CleanAtlantic

Tackling Marine Litter in the Atlantic Area

Characterization of microplastics ingested by mussels

Toward the determination of a bio-sentinel species of the marine environment contamination by microplastics?

WP 5.3: Monitoring the interaction of marine litter with fauna

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Summary

As in the rest of the oceans, MicroPlastics (MPs) are ubiquitous in the North Atlantic and setting up systems to monitor this contamination appears to be a necessity. The impact of MPs on organisms depends on a combination of specific variables and in particular the biological parameters of the species. Many species eaten by humans can ingest MPs and therefore represent a potential health hazard. Mussels are used as bioindicators for chemical contamination and, due to their nature as filter-feeding organisms and their geographical distribution (ubiquitous species), also appear suitable to be used as bioindicator species for MP pollution. The aim of this Interreg CleanAtlantic project task was to test the use of mussels for monitoring microplastic contamination on an Atlantic Area network consisting of the UK, France and Spain. The results indicate that MPs are present in mussels from all three countries, confirming that MPs are ubiquitous in the North Atlantic. The average quantities of MPs ingested by mussels in this study were 0.063 items.g^{-1}w.w for the UK, 0.094 items.g^{-1}w.w for France and 0.337 items.g^{-1}w.w for Spain. The use of mussels as an indicator of MPs contamination along the European Atlantic coastline appears, in this study, to be consistent. However, the work needs to be confirmed with a larger dataset across a time series (multiple years).

Acknowledgements

This work would not have been possible without the participation of the various partner laboratories that took mussel samples throughout the Spanish, French and British monitoring networks. We acknowledge the support of the Food Standards Agency (UK) for providing the British mussels. We would like to extend our warmest thanks to each and every sampler.

List of Abbreviations

ATR: Attenuated total reflectance

Cefas: Centre for Environment, Fisheries and Aquaculture Science, UK

CEMP: OSPAR's Coordinated Environmental Monitoring Programme

CI: Confidence interval

EU: European Union

FSA: Food Standards Agency, UK

FTIR: Fourier Transform Infrared spectroscopy

IEO: Spanish Institute of Oceanography (CSIC)

Ifremer: French national Institute for Ocean Science

ML: Microlitter

MPs: Microplastics

MSFD: Marine Strategy Framework Directive

NB: NumBer of individuals

NI: Not identified

OSPAR: (OSlo-PARis) Convention, Convention for the Protection of the Marine Environment of the North-East Atlantic

PA: Polyamide (nylon)

PAN: Polyacrylonitrile

PDMS: Polydimethylsiloxane

PE: Polyethylene

PES: PolyESter

PP: PolyPropylene

PS: PolyStyrene

PU: PolyUrethane

RO: Reverse Osmosis

ROCCH: Chemical contamination monitoring survey

UK: United Kingdom

1 Introduction

Plastic pollution has become a global issue causing major threats to the marine environment (Beaumont, *et al.*, 2019;). A study quantified that more than 12.7 million tons of plastic can enter the marine environment each year (Jambeck *et al*., 2015). Plastic properties such as its lightweight, durability, and low cost explain the increase in production, to 400 million tonnes in 2022 (PlasticsEurope, 2023), that have led to long-lasting contamination and accumulation in the marine environment (Andrady, 2011; Pirsaheb *et al.*, 2020). Plastic buildup poses many threats to marine ecosystems by direct pollution (Sutherland *et al.*, 2010), but it can also impact species inhabiting them by causing, for example, strangulation and suffocation problems (Darmon *et al.*, 2017; Fossi *et al.*, 2018). Indeed, plastic pollution is characterized by both macro - and mesoplastic debris and smaller plastic particles known as microplastics (MPs). These smaller plastic particles (<5 mm) can be ingested by marine organisms, resulting in various harmful effects (Giani *et al.*, 2019; Kumar and Prasannamedha, 2021) such as chemical contamination, endocrine disruption and altered immune system responses. To date, studies estimate that more than 220 species ingest MPs, affecting animals ranging from bivalves such as mussels to marine mammals (Li *et al.*, 2019). MPs can also have many ecological impacts (Khalid et al., 2021), as well as act as an economic and societal pressure (Arabi *et al.*, 2022; Ghosh *et al.*, 2023).

Through trophic transfer and direct ingestion, MPs are found throughout the food chain (Wang *et al.*, 2019; Justino *et al*., 2023; Parolini *et al*., 2023). The likelihood of MP ingestion and their resulting impact on organisms depends on a combination of specific parameters, such as the position of these particles in the water column (Van Cauwenberghe *et al*., 2015; Choy *et al*., 2019; Gao *et al*., 2023), physical characteristics, such as polymers additives and other associated chemicals, shape and size (Ahmed *et al*., 2023; Corella-Puertas *et al*., 2023), and organism ingestion systems and route of exposure, e.g., ingestion, inhalation (Liang *et al*., 2023). Many species are concerned, such as zooplankton, mussels, oysters, corals, fish, turtles and even seabirds (Andrady, 2011; Wesch *et al*., 2016), some of which are consumed by humans, representing potential impacts on human health (Huang *et al*., 2020). Although several studies have already shown that MPs are ingested by different species, their mechanisms and effects are still poorly understood. Plastics decrease in size with weathering and fragmentation, making them accessible to a wide range of organisms. For example, MPs are similar in size to zooplankton (0.3 mm–5 mm), which can lead to confusion for predators feeding on planktonic preys of this size. Therefore, they can be ingested and thus easily enter the marine food chain (Lusher, 2015; Van Cauwenberghe *et al*., 2015; Ward *et al*., 2019). It is therefore essential to improve our knowledge of the mechanisms involved.

The abundance and harm associated with marine litter has led to the development of directives to address it.

One such case is the Marine Strategy Framework Directive (MSFD, 2008/56/EC) in the EU, where marine litter monitoring (including microplastics) is implemented to achieve or maintain the Good Environmental Status. This is carried out by studying the spatial distribution, concentration and composition of marine litter in all the compartments of marine environment (e.g., water, sediment, biota), as well as observing the adverse effects on marine species such as through the ingestion of MPs. As yet, there is no agreed bioindicator for microplastics. Mussels, which are used as bioindicators for various chemical contaminants (ROCCH, OSPAR CEMP…), have been proposed as a suitable species. They are widely distributed, abundant and able to ingest

small particles as filter-feeders. Additionally, they are of commercial importance, are easy to sample and are sedentary, unlike fishes which have also been proposed as sentinel species. Although some studies have reported microplastic ingestion in mussels the use of mussels as a bioindicator is hindered by a lack of standardised and harmonised protocols (*e.g.* on the processing times, exposure, extraction protocol…). It has been highlighted that more comparable studies are required to assess their suitability (Li *et al*, 2021; Li *et al*, 2019).

The objective of this study was to implement a harmonised assessment protocol for the extraction of microplastics from mussels and compare concentration between three regions in the North Atlantic under the framework of the extension of CleanAtlantic Project, under task 5.3 dealing with monitoring the interaction of marine litter with fauna. Through these comparisons, the suitability of mussels as a bioindicator was assessed. Cefas, IFREMER and IEO worked on the characterization of pollution by MPs/microfibers in the marine environment, testing mussels as bioindicators along Spanish, French and British coastlines.

The results of this study will improve knowledge to build a future D10C3 MSFD indicator (microplastics in biota), currently under development, feed into development of OSPAR regional indicators and also national level. Furthermore, it may extend the spatial coverage of microplastic sampling in coastal areas to map and validate models.

2 Materials and methods

2.1 STUDY AREAS AND SAMPLING

To test the feasibility of using mussels as a bioindicator species for contamination by MPs, each partner relied on their existing national networks for coastal monitoring. As recommended by Bakir *et al*. (2020), to perform replicates at each site, a minimum sample pool of 20 to 25 mussels (ranging from 4 to 7 cm in size and from at least 5 to 8 sites per country) were sampled during Autumn 2022.

In the UK, mussels were collected monthly by Cefas Weymouth laboratory, as part of an FSA survey into harmful algal blooms. Cefas Lowestoft laboratory utilized the collection of surplus mussels (*Mytilus edulis*) between November 2022 and February 2023. Each month 7 to 13 sites were sampled. For the present study, 7 sites sampled in November 2022 were selected. Mussels were frozen prior to MP extraction.

In France, Ifremer coastal environment laboratories contributed in sampling 16 locations from 11th October 2022 to 5th November 2022.The sites sampled correspond generally to those included in the French annual chemical contamination monitoring program (ROCCH) and meeting with OSPAR Coordinated Environmental Monitoring Programme (CEMP) specifications. Analysis is carried out on the species *Mytilus edulis*. Exceptional sampling was carried out at two sites, due to the drying up of mussel beds on the south Atlantic coast *Estacade de Cap Breton* and *Bouée d'atterrissage du port de Bayonne*. Mussel samples were frozen at -20°C and sent to Ifremer LER-PAC laboratory to be analysed.

In Spain, IEO took advantage of the OSPAR CEMP to take wild mussels for analysis of MPs. The OSPAR CEMP takes place annually in late autumn and early winter in this area. Samples from 5 coastal stations along the north of Spain were collected. The samples were kept frozen before analysis.

Sampling locations for each country are shown in Figure 1 below. For the purpose of this study, MPs were defined as plastic particles ranging from 20 µm to 5 mm on their longest dimension.

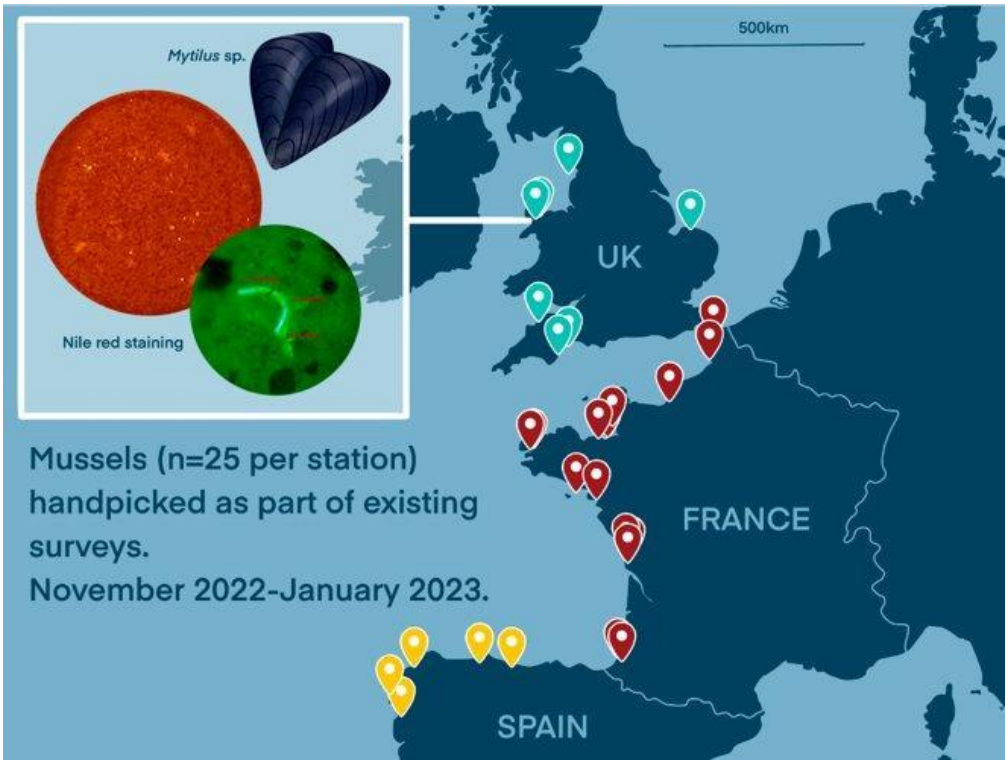

Figure 1 : Map of the sampling locations for the UK (blue), France (red) and Spain (yellow), see details of the sites in annex 1

2.2 LABORATORY ANALYSES

2.2.1 British samples - Cefas analyses

2.2.1.1 *Microplastics extraction processing*

Mussels were measured in the longest dimension. The tissue was removed from the shell and rinsed with filtered RO water and the byssal threads were removed. This follows the recommendation of Kolandhasamy *et al*. (2018). The mass of the tissue (wet weight, w.w) was then recorded. Mussels were placed in individual clean glass beakers capped with glass lids and covered with 40 mL of potassium hydroxide/sodium hypochlorite solution (15% KOH/2% active chlorine) and sonicated for 5 minutes (USC200T, VWR, UK). The beakers were then placed into an orbital incubator (Incubating mini shaker 980151UK, VWR, UK) at 40 °C 120 rpm for three days. Following this, 40 mL of degreaser (Elbow Grease, UK) was added to remove any fatty residue and the samples returned to the incubator for a further 24 hours. Once digestion was complete, samples were filtered over GF/D filters (Whatman ø 45 mm, 2.75 µm pore) using a vacuum manifold and glass funnels. Following this, the filter was flushed with 100 mL of RO water and the sides of the funnel rinsed. Filters were stained with Nile red for 30 minutes, and as once again flushed and rinsed with RO water.

2.2.1.2 *Microplastics characterization processing*

Filters were examined with blue and white light under a binocular microscope (MZ10F, Leica) with blue light attachment (FluoIII, CoolLED) and USB camera (GXCAM-U3PRO-20). Fluorescent particles identified as suspected microplastics were imaged and measured using GX Capture-T (version x64, 4.10.16968.20200415) and then transferred to a 25 mm ø 0.2 µm pore anodisc (VWR, UK) for FTIR analysis. The anodisc was placed in a drying cabinet (100L S/S, LTE, UK) at 40 °C for a minimum of 24 hours.

2.2.1.3 *Controls and contamination*

UK samples were processed in a clean environment. At *Cefas*, samples were kept covered by glass Petri dishes and all laboratory processes were carried out under a biological safety cabinet (Guardian MSC T1200, Monmouth). Additionally, 100% cotton lab coats and nitrile gloves were worn. The lab coat was dyed purple to aid in the identification of contamination from the garment. All reagents and solutions were filtered through a 0.2 µm regenerated cellulose filter (Whatman) prior to use. Glassware was triple rinsed with filtered RO water and covered with rinsed foil or glass Petri dishes.

Laboratory controls (also known as procedural blanks or negative controls) were collected for all lab processes. Three controls were collected per sample site (n = 25 mussels). After FTIR analysis, the average contamination from the blanks was removed from each station.

2.2.1.4 *Polymer processing*

Polymer identification was conducted using a Lumos II µFTIR (Bruker, UK). A subset of items was selected and analysed using ATR-µFTIR with a liquid nitrogen cooled MCT detector. A total of 32 scans were collected in reflectance mode in the range 4000–500 cm^{-1} at a resolution of 4 cm^{-1}. Polymer identification was verified by the percentage match score against polymer libraries (ATR-FTIR-library complete, vol. 1-4; Bruker Optics ATR-Polymer Library; IR-Spectra of Polymers, Diamond -ATR, Geranium-AT & IR-Spectra of Additives, Diamond-ATR). Only matches above 60% were selected for positive microplastic validation and polymer identification (Leistenschneider et al., 2021).

2.2.2 French and Spanish samples - Ifremer analyses

2.2.2.1 *Microplastics extraction processing*

For the French samples, 3 replicates were produced for each location. The mussels were defrosted for 2 hours and the intervalvular liquid was kept, the byssus was removed and the soft tissue was extracted from the shell. Tissue wet mass (w.w) and number of individuals were recorded. Each replicate was digested with 10% KOH with magnetic stirring and heated at 40°C for 12 hours. After full digestion, the microplastics were recovered by sieving (100 µm mesh sieve) and vacuum-filtered through a GF/A glassfiber filter (Whatman ø 47 mm, 1.6 µm pore).

For Spanish samples (analysed at Ifremer LER-PAC laboratory), mussel digestion was identical to French samples. Once the mussels have been digested with KOH, the solution was decanted, the supernatant kept and the rest (deposit) decanted again. As the French samples had been difficult to analyse due to the organic matter load, an additional step was added for the processing of the Spanish samples, in order to improve the condition of the filters for their analysis. The deposit was supplemented with a 50% potassium iodide (KI) solution to facilitate separation of the MPs by density. Once sedimented, the deposit was discarded and the supernatant added to the previous one. The solution containing all the MPs was then filtered in the same way as the French samples.

2.2.2.2 *Microplastics characterization processing*

For the French and Spanish samples, the filter analyses to characterise the microplastic were examined under an epifluorescence stereo microscope (Zeiss - Discovery. V12). Once the Nile red solution was sprayed on the

filter, fluorescent particles (considered as plastic) were counted (number), categorised (fragments, fibres, foam, pellet, etc) and measured (only particles > 100 µm).

2.2.2.3 *Controls and contamination*

The glassware used was washed in a dishwasher, then rinsed with ethanol and three times with ultrapure milliQ water (water filtered through a 22 µm filter). During the digestion process, the samples were covered with suitable aluminium caps. In addition, 100% cotton lab coats and nitrile gloves were worn by the operators, and analysis (digestion and extraction) were carried out under a clean chemical hood.

Three blanks were done throughout the sample treatment process in the laboratory. After fluorescence analysis, the contamination of the blanks was removed from the results.

2.2.2.4 *Polymers processing*

For the French samples, polymer analysis of the particles was carried out at Ifremer's Détection, Capteurs et Mesures laboratory (LDCM) with an AlphaR 300 Raman micro-spectrometer (µRaman).

Due to the state of the filters (significant soiling by organic matter despite the digestion steps) and the analysis time required by the µRaman, only the first replicate from each station was analysed. On each filter, particles were selected in random mode in order to be able to characterize a portion of the polymers ingested by the mussels.

For the Spanish samples, no polymer analysis could be completed during the project period.

2.2.2.5 *Data normalization*

Microplastic data were normalized according to the mass of soft tissue extracted *i.e* number of microplastics per weight analysed (item.weight^{-1} (w.w)) and the number of microplastic per individual (item.ind^{-1}).

2.2.2.6 *Correlation test*

In order to determine the existence of a relationship between the variables "number of individuals" and "weight" to characterize the impact of contamination, a correlation test (Pearson) is used, where n represents the number of individuals in the sample, R the correlation coefficient and p the significance threshold of the test.

2.2.3 Comparisons between France, Spain and the UK

It was assumed that the differences in methods did not affect the results. Statistical analysis was completed at Cefas. A bootstrap analysis was used to create 95% confidence intervals. A non-parametric Kruskal-Wallis ANOVA was used to compare microplastic contamination in mussels between the three countries.

3 Results

3.1 BRITISH SAMPLES - CEFAS ANALYSES

3.1.1 Controls and contamination

Negative controls were contaminated with an average 1.48±1.66 items sample^{-1}. These were primarily white and clear fibres.

3.1.2 Material collected

Seven sites were selected from mussels collected in November 2022. For each 25 mussels were investigated. A total of 175 mussels were analysed, ranging 39.66-69.43 mm in length (56.4±5.9 mm, mean ± SD). The wet weight of the mussel tissues was between 1.24 g and 12.89 g (4.3±1.9 g).

3.1.3 Visual identification and counting

Fluorescent items were recovered from 13% of mussels. A total of 49 items were recovered, with between 0 and 14 items recovered per mussel (0–2.73 items.g^{-1}). Particles were primarily white/clear (n=45), with fragments (n=25) and fibres (n=17) being the most common shape (Figure 2). Pink/red/purple, black, brown and orange items were also recovered, as were films and a microbead. Items were on average 527±693 µm long (26.7–3,051.1 µm) and 68±95 µm wide (11.9–414.5 µm) (Figure 3). Two mesoplastic items were found (>5mm). These were removed from analysis as they were not within the scope of the present study. Some particles were not measured as they could not be imaged.

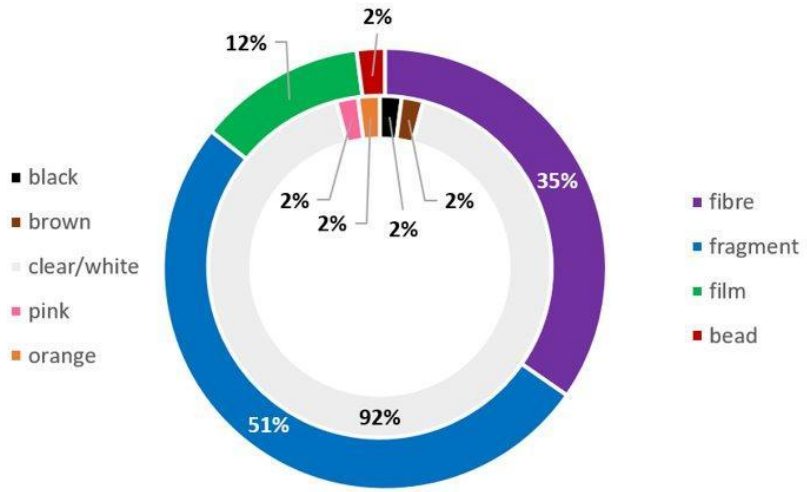

Figure 2 : The colour and shape of recovered, visually-identified particles from UK mussels.

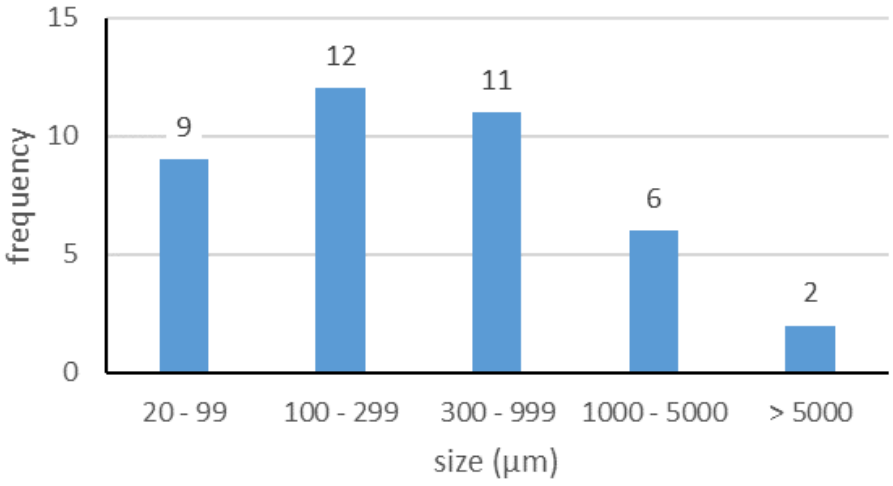

Figure 3 : The size of particles recovered from UK mussels (n=40).

3.1.4 Polymer identification with µFTIR

Of the visually-identified particles 31% of visually-identified particles (n=15) were analysed by µFTIR. Of these, 13% (n=2) could not be identified and were assumed to be natural. A further 7% (n=1) was identified as natural. The remaining 12 items were confirmed as microlitter items (Figure 4). Of these, 75% (n=9) were plastic, with acrylic (n=2), PES (n=2) and PP (n=2) most commonly identified. Rayon was the most abundant semi-synthetic/cellulosic material (n=2).

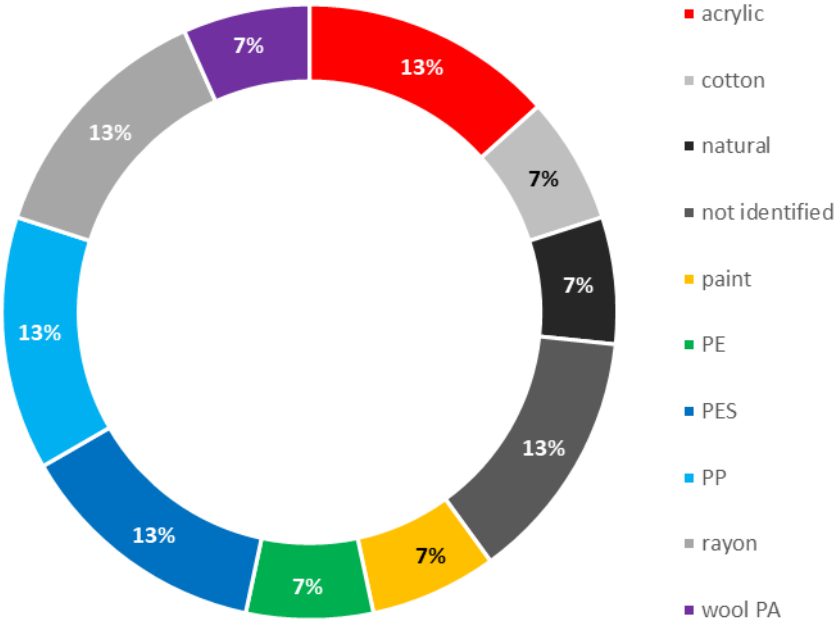

Figure 4 : A subset (n=15) of fluorescent particles picked from UK mussels were analysed by FTIR. The majority were plastic with some cellulosic and natural items.

3.1.5 *Microlitter abundance in mussels around the British coast*

The average number particles ingested per individual mussel in the UK was 0.269±1.228 (see §3.4 - Table 3). Abundance, however, varied between sites (Figure 5). Fishcombe (station 3221) was the only site where no mussels ingested microplastics. The greatest mean ingestion of litter was at the East Menai Strait (station 3222), followed by Foulney (station 3077). After Fishcombe, the West Menai Strait, had the lowest observed microlitter ingestion.

Figure 5 : Mean number of microlitter per individual in the UK.

3.2 FRENCH SAMPLES - IFREMER ANALYSES

3.2.1 *Results from MPs characterization by fluorescence stereomicroscope method*

The MPs characterization in mussels along French coast was carried out by two methods:

- Nile red analyse method carried out for all samples and replicates for particles larger than 100 µm,
- Raman spectroscopy analyse method only for replicate 1 of all the stations, for particle sizes between 50 and 200 µm, and on a quarter of a filter which represents on average the 25 % of the total number of particles detected.

Concerning the contamination of mussels in France (average of all stations combined), MP densities (in items.g^{-1} w.w) for all stations combined, ranged from 0 items.g^{-1}w.w to 0.310 items.g^{-1}w.w, with a mean density of 0.092 ± 0.106 items.g^{-1}w.w and a median of 0.045 items.g^{-1} w.w (Table 1). MP densities (per individual) for all stations combined, ranged from 0 items.ind^{-1} to 1.380 items.ind^{-1}, with a mean density of 0.243 ± 0.352 items.ind^{-1} and a median of 0.110 items.ind^{-1} (Table 1).

Table 1 : items.g^{-1} (ww) and items.ind^{-1} density statistics for all French stations combined

	MP concentration (items.g^{-1}(w.w))	MP concentration (items.ind^{-1})
Min	0	0
Max	0.310	1.380
Mean	0.092	0.243
Median	0.045	0.110
Standard deviation	0.106	0.352

Concerning the contamination of mussels by station (items.g^{-1}), four stations had no microplastic : Galon d'Or, Pointe aux Oies, Anglet (Pointe de Bayonne) and Baie d'Arguenon (Figure 6).

The lowest microplastics contamination was at Large de Boyard station and Pointe de St Quentin with respectively 0.01 items.ind^{-1}, followed by Roche du Port (0.03 items.ind^{-1}), Houat (0.04 items.ind^{-1}), Plage de St Enogat (0.05 items.ind^{-1}) and Verville (0.08 items.ind^{-1}). The highest microplastics contaminations were at Le Passage station (0.31 items.g^{-1}w.w), followed by Chausey station (0.30 items.g^{-1}w.w).

Regarding contamination by individuals (in items.ind^{-1}), the results are correlated with the results of concentration by weight (positive correlation, R = 0.77, p<0.001, n=16). So, no contamination in the mussels was observed at the Galon d'Or, Pointe aux Oies, Anglet and Baie d'Arguenon stations (respectively 0 items.ind^{-1}) (Figure 7). The minima and the maxima are also observed on the same stations with the lowest concentrations at Large de Boyard station with 0.03 items.ind^{-1} and St Quentin station (0.04 items.ind^{-1}). The highest concentrations were also located at Le Passage station (1.38 items.ind^{-1}) followed by Chausey station (0.15 items.ind^{-1}), even if the concentration per individuals is higher for Le Passage than at Chausey, while their contamination value by weight was very close, explained by the different number and the size of individuals in the analysis (with 41 and 4 individuals for Le Passage and Chausey, respectively).

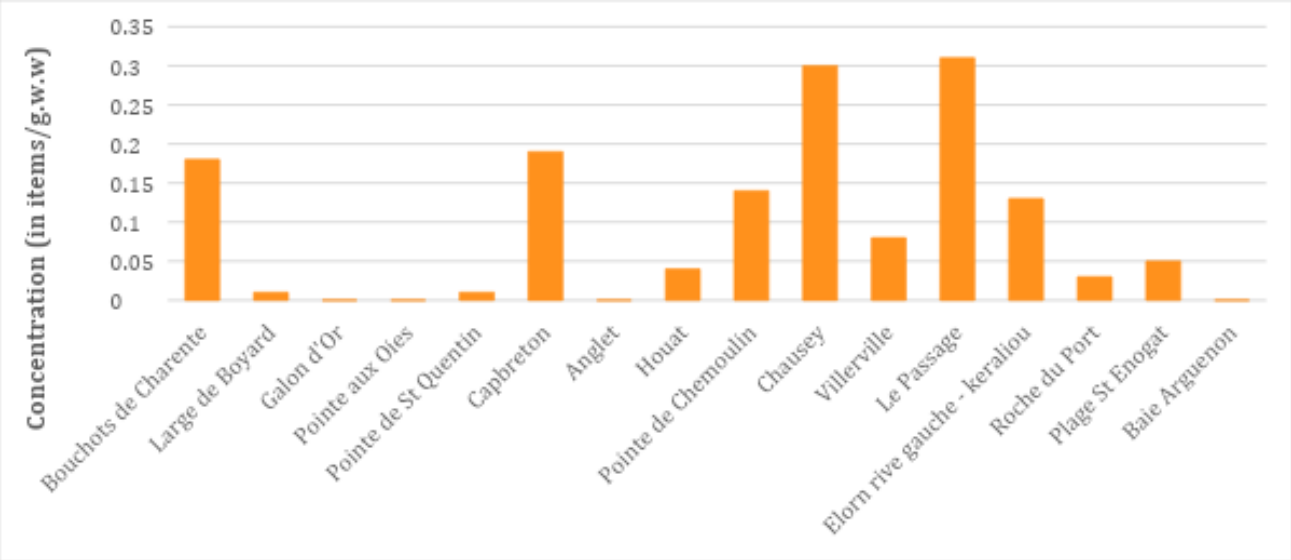

Figure 6 : Microplastics concentration in mussel in items.g^{-1}w.w for each French station (corresponding to the mean from a pool of 3 replicates)

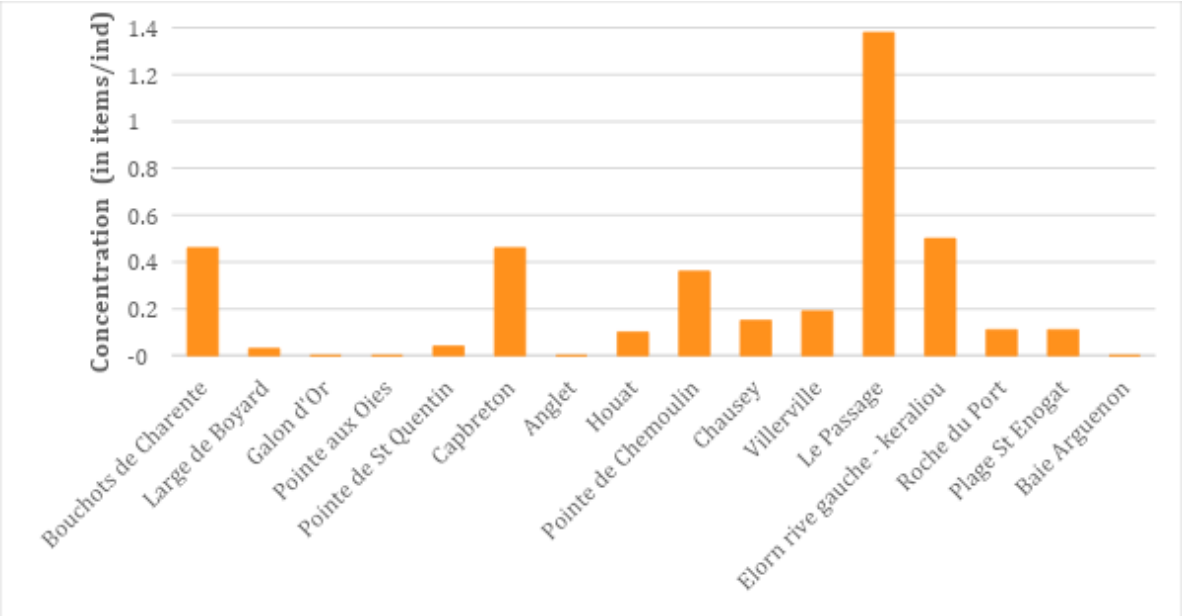

Figure 7 : Microplastics concentration in mussel in items.ind^{-1} for each French station (corresponding to the mean from a pool of 3 replicates)

Figure 8 : Mean number of microplastics per individual (items.ind^{-1}) at French sample sites

As far as MPs typologies are concerned, only fragment typology was found in all stations by the Nile Red identification method. The number of fragments ranged from 0 to 6 items, with an average of 0.320 ± 1.105 fragments per station. Chausey and Le Passage stations presented the highest fragment numbers (Figure 9) with 6 fragments counted for each sample pool, giving contamination concentration of 3.35 and 3.25 fragments.g^{-1} w.w.

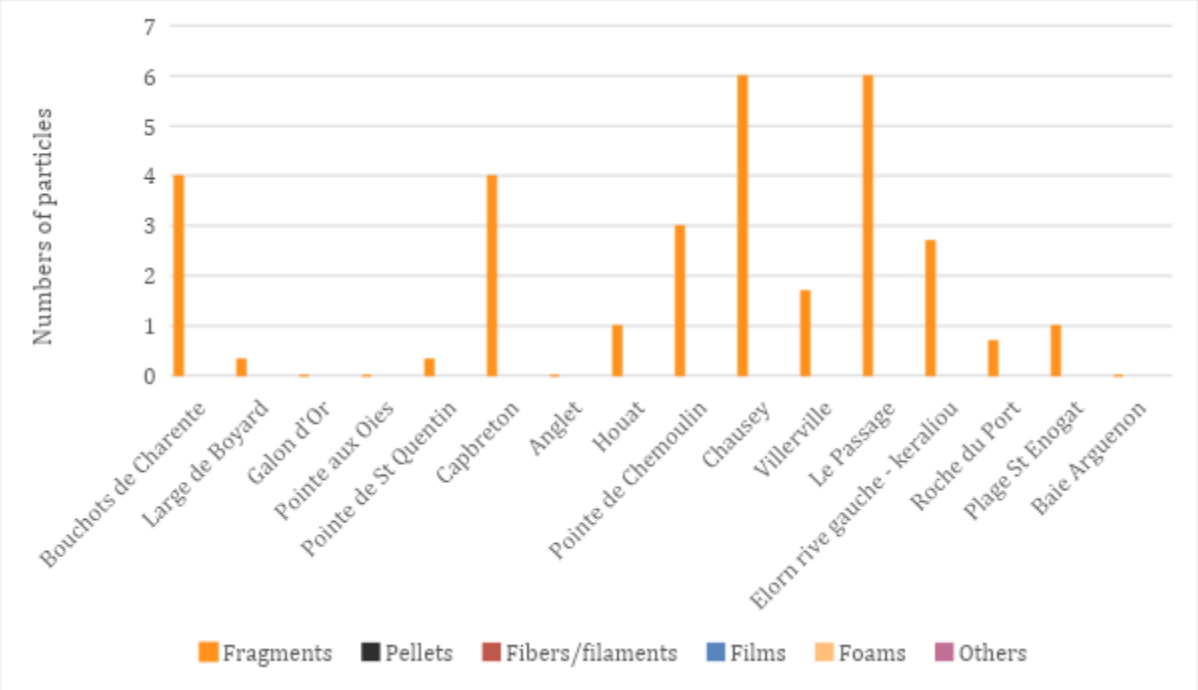

Figure 9 : Microplastic typologies for each French station

3.2.2 Results from MPs characterization by µRaman method

Due to the state of the filters (significant soiling by organic matter despite the digestion steps) and the analysis time required by the µRaman, only the first replicate from each station was analysed. On each filter, particles were selected in random mode in order to be able to characterize a portion of the polymers ingested by the mussels. Each filter is analysed, with a selection of around 25% of the number of particles (in this case the results are expressed in item/filter). Then, the concentrations are expressed per station by bringing the analysis result by 25% to 100% and by normalising for the weight of replicate 1 (expressed in items/ g.w.w).

The count of particles between 50 and 200 µm is 34,425 particles on 16 filters with an average of 2,152 ± 996 particles (table annex 2.1). In total out of the 16 filters, 9,106 particles were selected in random mode (random selection which represents on average 25% of the total number of particles detected by size class, table annex 2.1, column "percentage", the recovery rate of particles is between 18 to 66%), of which 8,549 made it possible to obtain an analysable signal: Raman spectra or fluorescence signal (indicated by NI for not identify in the table annex 2.1 to 2.3). It should be noted that the excluded particles induced saturation of the detector under the analysis conditions. This represents an average of 534 ± 273 spectra per filter. For this analysis, particles in the size class between 50 and 200 µm are favoured, which makes it possible to provide a complementary analysis to the results provided by the analysis of particles with Nile Red (size class > 100 um).

Few plastics are observed with this method (Figure 10 and Annex 2), where the average is 3.3 ± 4.8 items. Estimated density values of microplastic contamination in mussels range from 0 to 0.74 items.g^{-1}w.w. The highest value of estimated density is encountered for Villerville (0.74 items.g^{-1}(w.w)), Chausey (0.48 items.g^{-1}w.w), and Anglet (0.42 items.g^{-1}w.w) stations. Many stations didn't have MPs: Large Boyard, Pointes aux Oies, Pointe de Saint Quentin, Pointe de Chemoulin, Keraliou, Roche de Port and Baie Arguenon. However, it is important to remember that these results correspond to an estimated density made from only four filters analysed for only replicate (n=1). Some stations do not display any plastic particles from the results obtained

by Raman analyses but display contamination by MPs from the results obtained by fluorescence characterization.

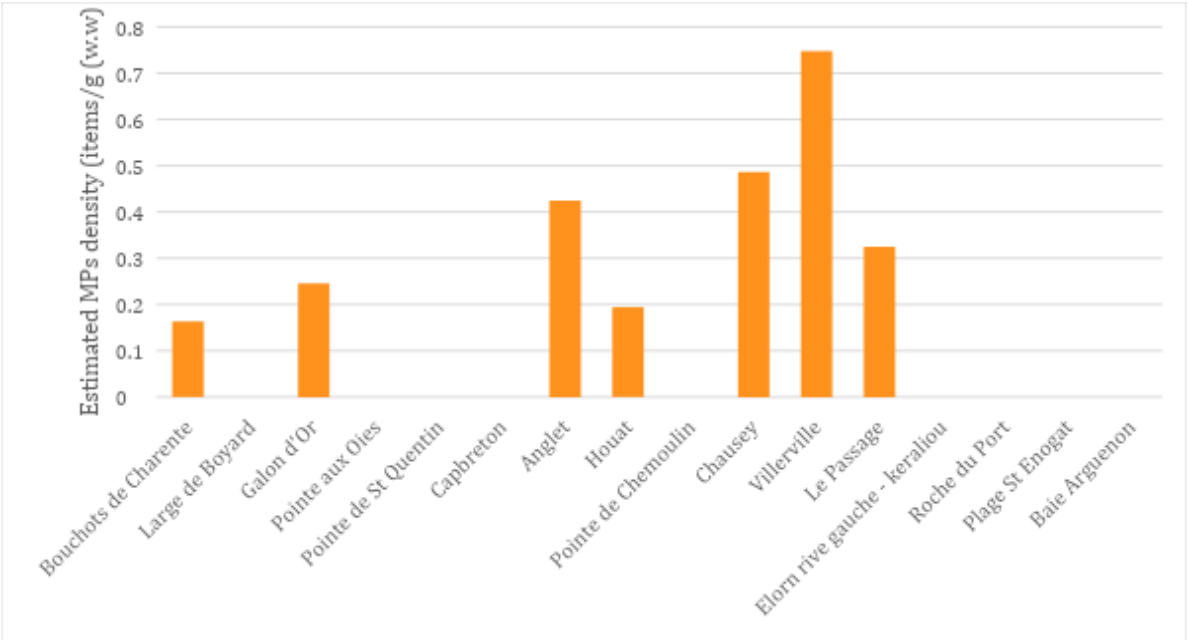

Figure 10 : Microplastics estimated concentrations in mussel in items.g^{-1} (w.w) for each French station

The µRaman analyses showed that the 15 plastic particles found displayed a wide diversity of polymers: PDMS, at 2 %, PE, at 4 %, PAN, at 13 %, PES at 2 %, PP, at 2 %, PS, at 4 %, PU, at 2 %. For 37 particles, only a pigment of synthetic origin could be identified (Figure 11 and annex 2). The pigment category corresponds to particles of anthropogenic origin whose polymer matrix couldn't be identified with certainty. The wide variety of polymers identified is consistent with the size range. Indeed, it has already been observed that as the size range decreases, the variability of the chemical nature of the polymers increases. The large number of not identify (NI) particles (n = 8,002) can be explained by the very high presence of fluorescence-inducing inorganic and organic particles, or particles that do not meet our analysis conditions.

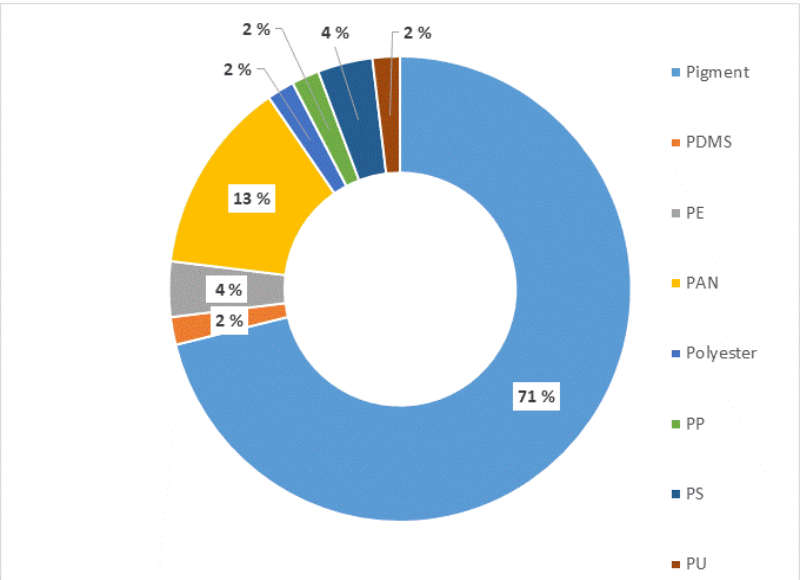

Figure 11 : Percentage of polymer and pigment found for the total French stations from the µRaman analyses (only a few parts of Replicate n°=1 filter has been analysed)

3.2.3 Microlitter abundance in mussels along the French coast

This study showed that the two stations where mussels are most contaminated by MPs are located in the Bretagne region. However, this region also includes one station with no contamination. The other stations with no contaminated mussels are spread across several regions (Nouvelle-Aquitaine, Hauts-de-France). At this stage of the research, it is not yet possible to identify a trend in the variability of contamination. MPs concentrations in mussels probably depend on anthropic pressure in the vicinity of the stations. Currents may play a role too in particle dispersion and should also be considered for a more detailed analysis of contamination.

3.3 SPANISH SAMPLES – IFREMER ANALYSES

Due to the lack of availability of the equipment used for analysis in the IEO and in order to meet the project deadlines, the samples taken by the IEO in Spain were sent to IFREMER for processing and analysis.

3.3.1 Controls and contamination

One negative control was processed along with the batch of samples. It contained 3 fragments (300 µm – 1mm). These were extracted from the results of the samples.

3.3.2 Material collected

Five sites were sampled between November 2022 and January 2023 along the northern coast of Spain (see Figure 13), from west to east: Vigo, Muros, Ferrol, Pravia and Ribadesella. At each site a minimum of 16 and a maximum of 24 mussels were taken (depending on availability). At the laboratory, the mussels were divided into 3 replicates per site with an average of 7 mussels each (and 20 g total weight).

3.3.3 MP characterization by fluorescence stereomicroscope method

The analysis of the particles in the mussels from Spanish sites were carried out using Nile red dye and visual identification under fluorescence stereomicroscope, following the same method as explained for the French samples.

Considering the 15 replicates analyzed (*i.e*. 3 replicates x 5 sites) and after the correction with the negative control, particles were detected in 12 of them. These 12 replicates contained a total of 95 items, being 94 fragments between 300 µm and 1 mm. The last item was classified as “other”; thus, it did not correspond to any of the other categories considered (fibers, fragments, films, pellets or foams).

The average amount of particles found was 0.337 items.g^{-1} w.w. and 0.881 items.ind^{-1} (see table 2 in section 3.4), however differences were found among sites. The results obtained per site are shown in number of items per individual (Figure 13) and in number of items per gram (Table 2 and Figure 14).

Figure 13 : Mean microplastic contamination per individual for Spanish sample sites (±SD).

The sites with the lowest concentration of particles (per gram w.w.) were Vigo and Ferrol, with no statistically significant differences between them (Mann Whitney U test, $p \geq 0.82$). These values were significantly lower than the concentrations found in Pravia and Ribadesella (Mann Whitney U test, $p \leq 0.05$), being Pravia the location with the highest concentration of the two (Mann Whitney U test, $p = 0.05$). Due to the high variability between replicates, no differences were found between Muros and the rest of the sites (Mann Whitney U test, $p \geq 0.12$).

Table 2 : Concentration of particles (nº of items per gram wet weight) in the 5 sampling sites selected in Spain.

Sites	Mean	SD	Median	Maximum	Minimum
Vigo	0.05	0.08	0.00	0.13	0.00
Muros	0.42	0.47	0.24	0.95	0.05
Ferrol	0.06	0.06	0.04	0.13	0.00
Pravia	0.74	0.21	0.74	0.95	0.53
Ribadesella	0.29	0.04	0.29	0.34	0.25

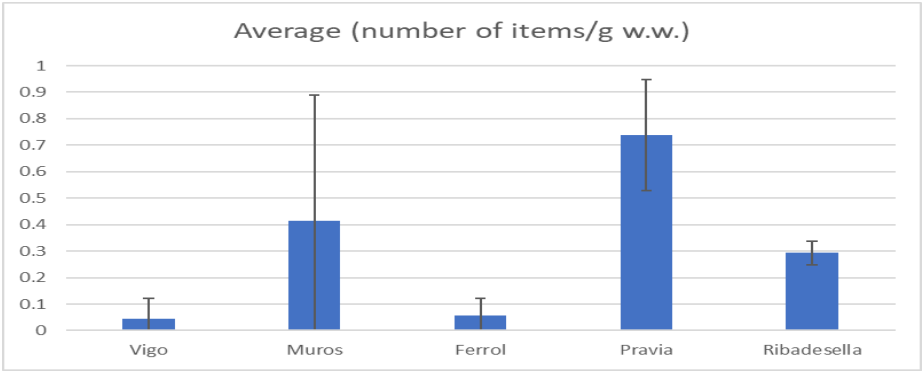

Figure 14 : Average particles concentration (number of items per gram in wet weight) in the Spanish sample sites (error bars represent the standard deviation).

The results found in this study are in line with the levels of contamination published by Reguera et al. (2019), who recorded concentrations of 1.59±1.28 MPs/g w.w. in the area of Vigo and of 2.55±2.80 MPs/g w.w. in the Cantabrian Sea (covering the area of Ferrol, Pravia and Ribadesella). Differently from that publication, in the present study the 99% of the particles detected were fragments, however Reguera et al. (2019) registered 33% and 30% of fragments in Vigo and the Cantabrian Sea respectively, being the rest of the particles classified as pellets, fibres, films and foams.

3.4 COMPARISONS BETWEEN COUNTRIES

The mean amount of microlitter for each country was calculated and 95% confidence intervals were determined with bootstrap analysis (Table 3). The Kruskal-Wallis ANOVA produced a p value of 0.12 for per individual comparisons. The individual p scores per country comparison were as follows: UK vs France (p=0.48); UK vs Spain (p=0.15); France vs Spain (p=0.062). Whilst no statistically significant difference was found between the countries, Figure 15 illustrates that Spanish mussels were generally more contaminated than French and British mussels, though some stations do show comparable levels. More stations or future years of data are needed to improve comparisons.

Table 3 : Summary of comparisons between countries with 95% confidence intervals from bootstrap analysis. Where ML = MicroLitter

	Number of stations	Mean ML per gram per station (95% CI)	Mean ML per gram per individual (95% CI)
UK	7	0.063 (0.030, 0,101)	0.269 (0.104, 0.463)
France	16	0.094 (0.048, 0.142)	0.258 (0.107, 0.458)
Spain	5	0.337 (0.121, 0.556)	0.881 (0.332, 1.429)

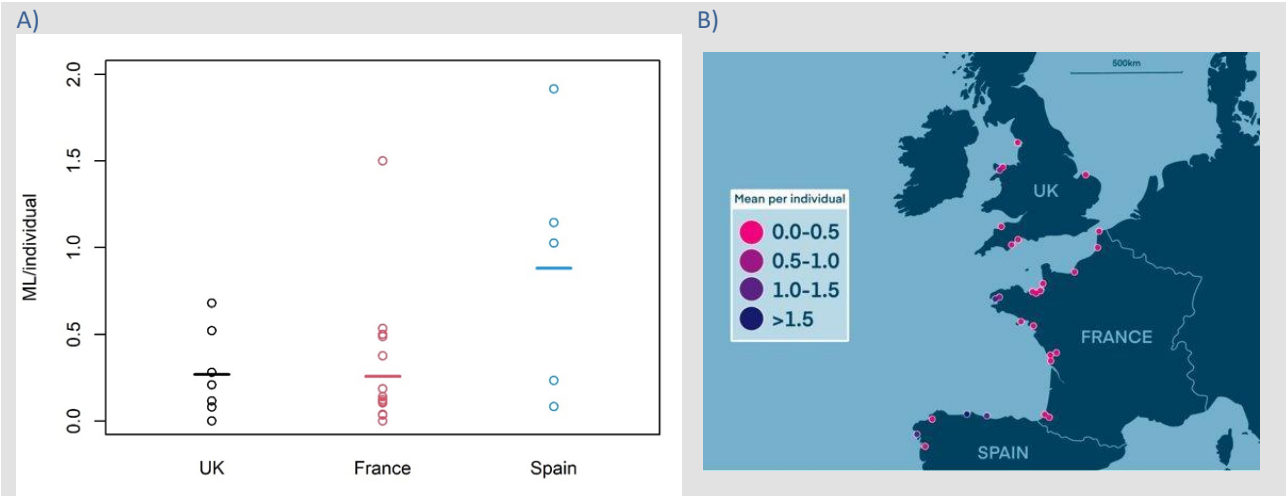

Figure 15 : Station means of microlitter per individual for each country, A) as a plot with overall mean (horizontal line), B) as a geographical representation where pink represents a low mean and purple represents a higher level of microlitter.

4 Discussion

Applying harmonized approaches utilising the low-cost screening method with Nile red demonstrated that mussels are ingesting microplastics, with particles detected as small as 26.7 µm. Mussels were found to ingest microplastics in all three countries, confirming that microplastics are ubiquitous in the North Atlantic. The location of the selected sampling sites falls within three OSPAR monitoring zones (Greater North Sea, Celtic Seas, Bay of Biscay and Iberian Coast) allowing for regional comparisons with seafloor litter abundance, macro- and mesoplastic ingestion and soon seafloor microplastic abundance (in development). The wide distribution of *Mytilus edulis* and other mussel species is one reason that this group make ideal bioindicators for microlitter and microplastics monitoring (Table 4). The results of the present analysis find that there is a statistical significance in microplastic abundance between the three countries and was able to determine potential hotspots. Whilst further years of data are needed to draw stronger conclusions, the preliminary dataset supports the use of *Mytilus edulis* as a regional bioindicator for marine microlitter.

Table 4 : Mytilus sp. make good bioindicator species as they meet several of the necessary criteria (marked with ×). [1] Ward and Kach (2009), [2] Catarino et al., (2017), [3] Brett and Grooves (1979).

Criteria for good bioindicators	Mussels (*Mytilus* sp.)
Wide geographical range	×
Representative of specific monitoring area	×
Species are not protected or endangered	×
Suitable particle retention time	72 hours [1, 2]
Already used as a bioindicator	×
Ability to ingest small and large particles	<1 mm [3]
Sedentary/can be stored in cages	×
Invertebrate (less training for handling)	×
Can be sampled cost effectively	×
Commercially important	×
Can be analysed with rapid Nile red screening	×

Globally, various studies have investigated microplastic ingestion in mussels. Though few studies on wild mussel have been conducted in Europe: UK (Scott *et al.*, 2019; Li et al., 2018; Catarino *et al.*, 2018; Courtene-Jones *et al.*, 2017), France (Hermabessiere *et al.*, 2019; Phuong *et al.*, 2018a, b), Spain (Reguera *et al.*, 2019). In the UK, the results of the present study are low with all previous studies reporting means greater than one particle per individual, and Scott *et al.* (2019) reporting means of between 1.43 and 7.64 items per individual. It is expected that including non-fluorescent litter items would increase the amount of litter recorded in these samples, making the results more similar.

The Spanish results presented in this report are similar to previously published estimates by Reguera *et al* (2019) who reported averaged values of 2.07 ± 2.21 MPs.g^{-1} w.w in mussels collected from the same area (Ría de Vigo and the Spanish Cantabrian coast).

For the French results, the Raman analysis and the analysis by fluorescence microscope are complementary, as they did not analyse the same size classes. French samples were more in line with previous estimates of microplastic contamination in mussels, with reported means of between 0.23 (Phuong et al., 2018b) and 0.76 (Hermabessiere *et al.*, 2019). On a wider geographical scale, all samples in the present report fall within the range of previous estimates in molluscs, which ranged from 0–10.5 MPs.g^{-1} (Danopoulus *et al.* 2020) and 0.04–4.0 MPs.g^{-1} (Ding *et al.*, 2022). This latter review also noted that wild and farmed bivalves are exposed to different concentrations of microplastics. Indeed, a review by Vandermeersch *et al.* (2015) found that some farmed mussels contained more MPs than wild individuals from a similar area. For monitoring purposes, we propose the use of wild mussels only. This work enables us to make a number of recommendations for future studies on the same theme.

Whilst contamination per individual may appear more meaningful in terms of risk to biota, human exposure and use in ecotoxicological assessments, it is imperative to also express the results in items per gram of flesh, which better relates the level of contamination of the organisms. This is illustrated by the examples of the French sites Chausey and Le Passage, both of which show a high level of contamination when the results are expressed in items.g^{-1} w.w., whereas Chausey's contamination is not as high when the results are expressed in items.ind^{-1} (due to the number of mussels used for the analysis: 41 for Chausey versus 4 for Le Passage, in order to obtain around 20 g of flesh w.w.). Despite recommendations in the bibliography to use a standard mussel size for analysis, it is not always easy to obtain individuals of identical calibrated size, depending on the marine region and therefore the richness of the waters, particularly when the spatial scale extends over several European sites. Indeed, mussels of a standard size (shell length) does not equate to an equal mass. However, the expression of results in items.g^{-1} w.w. appears to be perfectly coherent within the framework of a monitoring network for MP contamination in mussels, whether on a national or European scale.

25 individuals analysed per station

As recommended for small pelagic fishes as a monitoring tool for MPs, 25 individuals per sample site were analysed (Bakir et al., 2020). This number of individuals was suitable to find consistent contamination at a regional North Atlantic scale. It should be noted, however, that variation of ingestion in biota is often high and sometimes higher sample sizes are recommended. Ding et al. (2022) proposed 50 individuals as the minimum sample size for assessing MP contamination in bivalve molluscs, finding that studies using fewer individuals often reported more plastic. Given the concentrations per gram reported here were well within the range of previously published studies and were often lower, this is unlikely the case here. Furthermore, given the time, cost and impact of removing additional individuals, it is advised that more years of data be collected with 25 individuals per site to determine its suitability.

Mussels should be externally rinsed

Kolandhasamy *et al.* (2018) reported that 50% of microplastics reportedly ingested by mussels in the literature were in fact present on the external tissue surface. By rinsing the tissue before extracting microplastics, reported concentrations were halved. Indeed, the UK mussels in the present study were rinsed prior to analysis to remove any particles adhered to the surface of the tissue. This practice is not implemented in many studies and thus reports of ingestion are often overestimated. Perhaps explaining the lower recorded ingestion from British samples in the present study. Results not presented here, as items were mainly non-fluorescent, demonstrate that by rinsing the British mussels before extraction significant numbers of items are removed. This is an essential step in all future microplastic studies on molluscs or indeed any species that is digested whole.

Nile red is an effective screening tool

A growing number of MP studies are using Nile red to detect particles in biota (Bakir, *et al.*, 2020; Nalbone *et al.*, 2021; Battistin *et al.,* 2023; Imasha and Babel, 2023), demonstrating its effective use as an affordable and fast screening tool for microplastics. It is recommended that Nile red be implemented in future studies to increase the recovery of otherwise difficult to visually detect particles, such as small white fragments. It must however be coupled with spectroscopy to determine the polymers present in samples.

5 Conclusion

MPs are ubiquitous in the North Atlantic and setting up systems to monitor this contamination appears to be a necessity. However, as the concentration of floating microplastics is very dependent on currents, the monitoring of this indicator in water samples is not optimal for translating one single measure to averaged contamination levels. Monitoring microplastic contamination in sediment and biota would appear to provide more stable indicators and allows for the development of risk assessments based on the bioavailable fraction of MPs. The MFSD, and the regional OSPAR convention, have developed expert groups to work on these issues. The Interreg CleanAtlantic project has made it possible to research the potential mussels as an indicator of MPs. In this work, it is proposed that this indicator is expressed, at a minimum, as number of MPs per gram (wet weight) to control for the variation in sizes of individuals, with contamination per individual being secondarily presented. The high variability of the data avoided any difference between the locations sampled in the three countries, showing averaged concentrations ranging from 0.063 to 0.337 $MP.g^{-1}$ w.w. (UK and Spain respectively).

The use of mussels as indicators of MPs contamination along the European Atlantic coastline appears, in this study, to be consistent. However, the work needs to be confirmed with a larger dataset. This study has been proposed as part of the new Interreg Atlantic project called FREE-LitterAT and will be considered at regional and national levels, e.g., OSPAR.

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Annex 1: Details of mussel sampling stations for sites

Figure 16 : Details of mussel sampling stations for sites located in UK

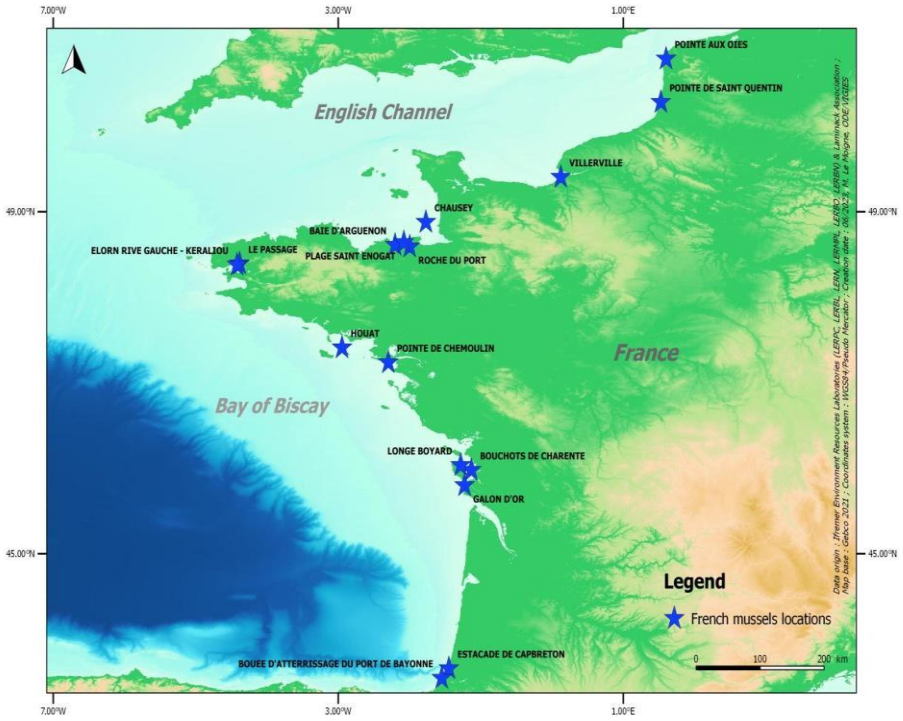

Figure 17 : Details of mussel sampling stations for sites located in France

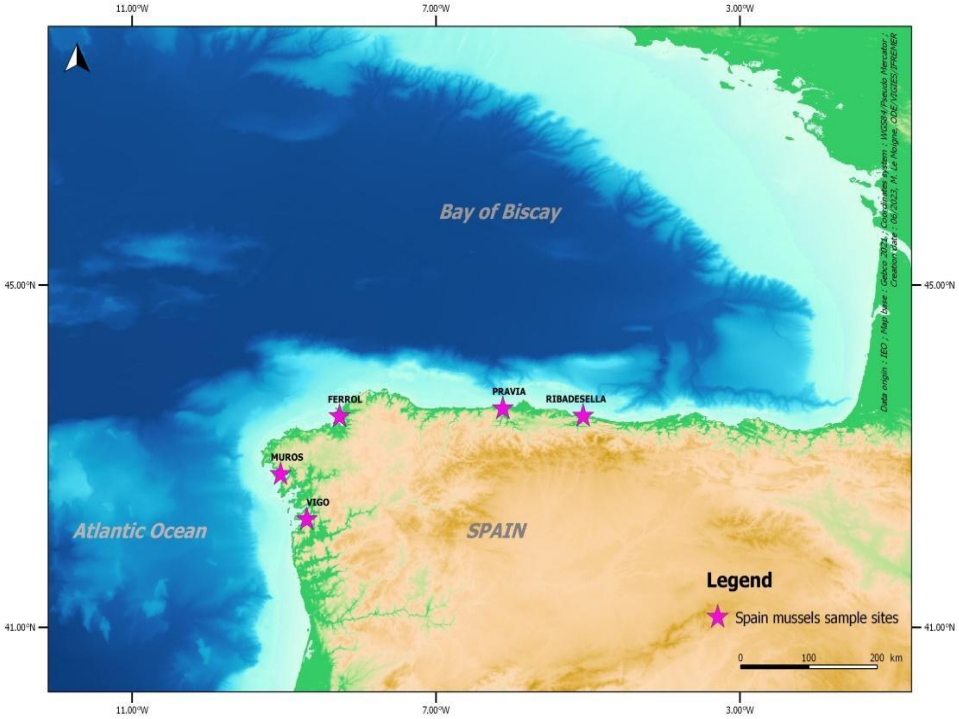

Figure 18 : Details of mussel sampling stations for sites located in Spain

Annex 2: Results from MPs characterization by µRaman method

Table annex 2.1 : synthesis of the number of MPs identified by the Nils Red technique, the number of particles counted via the ParticleScout software (PaSc), the number of particles analyzed having given a spectrum as well as the associated percentage, the number of MPs identified and estimated.

						MPs numbers		Anthropogenic particles	
Stations	**Replicat**	**Number of MPs found by Nile Red analyzes**	**Number of particles (50-200 µm) by ParticleSouct (PaSc) identification software**	**Number of particles by ParticleSouct (PaSc) RAMAN identification**	**Percentage**	**MPs Found**	**MPs estimated**	**Polymers and pigments**	**Anthropogenic particles found**
Bouchots de Charente	R1	0	586	137	23.4	1	4	4	17
Large de Boyard	R1	0	862	217	25.2	0	0	1	4
Galon d'Or	R1	0	3310	672	20.3	1	5	15	74
Pointe aux Oies	R1	0	1962	458	23.3	0	0	6	26
Pointe de St Quentin	R1	1	1479	373	25.2	0	0	2	8
Capbreton	R1	0	2879	692	24	0	0	5	21
Anglet	R1	0	3935	894	22.7	2	9	4	18
Houat	R1	1	1769	437	24.7	1	4	5	20
Pointe de Chemoulin	R1	0	2357	587	24.9	0	0	1	4
Chausey	R1	0	2368	542	22.9	2	9	1	4
Villerville	R1	0	4125	1011	24.5	4	16	1	4
Le Passage	R1	12	1597	1069	66.9	4	6	2	3
Elorn rive gauche - keraliou	R1	3	1835	416	22.7	0	0	2	9
Roche du Port	R1	0	2064	375	18.2	0	0	1	6
Plage St Enogat	R1	0	1355	260	19.2	0	0	1	5
Baie Arguenon	R1	0	1942	409	21.1	0	0	1	5
Total		17	34425	8549		15	53	52	228
Moyenne		1.1	2151.6	534.3	25.6	0.9	3.3	3.3	14.3
STD		3.0	995.9	273.2	11.2	1.4	4.7	3.6	17.6

Table annex 2.2: Summary table of the number of MPs particles identified for replicate number 1 by station, for the particles size included between 50 and 200 µm, and on a quarter of a filter (column 1) which represent on average at 25 % of total number of detected particles (allowing us to deduce the number of particles estimated for the sample, column 2) and calculate the MPs concentrations for the sample (column 4).

	MPs number			MPs concentration
Stations	Number of MPs found by RAMAN analyzes	Estimated number of MPs for replicat n°=1	Wet weight of mold fabrics for replicat n°= 1	Estimated density (items/g (w.w) for the replicat n°= 1
Bouchots de Charente	1	4	24.5	0.163265306
Large de Boyard	0	0	28.3	0
Galon d'Or	1	5	20.4	0.245098039
Pointe aux Oies	0	0	24.4	0
Pointe de St Quentin	0	0	26.9	0
Capbreton	0	0	20.6	0
Anglet	2	9	21.2	0.424528302
Houat	1	4	20.6	0.194174757
Pointe de Chemoulin	0	0	20.8	0
Chausey	2	9	18.5	0.486486486
Villerville	4	16	21.4	0.747663551
Le Passage	4	6	18.5	0.324324324
Elorn rive gauche - keraliou	0	0	20.1	0
Roche du Port	0	0	20.1	0
Plage St Enogat	0	0	22	0
Baie Arguenon	0	0	21.1	0
Total	15	53		2.585540767
Mean	0.9375	3.3125		0.161596298
Standard deviation	1.39	4.73		0.23

Table annex 2.3: Summary of the different identifications of the chemical nature of particles on replicate samples R1

Stations	Pigment	PDMS	PE	PAN	Polyester	PP	PS	PU	Total
Bouchots de Charente	1						1		2
Large de Boyard	1								1
Galon d'Or	1			1					2
Pointe aux Oies	1								1
Pointe de St Quentin	1								1
Capbreton	1								1
Anglet	2			2					4
Houat	4					1			5
Pointe de Chemoulin	2								2
Chausey	2	1			1				4
Villerville	1		2	1			1		5
Le Passage	11			3				1	15
Elorn rive gauche - keraliou	1								1
Roche du Port	6								6
Plage St Enogat	1								1
Baie Arguenon	1								1
Total	37	1	2	7	1	1	2	1	

Table annex 2.4: Summary of the different identifications of the chemical nature of the fibres on samples from replicate 1

Site	Polymer and synthetic pigment			Natural materials			Filter and NA		Total
	Polyester	PET+Pigment	Pigment	Cellulosic derivates	calcite	Mineral	Filter (DVPP)	NA	
Anglet			1	10	1			3	15
Baie-Arguenon		1	2	2				5	10
Bouchot-Charente			1	4			1	4	10
Capbreton			1	17		1		1	20
HOUAT				1				2	3
Keraliou			1	5				1	7
Large-de-Boyard	3		3	5	1			8	20
Plage-StEnogat			1	6				3	10
Pointe-Chemoulin		1		12	3	1		3	20
Pointes-aux-oies	1	1		2		1		5	10
Pointe-StQuentin				2	1			4	7
Roche-du-Puit		2	1	1	1			3	8
Villerville			3	8	2			2	15
Total	4	5	14	75	9	3	1	44	155