CleanAtlantic

Tackling Marine Litter in the Atlantic Area

Towards a protocol for the observation of microplastics in Biota

WP 5.3: Indicator for ingestion of microplastics

Cefas

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DISCLAIMER

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I. Introduction

Microplastics are widespread in the environment at a global scale with largely unknown related ecological and ecotoxicological effects (GESAMP, 2015). Currently, there is no widely accepted single protocol for the monitoring of microplastics in biota (or sediment or water) which makes comparison between data sets difficult. This could lead to errors in data interpretation, flawed conclusions and potentially misinformed regulatory actions. There is also a discrepancy between the type, size and number of microplastics being monitored in biota and laboratory-based dose-response ecotoxicological experiments on which risk assessment and regulatory concentrations are being based upon. As an example, laboratory based ecotoxicological experiments often rely on published monitoring data to relate to "environmental relevant concentrations" but due to the large variations between methods, generated concentrations might not be representative of "relevant environmental concentrations" (Maes et al., 2020). There is thus an urgent need to harmonise protocols for the detection of microplastics in environmental samples and to understand the extent of microplastic contamination in the Atlantic area as a first step for the development of long-term, integrated monitoring programmes.

This report discusses a number of publications which document the ingestion of microplastics by biota (Enders *et al.*, 2017; Hermsen *et al.*, 2017; A. Lusher *et al.*, 2017; Hermsen *et al.*, 2018; Bråte *et al.*, 2018; Bessa et al., 2019; GESAMP, 2019) comparing the methodology for detection and quantification of microplastics in biota and a suitable and sustainable indicator species for the monitoring of microplastics in biota in the Atlantic area.

I.1. MSFD Context

Marine litter affects marine life at several organizational levels and their impacts vary depending on species or populations, activities-sources, environmental conditions and the region or country considered. Descriptor 10 is defined as "properties and quantities of marine litter do not cause harm to the coastal and marine environment".

I.2. D10C3 Criteria – Ingestion Indicator

D10C3 corresponds to the criterion "The amount of litter and micro-litter ingested by marine animals is at a level that does not adversely affect the health of the species concerned. Member States shall establish threshold values for these levels through regional or sub regional cooperation''.

II. Method

This section summarises a proposed protocol for the detection and quantification of microplastics in biota using fluorescence tagging of polymers with Nile red (Maes *et al.*, 2017) coupled with an automated particle counting software developed at Cefas. This method was tested and improved on during the Clean Atlantic project. Data were generated for a bivalve (mussels), a pelagic fish (mackerel) and a demersal fish (dab).

Chemicals

The chemicals used in this study are listed in Table 1.

Table 1. List of chemicals, manufacturers and suppliers

Chemicals	Molecular formula	Manufacturer/Supplier	Purity
Potassium hydroxide	KOH	VWR/VWR	-
Sodium hypochlorite	NaClO	VWR/VWR	14% active chlorine
Nile red	C20H18N2O2	Acros organics/Thermo Fisher scientific	99% purity
Ethanol	C2H6O	Acros organics/Thermo Fisher scientific	95% purity

Chemical digestion

Two alkaline digestion methods were investigated in this study. A 10% KOH digestion solution (A. L. Lusher *et al.*, 2017) was applied and compared to a 30% KOH:NaClO solution (Strand and Tairova, 2016; Enders *et al.*, 2017). The beakers, containing the biota samples and the digestion solution, were placed in an incubator at 40°C for 3 days under constant agitation at 120 rpm after a 5 min sonication step. Full digestion was observed for all the materials selected with little residues for the mussels and the Mackerel stomachs. For the Dab samples, the intestines still contained undigested materials mainly composed of sediment grains. As a result, each digest had a substantial number of undigested residues left after the digestion process. Samples were then filtered on a GF/D grade Whatman glass microfiber filter with a 2.7 µm porosity. An extra step, using pre-filtered industrial strength degreaser using a 0.2 µm regenerated cellulose filter, was also used with samples with fat residues. Action of the degreaser was found to be optimum when added during the last hour of the incubation process (40°C under constant agitation).

Method blanks

Several studies have reported the occurrence of microplastics in biota, especially mussels and fish (Foekema *et al.*, 2013; Rummel *et al.*, 2016; A. Lusher *et al.*, 2017; Beer *et al.*, 2018; Bråte *et al.*, 2018). A recent study from Hermsen et al. (2017) highlighted the need for strict quality control processes for the extraction and quantification of microplastics due to their low occurrence in biota. Using strict quality control criteria, they investigated the occurrence of microplastics in 400 individual fish of four North Sea species: Atlantic Herring, Sprat, Common Dab, and Whiting on ingestion of >20 μm microplastic. Two plastic particles were found in only 1 (a Sprat) out of 400 individuals suggesting a lower incidence of microplastics in fish species as previously suggested in the literature. For this method, an open beaker was left open to the air during dissection to compensate for atmospheric contamination. The beaker was then filled with the alkaline digestion solution (either 10% KOH or 30% KOH:NaOH) and filtered onto a filter as specified in the following section and in Figure 1. The number of particles that exhibited fluorescence were deducted from the final number of items detected in biota.

Isolation and detection of plastics by fluorescence tagging of polymers using Nile red coupled with an image recognition software.

In this section, a fast and cost-effective screening approach, based on the fluorescence tagging of polymers using Nile Red, for the detection of microplastics in biota is presented (Maes *et al.*, 2017). A schematic diagram of the developed protocol is presented in Figure 1. The main analytical steps can be described as biota selection and sample preparation, alkaline digestion, clean-up process, filtration, staining and visualisation, quantification and validation (Figure 1).

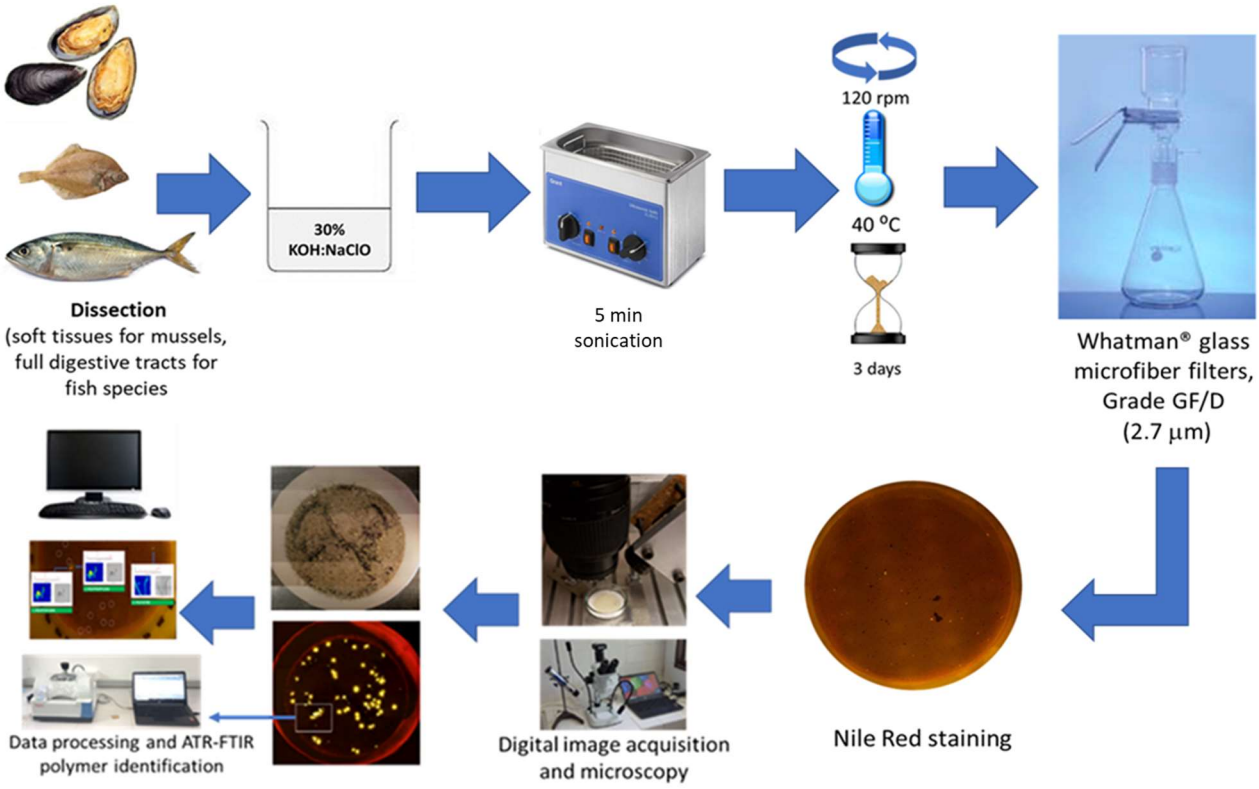


Figure 1. Proposed protocol for the extraction and isolation of microplastics from biota samples

Biota selection and sample preparation

Biota under investigation included mussels (a filter feeder), mackerel (large pelagic fish) and Dab (demersal fish). All the biota samples were provided by Cefas as part of already existing monitoring surveys with the exception for mussels which were sourced directly from a local supermarket. Particle retention time for fish can be relatively low with a gut retention time varying between 4 to 158 hours for fish. Similarly, microplastic gut retention time by mussels has been reported to be at least 72 hours (Ward and Kach, 2009; Woods *et al.*, 2018). As a result, the efficiency of the screening method was investigated for whole tissues (mussels), stomachs (mackerel) and for the gastrointestinal tract (Dab) (Table 2). A Standard Operating Procedure (SOP) for the collection of tissues from biota samples is presented in **Appendix 1.** Figure 2 shows the removal of the digestive tract from a Dab. Collected samples were transferred to clean 100 mL beakers and the weight was recorded.

Figure 2. Removal of gastrointestinal tract from Dab

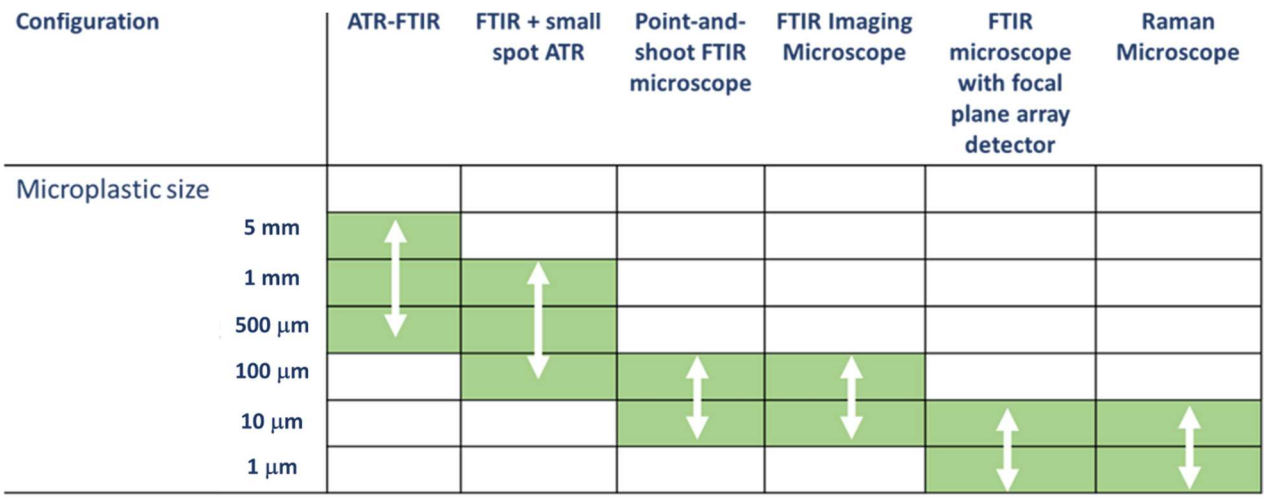
Table 2 Characteristics of the biota under investigation

Biota		Characteristics	Full tissues	Stomach	Gastrointestinal tract
Mussels	*Mytilus edulis*	Sessile filter feeders	X		
Mackerel	*Scomber scombrus*	Large pelagic fish		X	
Dab	*Limanda limanda*	Demersal fish			X

Quality control and polymer identification using Fourier-transform infrared spectroscopy (FT-IR)

Reducing ambient laboratory contamination is of concern for the investigation of microplastics due to their relative low occurrence in biota. Quality criteria for the analysis of microplastic in biota samples were compiled by Hermsen et al. and highlighted the need for strict contamination control procedures to be implemented. Such quality criteria were integrated in the proposed protocol for microplastic analysis in biota and listed in Table 7.

Particle characterisation and identification of the polymer type is necessary to validate the method and ensure the removal of false positives during analysis. A series of microscopic and spectroscopic methods (amongst other methods such as pyrolysis GC-MS) are currently being used for particle characterisation with contrasting applications, levels of technicality and costs (Figure 3). Most widely applied techniques are Attenuated total reflectance Fourier transform spectroscopy (ATR-FTIR) and micro-FTIR for smaller particles. Requirements can vary according to analytical needs, facilities and particle size being investigated (Figure 3).



Configuration	ATR-FTIR	FTIR + small spot ATR	Point-and-shoot FTIR microscope	FTIR Imaging Microscope	FTIR microscope with focal plane array detector	Raman Microscope
Level of technicality	Low	Low	Medium	Medium	High	High
Manual sample placement only	Yes	Yes	Yes	No	No	No
Automated analysis of filters	No	No	No	Yes	Yes	Yes
Compatible with fluorescence with Nile red	Yes	Yes	Yes	Yes	Yes	Yes
Relative cost	$	$$	$$$	$$$$	$$$$	$$$$

Figure 3. Currently applied spectroscopic techniques for the detection and characterisation of microplastics in environmental samples. Adapted from "Analytical instruments for microplastic analysis" (ThermoFisher Scientific, 2019).

Quantification and quality control

The microplastics processing tool ("Microplastics tool") is a semi-automated method to detect fluorescent microplastics and other small objects from microscope images. The tool utilises a two-step process called object-based image analysis (OBIA). The first step of OBIA is to segment an image into 'objects' which are of a similar size and shape to real world objects using a segmentation algorithm. Segmentation of the microplastic images was carried out within ArcMap using the Mean-shift segmentation tool which is a bottom-up segmentation algorithm, built using ArcMap Model builder (ArcGIS Desktop 10.5, version 10.5.0.6491). This operates by grouping adjacent pixels together that have similar spectral characteristics, thereby creating small objects. The process then runs iteratively, grouping larger and larger groups of pixels together until a threshold, either user defined or computed, is reached. The level of spatial and spectral smoothing can be adjusted by the user depending on the level of detail required from the segmentation. The second step is to then classify those objects into plastics and non-plastics based on their characteristics. Some of the advantages of OBIA over pixel-based classification include the use of shape and context in the classification of objects. Following segmentation, the image objects are used to extract basic statistics from the microscope images within ArcMap, including the mean colour and some geometric values. The statistics can then be used within a decision tree to identify microplastics from the other material in the image. Classified objects were exported as shapefiles and as a table for reference. The table includes the object statistics described in Tables A2.1 and A2.2 (**Appendix 2**) as well as the ratio of length: area and the fluorescence index [(R+G)/R]. The tool can be run on a single image or on a series of images giving the results of a whole plate as a single figure. Details on the data analysis ca be found in **Appendix 2**. Automated counting is also compared to manual counting as a subset for quality control and validation.

Uncertainties and evaluation of method

One of the main limitations of the fluorescence tagging of polymers using Nile red for environmental samples is the staining of chemically resistant natural organic materials (e.g. chitin) (Maes *et al.*, 2017). Uncertainties due to false positives was addressed by using an extra validation step using FTIR spectroscopy as well as data manipulation from the automated image recognition software (i.e. screening of the background fluorescence).

III. Results

Chemical digestion

The chemical digestion of organic matter and biota for microplastics was optimised to remove biological residues while avoiding degradation of plastic polymers in sediment. Visual inspection of the filters confirmed the presence of low density, chemical resistant materials. Initial protocol used a 10% KOH digestion at 40°C for 3 days. 10% KOH alone was not efficient for the removal of natural chemically resistant materials such as chitin which can produced a fluorescence when exposed with Nile red (Maes *et al.*, 2017). KOH:NaClO has also been applied in several studies as a chemical digestion solution for sediment and biota with no impairment of the identification of the tested polymers by Raman micro-spectrometry (Strand and Tairova, 2016; Enders *et al.*, 2017). Enders at al. (2017) developed and tested an alkaline digestion protocol to preserve small plastic particles while removing organic tissue materials. They concluded that using a 30% KOH:NaClO digestion solution was effective for the digestion of fish stomachs. The combination of KOH:NaClO was also found to be more effective than KOH and NaClO alone. As a result, a 30% KOH:NaClO was prepared and tested in the laboratory for biota samples. KOH and NaClO combined was effective at suppressing the fluorescence of some chemical resistant biological or natural residues while leaving intact the fluorescent behaviour of polymers (Figures A2.2 – A2.4, **Appendix 2**). As a result, a 30% KOH:NaClO was selected as the alkaline digestion method for biota samples. 5 ml of a 30% KOH:NaClO was added per g of wet tissue (Enders *et al.*, 2017).

Reporting units

There is still a requirement for a standardised reporting unit for the monitoring of microplastics in biota. It has been suggested that reporting data using multiple expressions of microplastics contamination is also necessary to relate to other studies (Lusher *et al.*, 2017). In this study, it was recommended that units should be reported as number of items per individual and number of items per g of material wet weight (w.w).

Fluorescence tagging using Nile Red staining

- Isolation and detection of plastic particles in mussels

The fluorescence tagging of polymers using Nile Red staining was applied to Mussels (*M. edulis*), sourced from a supermarket, destined for human consumption. No information on collection were available and

plastic contamination would have occurred between sampling, washing to packaging. As a proof of concept, only 3 individuals were processed. A single control was also used as indication of contamination. Filtration of the mussel digests was a slow process due to the low porosity of the filter initially used (0.2 μm regenerated cellulose filter). Other studies specified the use of filters varying in sizes ranging from 2 to 5 μm (Li et al., 2016; A. Lusher et al., 2017). The use of a Whatman® glass microfibre filter Grade GF/D with a 2.7 μm porosity was found to be the most effective in this study and was selected for biota.

Table 3. Preliminary results for the number of items per individual and per g wet weight for the particles isolated from mussels (n=3, ± SD).

Replicate number	Number of items per individual (blank corrected)	Number of items per g wet weight
Negative Control	2	
Mussel_Rep1	26	10
Mussel_Rep2	9	3.9
Mussel_Rep3	16	5.7
Mean	17	6.5
SD	9	3

- Isolation and detection of plastic particles in fish

Fluorescence tagging using Nile Red has also been applied to a mackerel (stomach) and a dab (digestive tract including stomach). Preliminary results are shown in Tables 4 and 5 for the dabs and mackerels, respectively. Negative (blanks) and positive controls (spiked blanks) were also prepared. Samples were analysed using visual and automatic counting. Recovery of the spiked blanks ranged from 80 to 90% from direct visual counting and from 79 to 90% using the Microplastics tool software. One dab sample was suspected to contain 9 suspected microplastics corresponding to 5 particles following correction with the negative control (i.e. empty filter). This resulted to an average number of items of 1 ± 2.2 items per individual for dabs (Table 4). One mackerel contained 9 suspected microplastics or 7 particles following correction with the negative control (i.e. empty filter). This resulted to an average number of items of 1.4 ± 3 items per individual for mackerels (Table 5).

Table 4. Preliminary results from the validation study for the number of items per individual and per g wet weight for the particles isolated from fish (Dab) (n=5, ± SD)

Replicate Number	Number of items – visual counting	Number of items- automatic counting	Number of items per individual (blank corrected)	Number of items per g wet weight
Spiked filter _10 items	9	9	-	-
Spiked filter_15 items	12	19	-	-
Negative control	4	4		-
Dab_Rep1	9	8	5	-
Dab_Rep2	0	0	0	-
Dab_Rep3	0	0	0	-
Dab_Rep4	0	0	0	-
Dab_Rep5	0	0	0	-
Mean			1	-
SD			2.2	

Table 5. Preliminary results from the validation study for the number of items per individual and per g wet weight for the particles isolated from fish (mackerel) (n=5, ± SD)

Replicate Number	Number of items per individual (blank corrected)	Number of items per g wet weight
Blank	2	-
Mackerel_Rep1	7	-
Mackerel_Rep2	0	-
Mackerel_Rep3	0	-
Mackerel_Rep4	0	-
Mackerel_Rep5	0	-
Mean	1.4	-
SD	3	

IV. Discussion

Validation of the protocol for biota

The fluorescence tagging of polymers using Nile Red staining was applied to a small number of Mussels (*M. edulis*) as a proof of concept. Digital images of the 2.7 µm porosity filters following staining using Nile Red were generated. Other studies specified the use of filters varying in sizes ranging from 2 to 5 µm (Li *et al.*, 2016; Lusher *et al.*, 2017). Preliminary results reported an average number of items per individual of 17 ± 9 corresponding to 6.5 ± 3 items per g wet weight (w.w) (Table 3).

Concentrations of microplastics in biota, including mussels, are widely available from the literature and are compiled in Table 6. Reported concentrations varied between studies due to the differences in extraction protocols and validation steps (e.g. use of controls or polymer identification as an extra validation step).

Lusher et al. (2017) reported from 0 to 14.67 items per individual and 0 to 24.45 items per g w.w for wild mussels (*M. edulis*) collected on the coasts of Norway (Table 6). For comparison, Li et al. (2016) reported a range of microplastics between 0.9 and 4.6 items per g w.w for the same test organism. These reported values were however well below the range reported by Mathalon and Hill, 2014 with a range of 34 to 178 items per individual. It would be recommended to validate these results using wild mussels collected directly from the field. Additional validation studies with mussels collected from pristine areas and spiked with a known concentration of microplastics should also be carried out.

The protocol was also applied to a mackerel (stomach) (n=5) and a dab (gastrointestinal tract) (n=5) as model species. The alkaline digestion process was found to be effective for both cases with complete digestion of the tissues after the 3 days incubation period. Microplastics were detected for both species however the method was only applied for a small population size for validation (n= 5) (Tables 4 & 5). The method was however further validated on an extended range of biota across different projects including commercially important species such as sardines (*sardinops sagax*), anchovies (*Engraulis encrasicolus*) and Redeye round herring (*Etrumeus whiteheadi*). Reported concentrations of microplastics for fish for the Atlantic area and for other locations are also available from the literature (Table 6). Foekema et al. (2013) investigated the occurrence of ingested plastic particles in the digestive tract of seven common North Sea species including herring, gray gurnard, whiting, horse mackerel, haddock, Atlantic mackerel and cod. 2.6% of examined fish contained plastic particles and in five of the seven species investigated. Reported particle size ranged from 0.04 to 4.8 mm indicating that fish species are able to retain larger sized particles than mussels (Foekema *et al.*, 2013). No plastics were found in gray gurnard and mackerel. Highest frequency of microplastics was found in cod from the English Channel. It is not surprising as cod is a demersal fish and is more exposed to sediments as compared to mackerels. As a contrast, Rummel et al. (2016) investigated 290 gastrointestinal tracts of demersal (cod, dab and flounder) and pelagic fish species (herring and mackerel) from the North and Baltic Sea for the occurrence of plastic ingestion. They detected plastic particles in 5.5% of all investigated fishes with a higher ingestion frequency in the pelagic feeders (Rummel *et al.*, 2016). Lusher et al. (2013) also suggested that microplastic ingestion appeared to be common, in relatively small quantities, across a range of fish species irrespective of feeding habitat. Another survey found no difference in the amount of ingestion when comparing all pelagic (38% with ingestion) and demersal (35% with ingestion) species (Lusher, McHugh and Thompson, 2013).

Other studies focused on top predators, including sharks, with the study from Maes et al. (submitted) investigating the impacts of microplastics to the endangered North-East Atlantic Porbeagle shark (*Lamna nasus*). Microplastics were detected in 9 out of 10 spiral valves at concentrations ranging from 0.48 to 10.4 particles per g wet weight (w.w.) content and from 1.5 to 9.5 particles per g w.w. tissue. No statistically significant correlations (95% significance) were found between the average number of plastic particles in spiral valve content and tissue and the Condition and Hepatosomatic Index of porbeagle sharks. The results of this research indicated that North-East Atlantic porbeagle sharks ingest microplastics, but further research is needed to investigate possible health effects of microplastic contamination.

Table 6. Number of items per g wet weight and per individual reported in the literature for several locations.

	Organism	Location	Number of items/g w.w.	Number of items per individual	Reference
Marine worms					
	A. marina	French, Belgian and Dutch North Sea coast	1.2 ± 2.8		(Van Cauwenberghe *et al.*, 2015)
Sea snails					
	C. abbreviatus	Western Pacific Oceans	0.16	2.9	(Abbasi *et al.*, 2018)
Prawns					
	P. indicus	Western Pacific Oceans	0.59	2.3	(Abbasi *et al.*, 2018)
Mussels					
	M. edulis	French, Belgian and Dutch North Sea coast	0.2 ± 0.3		(Van Cauwenberghe *et al.*, 2015)
	M. edulis	French Atlantic coast		0.61 ± 0.56	(Phuong *et al.*, 2018)
	M. edulis	China	0.9 – 4.6		(Li *et al.*, 2016)
	M. edulis	UK	0.7 – 2.9	1.1 – 6.4	(J. Li *et al.*, 2018)
	M. modiolus	UK	0.086 ± 0.031	3.5 ± 1.29	(Catarino *et al.*, 2017)
	M. edulis	UK	3.0 ± 0.9	3.2 ± 0.52	(Catarino *et al.*, 2017)
	M. edulis	Norway	0 – 24.45	0 – 14.67	(A. Lusher *et al.*, 2017)
	M. edulis	Germany	0.36 ± 0.07		(Van Cauwenberghe and Janssen, 2014)

	M. edulis	Canada		34 - 178	(Mathalon and Hill, 2014)
	Mytilus edulis, Perna viridis	Coastal waters of China	1.52 – 5.36		(Qu *et al.*, 2018)
	Bivalves	China		4.3 – 57.2	(Li *et al.*, 2015)
Oysters					
	Saccostrea cucullata	China	1.5 – 7.2	1.4 – 7.0	(H. X. Li *et al.*, 2018)
	C. gigas	Brittany, France	0.47 ± 0.16		(Van Cauwenberghe and Janssen, 2014)
	C. gigas	French Atlantic coast		2.10 ± 1.71	(Phuong *et al.*, 2018)
Fish					
Riverine fish		Amazon River estuary in North-eastern Brazil		1.75 (0 – 12.8)	(Schmid *et al.*, 2018)
Whiting	*S. Sihama*	Western Pacific Oceans	0.25	1.5	(Abbasi *et al.*, 2018)
Greater lizardfish	S. tumbil	Western Pacific Oceans	0.37	2.8	(Abbasi *et al.*, 2018)
Pelagic and demersal fish		English Channel		1.9 ± 0.10	(Lusher, McHugh and Thompson, 2013)
Adriatic fish mullet		Adriatic Sea, Italy		1 – 1.78	(Avio, Gorbi and Regoli, 2015)
Large pelagic fish	*Xiphias gladius, Thunnus; thynnus and Thunnus alalunga*	Mediterranean Sea		4 - 16	(Romeo *et al.*, 2015)
Demersal fish		Spanish Atlantic and Mediterranean coasts		1.56 ± 0.5	(Bellas *et al.*, 2016)

Pelagic and demersal fish		North and Baltic Sea		0.03 ± 0.18	(Rummel *et al.*, 2016)
Sunfish bluegill and Longear	*Lepomis Macrochirus & Lepomis megalotis*	Brazos River Basin, Central Texas, USA		10.1 – 13.9	(Peters and Bratton, 2016)
Demersal & pelagic fish		Northeast Atlantic around Scotland		1.8 ± 1.7	(Murphy *et al.*, 2017)
Flying fish	*C. rapanouiensis*	South Pacific coastal waters around Easter Island		1.0 ± 0.0	(Chagnon *et al.*, 2018)
Commercial fish		Mondego estuary in Portugal		1.67 ± 0.27	(Bessa *et al.*, 2018)
Sardines and Anchovy	*Sardina pilchardus and Engraulis encrasicolus*	Spanish Mediterranean coast		0 - 3	(Compa *et al.*, 2018)
Tuna					
Yellow Fin Tuna	*T. albacares*	South Pacific Ocean Coastal waters of the Eastern Island		5.0	(Chagnon *et al.*, 2018)
Whales					
		Northern Ireland		2.95	(Lusher *et al.*, 2015)
Sharks	*Lamna nasus*	North Atlantic Area	5.61 ± 0.78 (1.5 – 9.5)		Maes et al., submitted
SeaBirds					
Northern Fulmars	*Fulmarus glacialis*	Pacific and Grays Harbor counties, Washington		13.3	(Terepocki *et al.*, 2017)
Sooty Shearwaters	*Ardenna grisea*			19.5	(Terepocki *et al.*, 2017)

Table 7 Summary of the proposed method for the detection and quantification of microplastics in biota samples (modified from Hermsen et al., 2018).

Activity	Description	Comments	Reference
Sample collection	Preparation of biota and tissue collection	See **Appendix 2** for the SOP	CLiP SOP 012
Sample size	At least 50 individuals	Number of 50 individuals chosen arbitrarily by the International Council for the Exploration of the Sea (ICES) and the Technical Subgroup on Marine Litter (MSFD-TSGML), not based on statistical evidence	(Strategy Framework Directive, 2013; ICES, 2015; Hermsen *et al.*, 2018)
Sample storage	Storage at -20°C		
Laboratory preparation	Pre-rinse of the glass sample jars using MilliQ water and dried upside down in a biological safety cabinet. Pre-rinse glassware using Milli-Q water and cover with milliQ-rinse foil. Submerge dissection kit in a pre-rinsed beaker containing Milli-Q water.		
Laboratory contamination control	Use of cotton lab coats, use of biological safety cabinets for sample handling, filter all chemical reagents on 0.2 µm regenerated cellulose filters, use MilliQ water for reagent preparation and rinsing equipment, clean surfaces using lint free clean room cotton wipes		
Negative control	Use of a control during dissection process by opening a pre-rinsed sample jar for the same time necessary for the dissection of 1 sample.	The number of particles that exhibited fluorescence were deducted from the final number of items detected in biota.	
Positive control	Use of spiked GF/D filters with a known number of	Positive controls are also being used as	

	reference plastic materials of varying polymer type and sizes Used of spikes biota tissues collected from pristine areas for validation of method	recovery for the visual and automatic image recognition software	
Target component	Gastrointestinal tract, wet weight recorded		
Additional information	Individual body weight and body length		
Sample treatment	Digestion using 30% KOH:NaClO	Validated for sediment and biota	This study, (Strand and Tairova, 2016; Enders *et al.*, 2017)
Filter selection	GF/D grade Whatman glass microfiber filter with a 2.7 μm porosity	Rinse filter with MilliQ water before use, no interference from filter during staining with Nile red	
Sample clean-up	Use of pre-filtered degreasers for fat residues removal	Only for fat deposits, degreaser added during the incubation process at 40°C	
Microplastic detection	Staining of the particles using Nile Red		
Microplastic quantification	Digital imaging coupled with automated image recognition/Validation using microscopy		
Polymer identification	FTIR or Raman spectroscopy		
Reporting unit	Number of items per individual and number of items per g wet weight		

V. Selection of an indicator species for the monitoring of microplastics in the marine environment

One of the objectives of the project was to identify and propose a suitable and sustainable indicator species for the monitoring of microplastics in biota in the Atlantic area. Data were generated for a bivalve (mussels), a pelagic fish (mackerel) and a demersal fish (dab) as models. These species were selected due to their availability within Cefas as well as their widespread distribution around the UK and the Atlantic area (Figure 4). On-going work is focusing on the validation and optimisation of the protocol on a wider range of biota for a bigger population size (minimum of 50 individuals per species per location) across a range of national and international projects (e.g. Commonwealth Litter Programme or CLiP). Specific criteria, essential for the selection of an appropriate indicator species, were assessed in Table 8.

It is clear from Table 8 and Figure 4 that mussels (*M. edulis*) is fulfilling all the criteria defined for the identification of a suitable biological indicator for microplastics for the Atlantic area. A recent study from Bråte et al. (2018) also suggested that *Mytilus* spp. would be suitable organisms for the monitoring of microplastics in coastal waters. There is need for further studies as some uncertainties remain associated with mussel sizes which could influence ingestion, the role of depuration and other fate related processes (Bråte *et al.*, 2018). Further validation is therefore needed before implementing the role of mussel species in a regulatory context for the monitoring of microplastics in the Atlantic area.

Table 8 Recommended criteria for the selection of a bioindicator species for the monitoring of microplastics in biota for the Atlantic area.

Criteria	Biota		
	Mussel	Mackerel	Dab
Wide geographical distribution	✓	✓	×
Representative of a specific monitoring area	✓	×	×
Species that are not protected or endangered	✓	✓	✓
Suitable particle retention time within organisms (hours)	72 [1]	4 – 158 [2]	4 -158 [2]
Already used as bioindicator/biomonitoring species	✓	✓	×
Ability to ingest and retain small to larger sized particles	<1mm [3]	<5 mm [4]	<5 mm [4]
Species that can be kept in cages for easy field deployment and collection	✓	×	×
Invertebrate species, which require less staff training (cost-effective) for handling than vertebrate species	✓	×	×

Perform sampling in a cost-effective manner by synergies with pre-existing programs	✓	✓	✓
Commercially important species with public health implications	✓	✓	✓
Ease of sample preparation and validated analytical protocol using Nile Red polymer fluorescence tagging	✓	✗	✗

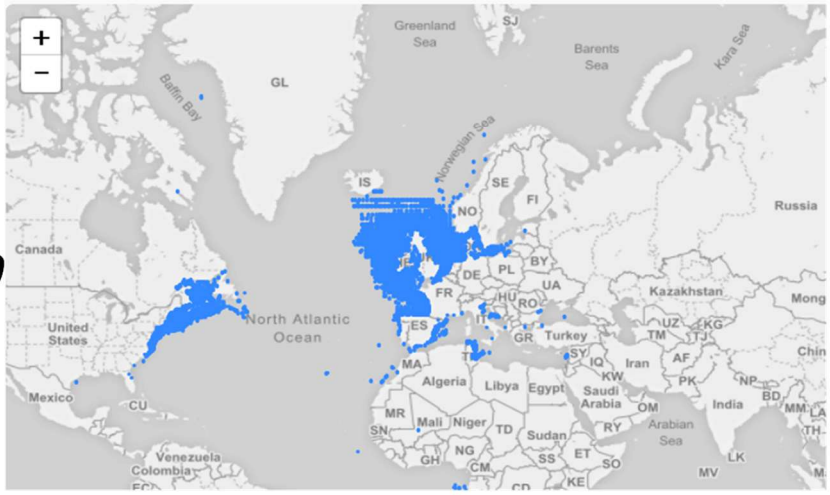
[1] (Ward and Kach, 2009; Catarino *et al.*, 2017); [2] (Brett and Grooves, 1979); [3] (Bråte *et al.*, 2018); [4] (Nelms *et al.*, 2018)

Common mussel

(Mytilus edulis)

Atlantic mackerel

(Scomber scombrus)



Dab

(Limanda limanda)

Figure 4. Distribution map of the common mussel (Mytilus edulis), the Atlantic mackerel (Scomber scombrus) and Dab (Limanda limanda) for the North Atlantic area (MarLIN, 2008).

IV. Conclusions

- A rapid screening approach using fluorescence tagging of polymers using Nile red and using an automated data processing was developed and validated for biota. This method needs continued validation using FTIR.
- Monitoring of microplastics in biota needs further investigation with a higher number of samples considering an extended range of sampling locations. The recommended number of individuals remains to be estimated to produce environmental concentration of microplastics in biota with a high level of certitude.
- It is recommended that reporting units for biota should be in number of items per individual and number of items per g wet weight.
- It is recommended to extract the whole of the gastrointestinal tract rather than either the stomach or the intestines due to the relatively short particle residence time for microplastics in the stomach.
- *Mytilus edulis* appeared to be a suitable organism for the monitoring of microplastics in coastal waters for the Atlantic area.

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Appendix 1 – SOP for collection of specimens for the monitoring of microplastics in biota (individual samples)

Contents

Cleaning-up

Note: **Keep covers on glassware and any other equipment that comes into direct contact with sample as much as possible to avoid ambient contamination.**

- Work in a clean environment (clean work top with water/alcohol/acetone/laminar flow when possible)
- Use the washing machine to wash dirty glassware and transfer the glassware to “tap water rinsed” container
- Rinse all the glassware with RO water and transfer the glassware to the RO container for transport to the lab
- Dry all the glassware under a biological safety cabinet (BSC).
- Place some foil between the pot and the plastic lid.
- Place a pre-cut waterproof paper between the foil and the lid for labelling

Sample preparation

1.1. Materials

- 120 mL glass pot with lid or sample vial.
- Wooden dissection board
- Gloves
- Dissection kit and metallic tweezers
- Glass beaker
- RO or MilliQ water
- Waterproof paper
- Waterproof label
- Foil

- Analytical balance (2 decimal places)

1.2. Quality control

- Use 2 blanks
- For the 120 pots, leave 2 pots exposed to the air to compensate for ambient contamination Cover the pots with foil while no activities are being taking place in the lab.
- For the small vials, leave 2 small vials exposed to the air to compensate for ambient contamination. Cover the vials while no activities are being taking place in the lab.

1.3. Precautions to take when collecting samples in the field

- Wear cotton clothes (lab coat), and minimise synthetic clothing
- Minimise air circulation, in case there is airflow mind the direction so possible fibres coming from clothes do not end up near the guts
- Wear blue nitrile gloves
- When plastic bags have to be used to store samples, choose a bright colour so it is easy to identify in case of contamination

1.4. Removal of the digestive tract

- Prepare a glass beaker with RO water or MilliQ water and cover in foil.
- Rinse the dissection kit and metallic tweezers in the glass beaker before and after use to avoid cross-contamination
- Prepare a wooden dissection board or cover the plastic board with foil (Figure A1.1)

Figure A1.1. Example of a dissection preparation area.

- Write down: species, where and when it was collected, (gender) and the length/weight of the animal.
- A minimum number of 50 individuals per species is recommended by OSPAR and MSFD
- Remove the whole digestive tract (if possible), stomach otherwise, or in case of sharks the spiral valve. If only part of the digestive tract has been collected, please make a note on the label (e.g. stomach only) (Figure A1.2)
- Avoid content getting lost by using metal clamps or cotton cord at the beginning and end of the digestive tract before cutting.
- Place the pot or vial, covered with some aluminium foil, on top of the analytical balance and zero it
- Transfer the digestive tract to the vial (for small sized guts) or pot quickly to limit losses via leaking (Figure A1.3).
- **Record the wet weight of the tissues in g**
- Place one digestive tract (or stomach) per pot/vial.

Figure A1.2. Removal of the digestive tract

Figure A1.3. Collected digestive tract in collection vial. Use pots for larger items.

1.5. Labelling

- 2 labels should be used:
    - Use a waterproof adhesive label on the outer part of the vial or pot.
    - Use a piece of waterproof paper between the foil and the plastic lid (only for the 120 mL pots).
- Labelling system: on the waterproof label and the waterproof paper add the following information:

1. Survey code_Date_Station nber_Species_sample number

1.6. Storing of the samples

- Place the pot on the tray and store in a freezer when not processed immediately.
- The sample is ready for digestion and extraction

Appendix 2 – Fluorescence tagging using Nile red, digital imaging and data processing

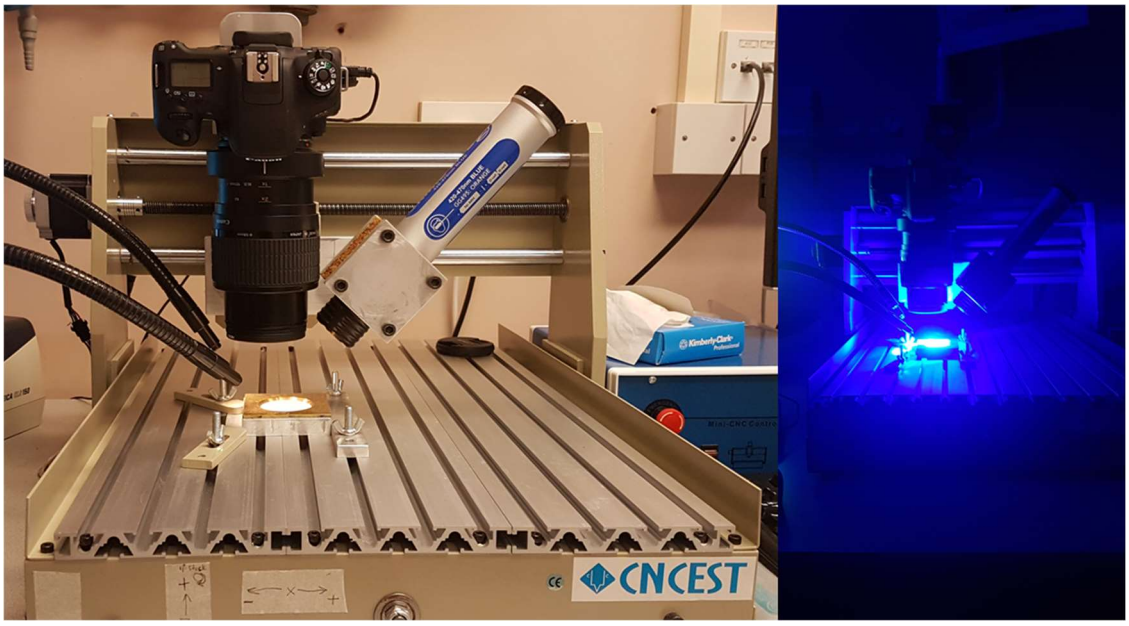

Figure A2.1 Experimental set-up of the digital imaging acquisition system using white light and blue light (420-470 nm)

Table A2.1 Object statistics extracted from the image

Statistic	Description
Mean_Red	The mean value for the red band of all the pixels within an object
Mean_Green	The mean value for the green band of all the pixels within an object
Mean_Blue	The mean value for the blue band of all the pixels within an object
Size	The total number of pixels within the object
Length	The length of the circumference of the object in pixels.

For this investigation the default values (Table A2.2) for the model parameters were used. The decision tree is displayed in Figure A2.1

Table A2.2 Microplastic identification tool parameters

Parameter	Value (Default)
Spectral Detail (segmentation)	18
Minimum total fluorescence (RGB)	60
Minimum object Mean Red	50
Minimum Object Mean Greed	20
Maximum within object range for Red	250
Minimum object size (pixel)	100
Maximum Object size (pixel)	4,000,000

Classified objects were exported as shapefiles and as a table for reference. The table includes the object statistics described in Table A2.1 & A2.2 as well as the ratio of length:area and the fluorescence index [(R+G)/R]. The tool can be run on a single image or on a series of images giving the results of a whole plate as a single figure.

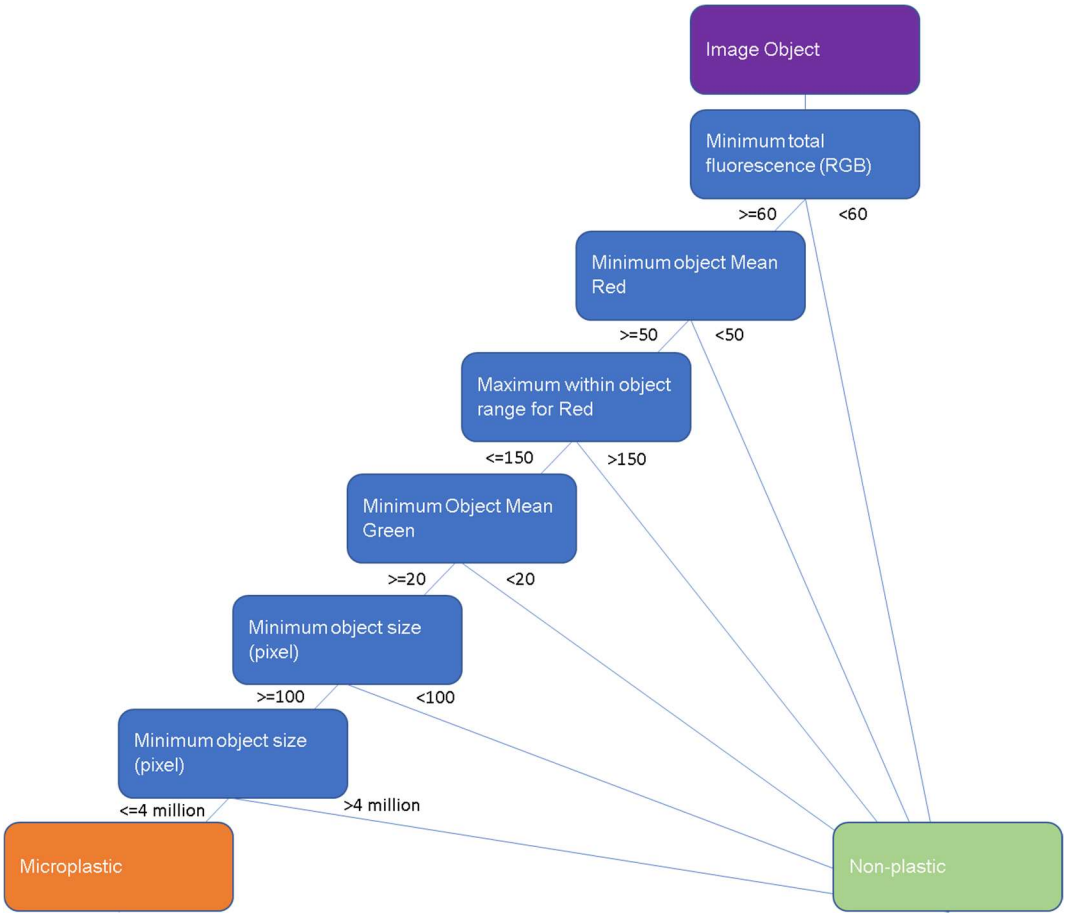

Figure A2.1 Decision tree for classification of objects as microplastics

Alkaline chemical digestion

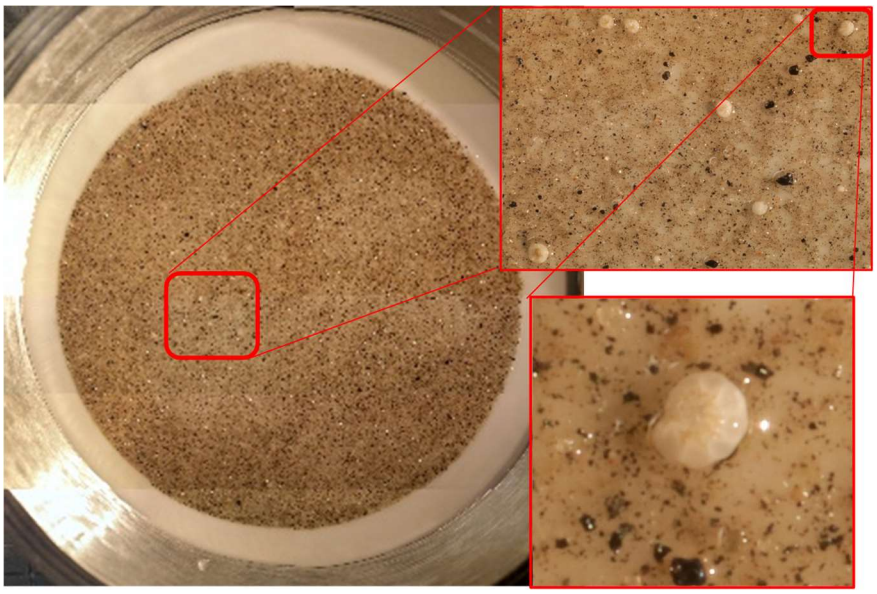

Figure A2.2 Chemical resistant materials on the surface of the filter following alkaline digestion at 40°C for 3 days under constant agitation at 120 rpm.

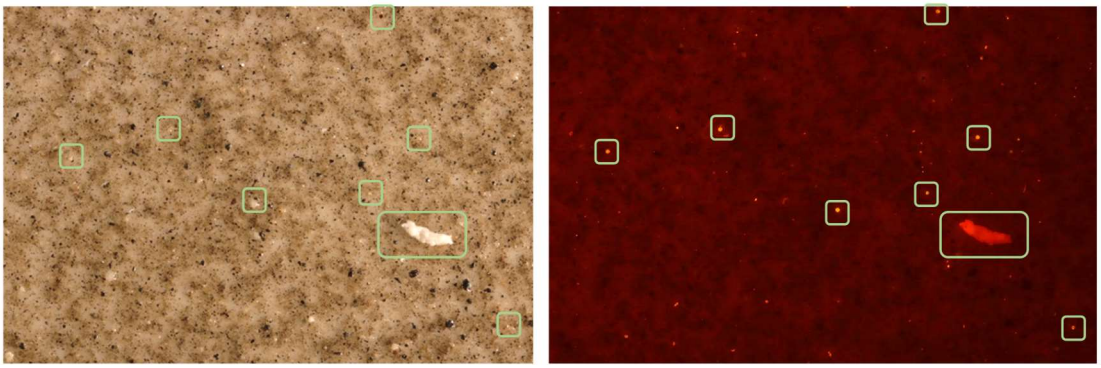

Figure A2.3 Fluorescent biological residues after fluorescence tagging using Nile red following a 10% KOH digestion process at 40°C for three days. In green are the chemical resistant biological residues.

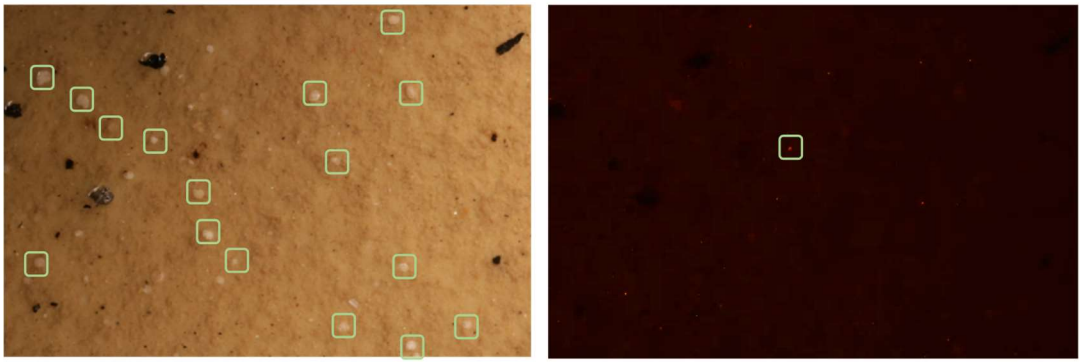

Figure A2.4 Fluorescent biological residues after fluorescence tagging using Nile red following a 30% KOH:NaClO digestion process at 40°C for three days.