; subsequently removed) was most prevalent (58%) followed by actinopteri (ray-finned-fish; 42% of Chordata and 19% of all sequences returned; Figure 4b). The three most common families

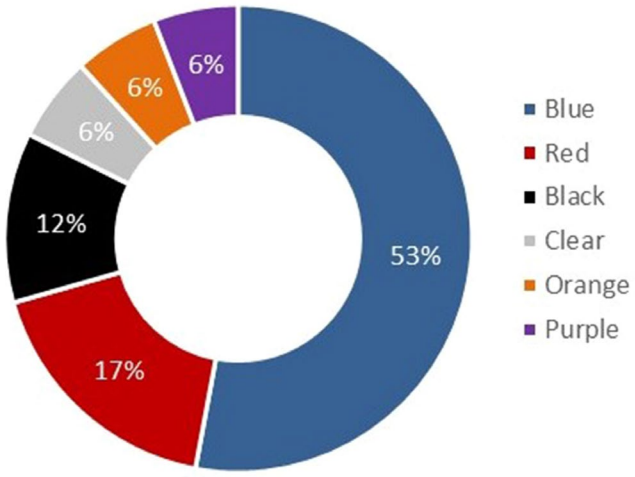

FIGURE 3 Doughnut plot showing proportions of microplastic colours detected in seal scats (blue = 53%, red = 17%, black = 12%, clear, orange and purple = 12%)

of ray-finned fish were gadidae (specifically Atlantic cod; 47%), pleuronectidae (righteye flounders; 45%) and paralichthyidae (large-tooth flounders; 5%; Figure 4c). Further details of the prey DNA analysis outputs can be found in Supplemental Information.

3.2.3 | Relationship between prey type and microplastics abundance

Individual GLMs were run for each prey family and the most appropriate model selected based on AIC scores and *p*-values. A significant positive correlation was found between the proportion of Gadidae and number of microplastics ($F_{1,13} = 2.063$, $p = .05$, Figure 5a), whereas a statistically negative (biologically not positive) correlation was observed for the two flounder families (Pleuronectidae $F_{1,13} = 0.177$, $p > .05$; Paralichthyidae $F_{1,13} = 10.95$, $p < .05$; Figure 5b,c).

4 | DISCUSSION

Marine top predators, such as marine mammals, ingest microplastics (Lusher et al., 2015; Nelms et al., 2019) but the pathways by which this occurs are less well understood. Aside from direct consumption of microplastics from the marine environment, trophic transfer is thought to represent a major route of ingestion for mid- and high-trophic level taxa (Hammer, Nager, Johnson, Furness, & Provencher, 2016; Nelms et al., 2018). Here, we present a novel and effective methodology pipeline that facilitates the simultaneous investigation of a more detailed aspect of trophic transfer – the relationship between specific prey types and the abundance of microplastics detected in scats from wild seals – using small sample volumes. To do so, we used DNA metabarcoding, a powerful molecular technique designed to identify taxonomic groups in complex samples (Bucklin & Lindeque, 2016), combined simultaneously with a microplastic extraction process. We believe that the methods described here could not only advance the development of our understanding of microplastic exposure experienced by these marine top predators, but could also help to elucidate the microplastic contamination status of the wider marine ecosystem by proxy. In addition, as microplastics have been detected in air, soil and freshwater environments (Dris, Gasperi, Saad, Mirande, & Tassin, 2016; Huerta Lwanga et al., 2017; Rillig, Ziersch, & Hempel, 2017; Windsor, Tilley, Tyler, & Ormerod, 2019), our method could be applied to a wide variety of taxa to investigate this issue across countless ecosystems.

TABLE 1 Overall number of Operational Taxonomic Units (OTUs) and sequences per Kingdom (eukaryote, fungi, bacteria and viridiplantae) detected in seal scats, and their percentage of the overall composition

Kingdom	No. OTUs	Total no. sequences	% composition
Eukaryota	4,881	934,586	65.238
Fungi	1,731	495,391	34.581
Bacteria	26	2,579	0.180
Viridiplantae	2	13	0.001
Total	6,640	1,432,569	100.000

The spiked trial demonstrated that the protocol used for Treatment A produced 100% recovery of purpose-made microplastics, and the extraction of sufficient DNA quantity and quality for metabarcoding analysis. Using this optimal protocol, it was possible to examine the feasibility of assessing prey composition in detail, and detecting microplastics in the scats, concurrently. This stream-lined methodology pipeline removed the necessity of performing both the DNA and microplastic extraction steps separately, which maximized time and resource efficiency and reduced the associated costs and sample required. These outcomes validate the pipeline and demonstrate its efficacy for extracting microplastics and high quality DNA from small volumes of fecal samples, further illustrating its applicability to species other than large marine vertebrates.

Our approach of using the 18S V9 region for metabarcoding diet assessment proved appropriate in the context of seal scats because the amplicon's relatively small size enabled the analysis of degraded DNA present in faeces, which can be difficult to amplify successfully (McInnes et al., 2017). Additionally, whereas some dietary metabarcoding studies use blocking primers to inhibit the amplification of predator DNA (McInnes et al., 2017; Peters et al., 2015), our methods negate this need, which is beneficial because blocking primers may also prevent amplification of some prey species (McInnes et al., 2017), particularly if the predators and prey are closely related, or if the predator is known to consume conspecifics (Bishop, Onoufriou, Moss, Pomeroy, & Twiss, 2016). The use of universal primers to amplify DNA in the gut contents along with predator-specific blocking primers can also introduce biases into the PCR by also blocking amplification of DNA from closely related species and therefore the analysis of predator diets (Piñol, San Andrés, Clare, Mir, & Symondson, 2014). Compared with the traditional approach of using hard-part analysis to examine prey composition, metabarcoding has the ability to detect greater species diversity as well as cartilaginous prey which leave no

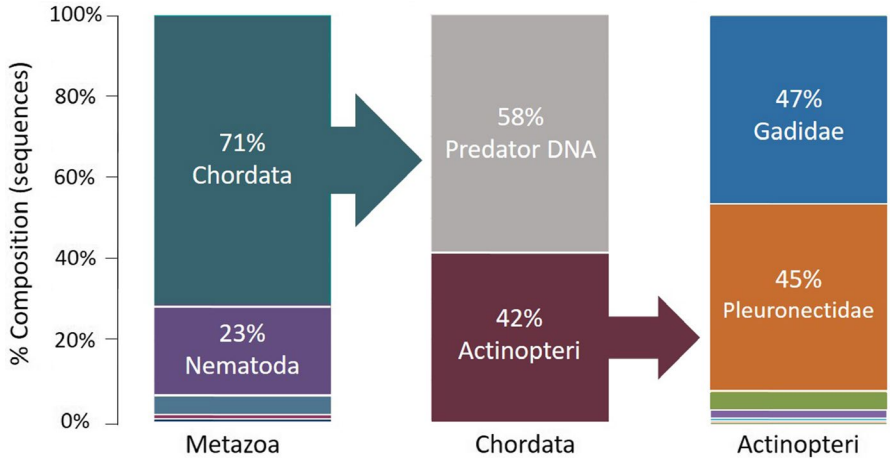

FIGURE 4 Stepwise process to identify prey (a) Percentage sequences by Phyla detected in Metazoa (Chordata (teal; 71%), Nematoda (purple; 23%) and Cnidaria (blue; 5%); (b) Percentage sequences by Class detected in Chordata, the most abundant Phyla (predator DNA (grey; 58%) and Actinopteri (burgundy; 42%); (c) Percentage sequences by Family (Gadidae (blue; 47%), Pleuronectidae (orange; 45%) and Paralichthyidae (green; 5%) detected in the Actinopteri, the most abundant when predator DNA was eliminated

obvious remains and are unlikely to be detected by eye (Deagle et al., 2009). In addition, a lesser sample volume is required which enables this technique to be used on smaller organisms (Bucklin & Lindeque, 2016). Deriving relative abundance data in diets from metabarcoding can, however, encounter issues such as, primer biases, quality of DNA, differential degradation of material during digestion and heterogeneity in the prey composition of scats (Deagle et al., 2005; Matejusová et al., 2008), so any outputs should be interpreted with these in mind.

Microplastics were detected in over half of the scat sub-samples analysed. There are few other studies on seal scats to compare our results to, but Nelms et al., (2019) found microplastics in the digestive tracts of all wild cetaceans (eight species; 43 individuals) and pinnipeds (2 species; 7 individuals) from British waters examined and 1–4 microplastics were detected in 48% of scats from captive grey seals fed on wild-caught Atlantic mackerel (*Scomber scombrus*; Nelms et al., 2018). Considering other species, Bråte, Eidsvoll, Steindal, and Thomas (2016) found that 3% of Atlantic cod stomachs from the Norwegian coast contained synthetic polymers and Rummel et al., (2016) detected plastic in approximately 1.2% (n = 2 of 162) of cod and 5.5% (n = 4 of 72) of flounder examined. The finding of greater numbers of microplastics in flounder is contradictory to our results here, in which higher proportions of cod were associated with greater microplastic abundances when compared to the two flounder families. These observations can be explained by a number of factors. Firstly, as this was a proof of concept study rather than a full environmental assessment, we used a small sample size to develop and test our methodology pipeline. Consequently, any potential relationships detected between prey composition and prey type are likely to be indications only and further work is required to investigate this fully (see methodological recommendations below). Secondly, the methods of examining the presence of microplastics used in the studies above differed from those employed here (i.e. fish digestive tracts vs. fish remains from scats) and therefore are likely to yield differing results. Thirdly, spatial variation in microplastic abundance and the overlap with local fish distributions – which also exhibit temporal (e.g. seasonal) and spatial (e.g. regional and depth) variation – may produce diverse patterns and trends. For example, the seals in this study predate fish in the Celtic Sea but the fish examined by Rummel et al., (2016) fed in the North and Baltic Seas where the abundance of microplastics, in both the marine environment and the species that inhabit it, might differ. Though little is known about the diet of grey seals in the Celtic Sea, where Skomer Island is located, a review by Brown et al. (2012) revealed that flatfish (e.g. flounders) contribute more to the diet of seals (grey and common; *Phoca vitulina*) in the neighbouring Irish Sea than in all other UK sea areas (Atlantic, North Sea Islands, Moray Firth, southern North Sea) investigated. Similarly Gadoids were a prominent food source in this area (Brown et al., 2012). These findings from hard part analysis correspond to and corroborate the dietary composition reported here obtained through metabarcoding analysis.

Our results are preliminary and not designed to serve as an assessment of microplastic abundance in wild seal diet but as an example of how our protocol could be used to do so accurately, and in a resource and time efficient way, on a larger scale across a wide variety of taxa. We therefore make a number of methodological recommendations to assist in the robust collection and analysis of samples;

1. Wherever possible, microplastic contamination should be minimized. Scats should be collected using non-plastic equipment (or scrapings of plastic equipment should be taken for comparison as a control) and a sample from the surrounding substrate should be collected to eliminate any obvious environmental sources of plastic. During sample processing, a subsample from the centre of the scat should be used to avoid any possible contamination of the external surfaces. Further information on contamination control can be found in Nelms et al., (2018).
2. To obtain the best DNA results, and therefore the most accurate representation of prey species present, the collection of fresh scats is optimal (Jeanniard-du-Dot et al., 2017). Additionally, samples should be stored at −20°C as soon after collection as possible to prevent DNA degradation (Albaina et al., 2016; Berry et al., 2017; McInnes et al., 2017).
3. To achieve ecologically representative results, we recommend that a systematic and extensive sampling approach be adopted. For example, regular sample collection across informative temporal and spatial scales will allow for any seasonal and geographical variations

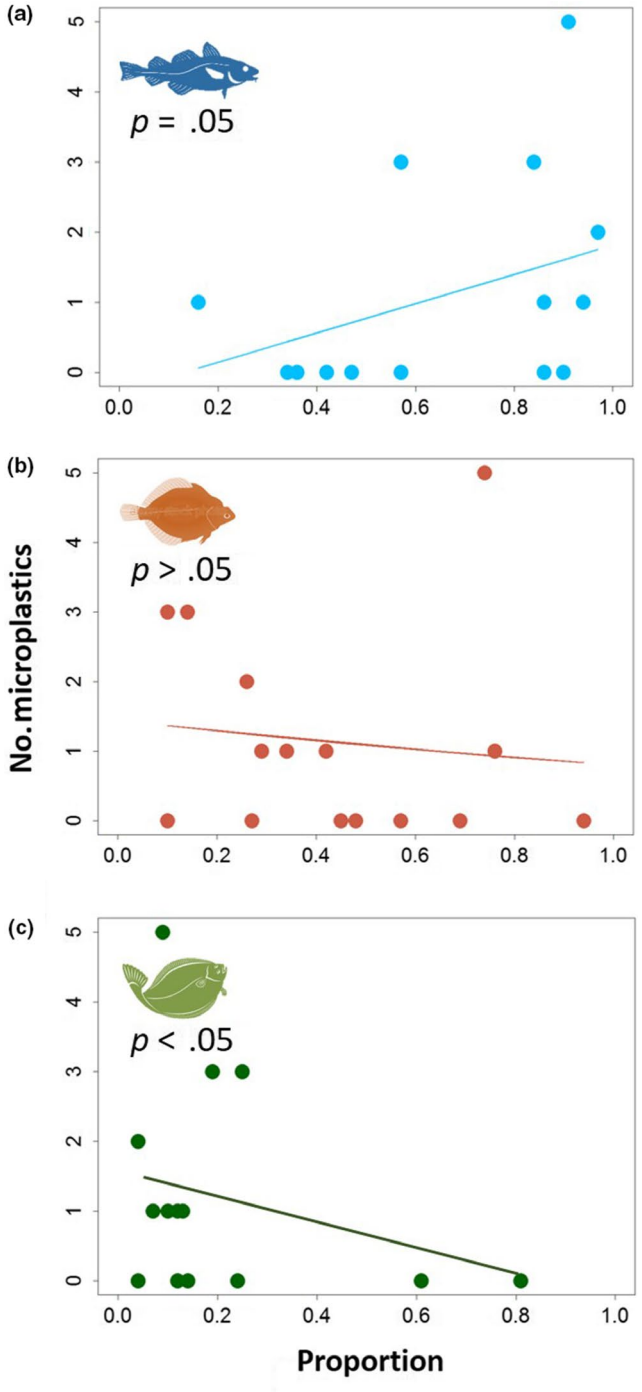

FIGURE 5 Scatterplots showing the correlation (as investigated using generalized linear models; GLMs) between the number of microplastics and the proportion of the top three most prevalent prey families (by proportion of sequences) (a) Gadidae (b) Pleuronectidae (c) Paralichthyidae

to be observed. The sample size should also be significantly expanded beyond the 15 analysed for exploratory purposes here.

4. To ground-truth any relationship between microplastic abundance and prey type detected from the scats it would be useful to examine the prey items directly, i.e. sample the gut content of fish species that are known to be consumed by the seals from same area that the scats are collected from. There would also be merit in examining water-borne microplastics and analysing for similarities in fish and scats. This would also reveal whether patterns relating to abundance and type of microplastics as detected in certain fish species, is related to those levels observed within their habitats.

By using non-invasive techniques to assess diet and the presence of microplastics, it is possible to glean insightful information from wild and representative animals, without the need to sample stranded individuals which may not have been feeding normally prior to death, as is often the case in microplastics studies focusing on marine megafauna. Though the methods described here were developed on seal scats, they are applicable to other predatory aquatic taxa where the question of microplastic ingestion may be linked to prey consumption, for which fresh faeces is accessible (such as birds and polar bears, or freshwater vertebrates, e.g. otters); or when gut content can be extracted from the digestive tract of dead animals, such as cetaceans, elasmobranchs, marine turtles, birds and large predatory fish, for example, tuna. Given that microplastics have been detected in air, soil and freshwater environments (Dris et al., 2016; Huerta Lwanga et al., 2017; Rillig et al., 2017; Windsor et al., 2019), the method developed here could be applied to a wide variety of taxa to investigate the relationship between microplastic ingestion and prey composition in most food web scenarios.

In conclusion, this novel study is the first to combine diet analysis using non-invasive, scat-based molecular techniques and the quantification of ingested microplastics for the purpose of investigating dietary exposure to microplastics in a marine top predator.

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AUTHORS' CONTRIBUTIONS

P.K.L., B.J.G., T.S.G. and S.E.N. conceived the project aim; P.K.L., H.E.P. and S.E.N. designed the methodology; K.A.B. obtained the samples; S.E.N. and H.E.P. carried out the sample processing work; D.S. provided expertise and access to essential equipment. S.E.N. carried out the analysis and led the writing of the manuscript. All

authors contributed critically to the drafts and gave final approval for publication.

DATA AVAILABILITY STATEMENT

Data available from GenBank (accession number for grey seal 18S V9 nucleotide sequence - BankIt2148050 seq MH845620, Nelms et al., 2018) https://www.ncbi.nlm.nih.gov/nuccore/MH845620.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

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