ECsafeSEAFOOD

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1. Summary

Microplastics are present throughout the marine environment and ingestion of these plastic particles (<1 mm) has been demonstrated in a laboratory setting for a wide array of marine organisms. Here, we investigated the presence of microplastics in biota and sediment originating from four European hot-spot locations: the Po estuary (Italy), Tagus estuary (Portugal), Ebro estuary (Spain) and Western Scheldt (Netherlands).

Microplastic body burden in biota was assessed using the traditional tissue digestion technique, as a preliminary exploration of micro-CT for the assessment of microplastics in tissues turned out to be unsuccessful for field organisms. Microplastic concentrations in *Mytilus edulis/Mytilus galloprovincialis* ranged from 0.08 ± 0.09 particles per gram of tissue (ww) (Tagus estuary) to 0.16 ± 0.12 particles.g^{-1} (ww) (Po estuary). The fillet of European sprat (*Sprattus sprattus),* collected in the Western Scheldt, contained no microplastics. This discrepancy between the two species investigated is mainly be attributed to differences in the type of tissue that was analysed. Mussels were analysed as a whole, meaning the digestive tract (containing ingested microplastics) was included in the analysis. For sprat analysis, only fillets were included, implying that only translocated microplastics (i.e. microplastics that are transported from the digestive tract to the tissues and circulatory system) were assessed. Also, during their lifetime, both species will face a different exposure to microplastics. Mussels display an extensive filter function, filtering very large volumes of seawater per day. As a result, they are directly exposed to the microplastics present in the seawater. Sprat on the other hand, are strict planktivores, and as a result will only be exposed to microplastics indirectly, through the consumption of microplastic containing prey. As these fish are exposed to far lower concentrations of microplastics, the chances for translocation to the tissues (here fillet) are greatly reduced.

Sediment concentrations between and within hot-spot locations showed high variability, a fact that is not new to microplastic research. Portugal showed the lowest levels of contamination, with concentrations ranging from 37 to 60 particles per kg of dry sediment. The Po and Ebro estuary showed higher levels of contamination with maximum concentrations reaching 147.6 ± 32.9 particles.kg^{-1} dry and 191.0 ± 13.9 particles.kg^{-1} dry, respectively. From the analyses it appeared that local sediment concentrations can be used as proxy for water column concentrations, as mussels originating from locations with lower microplastic pollution in the sediments showed a smaller body burden, and vice versa.

Animal body burden of plastic-specific chemicals (i.e. additives such as Bisphenol A and flame retardants) and plastic-associated contaminants adsorbed from the surrounding environment were assessed. Mussels and fish tissue contained significant levels of plastic-specific chemicals, but as analysis of the results indicated, the levels of these chemicals are not directly related to the microplastic body burdens of these animals. A trend between microplastics body burden and the levels of the plastic-associated contaminants PAHs was noticeable, as mussels from the Po estuary contained both the highest levels of PAHs in their tissues and the highest microplastic concentration. For the other two locations, no such trend was detected. Since PAHs are widely distributed environmental contaminants, present in both the water and sediment compartments of marine

ecosystems, the exposure of these animals is not only through the ingestion of microplastics, but also through direct exposure, making it very difficult to assess the contribution of microplastics to PAHs body burden.

The research performed in the framework of the ECsafeSEAFOOD project delivers important and new insights in the distribution of microplastics in marine biota, and by extension the human food chain, throughout Europe. The results presented here indicate that human food safety may be compromised by this type of marine pollution.

2. Background

Plastic debris is ubiquitously present in the world's seas and oceans. One aspect of this plastic pollution is the presence of microplastics (<1mm) in the aquatic ecosystem. Microplastics are the products of the degradation of larger plastic items into smaller fragments, or can originate from cosmetics and from synthetic fabrics such as polyester and polyamide (Andrady, 2011; Cole et al., 2011).

Because of their small dimensions, microplastics become available for ingestion to a wide range of marine organisms. Ingestion has already been demonstrated for organisms at the base of the food chain: a large variety of planktonic organisms, such as copepods, euphausiacea (krill) and larval stages of molluscs, decapods and echinoderms (Cole et al., 2013; Hart, 1991; Lee et al., 2013) will take up microplastics while feeding, as well as other invertebrates, such as polychaetes, bivalves, echinoderms and decapods (Graham and Thompson, 2009; Murray and Cowie, 2011; Thompson et al., 2004). The ingestion of microplastics by marine biota may result in a limited food uptake through the blockage of feeding appendages and the alimentary canal (Cole et al., 2013; Murray and Cowie, 2011). Moreover, ingested microplastics have the potential to be taken up by epithelial cells of the intestinal tract (von Moos et al., 2012) and even translocate through the intestine wall to the circulatory system of exposed mussels (Browne et al., 2008). Microplastic ingestion does not only cause physical harm but can act as vectors of additives incorporated during manufacture (e.g. polybrominated diphenyl ethers (PBDE)) and organic pollutants sorbed from the surrounding seawater (e.g. polychlorinated biphenyls (PCBs)) to biota (Teuten et al., 2009).

The increasing scientific evidence that numerous marine (invertebrate) species ingest microplastics is an indication that these microscopic plastic particles are entering the marine food chain. Taking into consideration that the global food supply of seafood, both from capture and aquaculture production, was over 125×10^6 tonnes in 2009 (FAO, 2012), consequences for human food safety need to be considered.

3. Optimization of existing techniques for microplastic quantification

Traditional microscopic techniques, such as light microscopy of histological sections, are commonly confronted with distortions due to intensive specimen preparation and difficulties in reconstructing samples. This invasive technique consequently leads to the loss of key information (Cnudde et al., 2008). These problems can be overcome with the help of CT-scanning, in which X-rays are used to create cross-sections of a sample from which a virtual model can than be recreated. Especially micro-CT provides a very high resolution (up to 1µm for small samples) and hence high accuracy. Additionally, this high spatial resolution can be obtained without destruction of the sample and without invasive sample preparation. Here, we explored the use of micro-CT as a novel technique in microplastic research.

Since X-rays interact with electrons in the sample, electron-dense structures can be visualized in a

CT-image as a contrast pattern against the background. However, biological soft tissue, as well as plastic, is composed of low-atomic-number elements (such as carbon, oxygen and hydrogen) which produce very little contrast. Therefore, both soft tissue as well as microplastics had to be labelled/treated to ensure an effective visualization of the soft tissues and microplastics. The model microplastic used in this study was a polyethylene powder composed of spherically shaped particles ranging from 10 to 20 µm in diameter (BSPMS-1.5, Cospheric LLC). These microspheres were embedded with barium sulphate, which acted as a radiocontrast agent, and as a result the particles were denser and hence better visible on the scan (Figure 1).

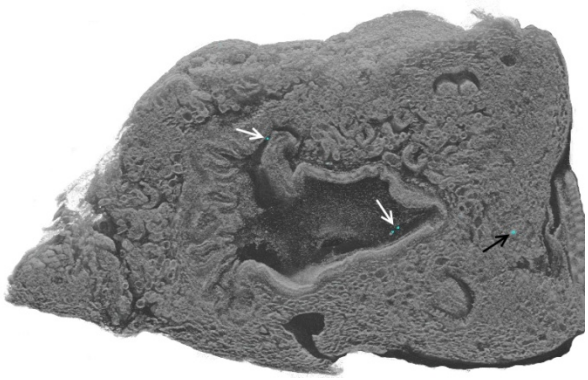

Figure 1: 3D-rendering of the digestive gland showing a barium sulphate embedded microsphere within the tissue of the digestive gland (black arrow), and two microspheres in the lumen of the stomach (white arrows). Micro-CT images were made by UGCT.

It was decided to limit the sample for micro-CT imaging to the digestive gland (instead of the entire mussel) since both the stomach and intestine are located in this gland and a higher resolution of the micro-CT images could be obtained by reducing the size of the sample. Using micro-CT we were able to identify microplastics ingested by the exposed mussels. These results indicate that the use of micro-CT imaging is a promising technique for microplastic research. However, as the density of "virgin" plastic is comparable to that of biological tissue, extra-dense microplastics (here, microplastics embedded with barium sulphate) have to be used. As a result, this technique is not applicable for the identification of microplastic in natural samples (i.e. organisms collected in the field), therefore traditional extraction techniques, i.e. tissue destruction) were used for the assessment of the microplastic burden in mussels.

4. Assessment of microplastics in biota and sediment from hot-spot locations

4.1 Methods

4.1.1 Sampling

Mussels (*Mytilus edulis* and *Mytilus galloprovincialis*) were sampled at three European hot-spot locations (Table 1). Originally, it was decided to analyse European Flounder from the Western Scheldt as a fish model species, but due to sampling restrictions (e.g. shortage of fish, pers. Comm. Michiel Kotterman IMARES) it was decided to use European sprat (*Sprattus sprattus*) for microplastic assessment in fish tissue. These fish were deemed appropriate as their feeding strategy expose them equally to microplastic ingestion. Mussel and fish tissue were sent to the Ghent University (UGent)

Laboratory of Environmental Toxicology as frozen (-20°C) samples.

Simultaneously, sediment was sampled at the same hot-spot locations, with the exception of the Western Scheldt (Table 1). Untreated sediment (2 kg, not sieved or mixed) were collected at 5 sub-locations within the three hot-spot areas. All locations were fully geo-referenced (See ANNEX 1). The exact locations were chosen by the sampling institutes. Sampling was performed by using a Van Veen grab on the top layer of sediment. In order to prevent contamination, no plastic containers were used, but samples were shipped in glass or aluminium containers.

Table 1: Specifications of the biota and sediment samples.

Species or matrix	Location	Target size	Sampling partner	N° ind. per pool
Mussel	Po Estuary	> 4cm	Aeiforia	100
	Tagus estuary	> 4cm	IPMA	100
	Ebro Estuary	> 4cm	IRTA	100
European Sprat	Western Scheldt	>10 cm	IMARES	50
Sediment	Po Estuary	Not Applicable	Aeiforia	Not Applicable (2 kg)
	Tagus estuary	Not Applicable	IPMA	Not Applicable (2 kg)
	Ebro Estuary	Not Applicable	IRTA	Not Applicable (2 kg)
	Western Scheldt	Not Applicable	IMARES	Not Applicable (2 kg)

4.1.2 Microplastic extraction

It is important to note that all materials used for during extraction and analysis were rigorously cleaned with filtered deionised water (0.8µm membrane filter, Supor®800, GelmanSciences) to prevent any contamination by airborne microplastics (especially fibres).

Sediment

Microplastics extraction was performed using the extraction technique developed by the Laboratory for Environmental Toxicology at Ghent University (Claessens et al., 2013). This extraction technique consists of two subsequent steps: first a volume reduction using elutriation, followed by a density separation using the high-density sodium iodide (NaI).

Volume reduction was achieved by adding 500g subsamples to an elutriation system. In this system, an upward water flow is created by forcing tap water through the system from below. This tap water passes through a 1mm and 38µm mesh first, to remove any microplastic contamination. At this point the sediment becomes fluidized. At the bottom of the column, aeration is provided to ensure efficient separation of plastic and sediment particles. The water flow, combined with the aeration, separates the lighter particles, including microplastics, from the heavier sand particles, and the rising water takes them to the top where they eventually flow over the edge and are retained on a 35µm sieve.

After this volume reduction, the 500g subsample is reduced to a sample of only a few milliliters of sediment. This material, collected on the 35µm sieve, subsequently undergoes a NaI-extraction. The solids are transferred to a 50mL centrifuge tube, and 40mL of a NaI-solution (density of

approximately 1.6 g cm^{-3}), is added. This is followed by vigorous (manual) shaking and centrifugation for 5 min at 3500g. After centrifugation, the top layer containing the microplastics is vacuum filtered over a 5µm membrane filter (Whatman AE98). This NaI-extraction is repeated three times to ensure that all plastic particles are extracted.

Biota

Microplastics extraction from animal tissue was performed using a traditional tissue destruction technique developed by the Laboratory for Environmental Toxicology at Ghent University (Claessens et al., 2013).

In short, mussels were thawed, and added to a glass erlenmeyer (triplicate). The wet weight of the organisms was determined. Then, 20 mL of concentrated nitric acid (69% HNO_3) was added to the erlenmeyer and left to stand overnight at room temperature. The erlenmeyers were covered with a clock glass to avoid contamination with airborne microplastics. The following day, the digest was heated for two hours to boiling temperature. The resulting mixture was then diluted to 200mL by warm (~80°C) filtered deionised water and vacuum filtered over a 5µm cellulose nitrate membrane filter (Whatman AE98) immediately after dilution. Fish fillets were analysed in a similar manner, except that the fillets of a single individual were added to an erlenmeyer.

Procedural blanks (i.e. samples containing no tissue) were included in every acid destruction performed, to account for any possible (airborne) contamination.

4.1.3 Microscopic analysis

Microplastics were identified under a microscope (Olympus BX41 at magnification 100×). Microplastics were identified according to shape, and the dimensions and colour were noted down. Three types of microplastics were discerned: fibres (equally thick throughout their entire length and non-segmented), fragments (irregularly shaped with tough and broken edges) and beads (spherical or ovoid in shape).

4.1.4 Interlaboratory validation

As microplastic assessment after extraction is based on a purely visual detection, reporting microplastic concentrations and abundances can be biased based on the assessor's experience and interpretation of the particles, but also even microscope quality. It was therefore decided to test whether the microplastic abundances observed for the hot spot *Mytilus* samples at the Laboratory for Environmental Toxicology at UGent is comparable to those observed by ILVO when performing their extraction and detection techniques.

Microplastic extraction according to ILVO protocol

All required solutions were filtered with a qualitative filter with particle retention 10–20 um (VWR, Grade 310) before starting the destruction protocol (De Witte et al., 2014). All laboratory glassware was cleaned with acetone and filtered with type 1 water before use, as recommended by Claessens et al. (2013). After opening the shells, the mussel body was rinsed with filtered type 1 water to remove the intervalve water. Extraction of microplastics from the mussel bodies was performed using an acid destruction with a mixture of nitric acid (VWR, 65%) and perchloric acid (VWR, 68%), HNO3:HClO4 (4:1 v:v). For an optimal digestion of the mussel bodies, 500 ml acid solution was used per 100 g tissue. The stronger perchloric acid helps to reduce the remaining greasy tissue fraction

after destruction. The mussel body was digested overnight at room temperature in a closed fume hood. The solution was covered with a clock glass to avoid contamination by air. The digest was boiled during 10 min, followed by a dilution of the digest with type 1 water. The solution was boiled a second time until the tissue was completely digested as observed by visual inspection. The acid digest was filtered over a qualitative filter (VWR, Grade 310) and the filter was transferred on a glass Petri dish for transport and visualization of microplastics under a stereo microscope (Leica M 20:5:1 or M 16:5:1 zoom). Observed microplastics were classified by category (fibre – film –spherule) and colour for each assessed mussel or blank sample. Each plastic fragment was verified as plastic with a hot needle. Synthetic polymer types were not identified.

One destruction batch was performed for each location, which consisted of 5 mussels and 3 blank analyses. For the blank analysis, the entire procedure was performed without mussel tissue. Results were evaluated after blank subtraction.

4.2 Results and Discussion

4.2.1 Microplastics in sediment

Microplastics were detected in every sample of each hot-spot location (Table 2). Microplastic concentrations varied considerably between the different hot-spot locations investigated. The highest concentration was observed in sediment from the Ebro estuary (191.0 ± 19.3 particles per kg dry sediment), while the lowest concentrations were detected in the Tagus estuary (ranging from 37 to 60 particles.kg^{-1} dry). While the concentrations observed in the different locations of the Tagus estuary are very comparable, the Po and Ebro estuary show a high variation in microplastic concentrations between sub-locations (identified by the different sample IDs, Table 2). The large difference in mean microplastic concentration between, but also within sub-locations (see STDEV in Table 2), can be attributed to the large spatial variation in microplastic distribution. It has been suggested that the variation within sampling locations can be as large as within sampling locations (Claessens et al., 2011; Cole et al., 2011; Ryan et al., 2009).

The majority of microplastics detected in sediment samples were fragments (74%). In contrast to the animal samples, fibres were detected in sediment samples. This microplastic type represented 14% of all observed microplastics. Beads represented 12%. In the samples from Po estuary a striking number of beads was detected: here, 41% of all microplastics were beads. In Tagus and Ebro estuaries, beads only represented 7% and 2%, respectively. Due to the high number of beads in Po estuary, fibres only represented 6% of the total microplastic count (in Tagus and Ebro estuaries this was 16% and 17%, respectively).

4.2.2 Microplastics in biota

Mytilus edulis/Mytilus galloprovincialis

Microplastics were detected in mussels from all hot-spot locations, albeit in quite low concentrations (less than 1 particle per gram of tissue) (

Table 3). The microplastic body burden of mussels originating from the three different hot spot locations were very similar, ranging from 0.08 to 0.16 particles per gram of tissue (wet weight). The highest average concentration of microplastics per gram of tissue was observed in mussels originating from Po estuary, while Portuguese mussels appeared to have the lowest average

concentration. However, as the standard deviations indicate, there is a high variability within the three hot-spot locations.

The majority of microplastics detected in the organisms were fragments (80% of all particles), while beads represented 20% of all extracted microplastics. No fibres were detected in the analysed individuals. It is important to note, however, that the microplastic concentrations reported here could be underestimations as the use of concentrated HNO_3 has a detrimental effect on (nylon) fibres. Using 69% HNO_3, results in the total destruction of this type of microplastic during extraction (Claessens et al., 2013). As a result, any fibre originally present within these animals will not be detected during microscopic analysis.

Table 2: Mean concentrations of microplastics (number of microplastics per kg dry sediment) in the three hot-spot locations. STDEV represents the standard deviation of the mean.

Location	Sample ID	Concentration	STDEV	Unit
Po Estuary	AEIFsed01 - A	97.6	33.4	items.kg^{-1} dry
	AEIFsed01 - B	147.6	32.9	items.kg^{-1} dry
	AEIFsed01 - C	142.1	36.4	items.kg^{-1} dry
	AEIFsed01 - D	97.3	11.3	items.kg^{-1} dry
	AEIFsed01 - E	117.2	42.7	items.kg^{-1} dry
Tagus Estuary	IPMAsed01 - A	59.8	10.7	items.kg^{-1} dry
	IPMAsed01 - B	38.5	8.2	items.kg^{-1} dry
	IPMAsed01 - C	44.8	8.4	items.kg^{-1} dry
	IPMAsed01 - D	36.7	13.5	items.kg^{-1} dry
	IPMAsed01 - E	40.1	8.0	items.kg^{-1} dry
Ebro Estuary	IRTAsed01 - A	191.0	13.9	items.kg^{-1} dry
	IRTAsed01 - F	104.1	14.8	items.kg^{-1} dry
	IRTAsed02 - A	87.3	16.0	items.kg^{-1} dry
	IRTAsed02 - F	122.0	14.0	items.kg^{-1} dry
	IRTAsed03 - A	52.6	2.4	items.kg^{-1} dry

It has to be noted, however, that the microplastic concentrations mentioned here are not purely tissue concentrations. As the organisms were frozen prior to shipment, it was no longer possible to let them clear their gut. As a result, the microplastic concentrations reported here are a combination of the microplastics still present in the gut and those present in the organism on a more permanent basis (e.g. translocation to the tissues).

Table 3: Mean concentrations of microplastics (number of microplastics per gram of tissue (ww)) in *Mytilus* originating from the three hot-spot locations. STDEV represents the standard deviation of the mean.

Location	Concentration	STDEV	Unit
Po Estuary	0.16	0.12	items.g^{-1} tissue (ww)
Tagus Estuary	0.08	0.09	items.g^{-1} tissue (ww)
Ebro Estuary	0.11	0.12	items.g^{-1} tissue (ww)

Ingestion of microplastics of different sizes and shapes by filter feeders has already been demonstrated several times in laboratory settings (e.g. Browne et al., 2008; Cole et al., 2013; Thompson et al., 2004; von Moos et al., 2012; Ward and Shumway, 2004). In a recent paper, Van Cauwenberghe & Janssen (2014) detected on average 0.36 ± 0.07 particles per gram of tissue in *Mytilus edulis* and 0.47 ± 0.16 07 particles per gram of tissue in *Crassostrea gigas* (both without gut clearance). These values are comparable to the microplastic body burden observed in this study. Yet, when venturing into literature, widely varying concentrations can be encountered as well. Mathalon and Hill (2014) detected fibres in wild and farmed mussels. Farmed mussels had significant higher concentrations of microplastics compared to wild mussels: on average 178 fibres per farmed mussel compared to an average of 126 fibres per wild mussel in the most polluted site. These plastic body burdens are several order of magnitude higher than the concentrations in mussels reported here. While the use of concentrated HNO_3 in the tissue digestion has detrimental effects on fibres (Claessens et al., 2013), Mathalon and Hill (2014) report a contamination of approximately 100 fibres per filter.

The differences in microplastic pollution that exist between hot-spot locations, with Portugal being least polluted, is reflected in microplastic concentrations observed in mussels originating from these locations. Parallel with the sediment concentrations, the Portuguese mussels show the lowest contamination, while Spanish and Italian mussels present higher levels of microplastics. So, even though mussels are not directly exposed to the microplastics present in sediments (as they are filter feeders, filtering particles from the seawater), the sediment concentrations appear to be good proxy for the exposure of biota in the water column, and hence water column concentrations.

Due to technical challenges, reports of microplastic concentrations in field organisms are highly underrepresented in scientific literature. As a result, the results presented here may provide interesting insights in the microplastic burden of field organisms.

Sprattus sprattus

The ingestion of large microplastics (5 – 1 mm) by fish has already been investigated on a number of occasions. Stomach content analysis has been used to demonstrate plastic ingestion by fish originating from different seas and oceans: the North Sea and English Channel (Foekema et al., 2013; Lusher et al., 2012), the Pacific (Boerger et al., 2010; Carson, 2013; Choy and Drazen, 2013; Davison and Asch, 2011; Jantz et al., 2013) and the Atlantic (Possatto et al., 2011; Ramos et al., 2012). In all studies, microplastics were detected in a fraction of the examined fish, with incidences ranging from 9% to over 36%. However, in scientific literature no reports of any assessments of the presence of microplastics in fish tissues (fillets) can be found. Yet, when considering the safety of seafood for

humans consumption fillets are of especially interest, as the whole fish (i.e. including the viscera) is hardly consumed.

Analysis of digested sprat fillet showed that this species contain no microplastics in their tissue. Sprat are planktivores (Casini et al., 2004), and unlike other members of the Clupeidae family, they do not change their diet with size. As a result they only include zooplankton in their diet throughout their entire life cycle. As Cole et al. (2013) demonstrated that various zooplankton taxa ingest microplastics, sprat should be indirectly exposed to microplastics by feeding on microplastic-containing zooplankton. However, compared to mussels, the exposure of European sprat to microplastics is considerably lower. Indeed, as mussels are extensive filter feeders, they are directly exposed to microplastics concentrations present in the water column. In contrast, Sprat are only exposed to microplastics that are suitable (shape and size) for ingestion by zooplankton. Hence, as no microplastics were detected in sprat fillets, transport of ingested microplastics to the tissues (i.e. translocation) might be non-existent or negligible in sprat.

Additionally, the analysis of microplastics in sprat differed from that of mussels. More specifically, during the fish analysis only fillets were included, while *Mytilus* analysis comprised the whole individuals (i.e. containing the digestive tract). This means that, if mussels had ingested microplastics prior to sampling, they would have been included in the assessment as they were still present in the stomach or intestine. Yet, while mussel and fish analysis differed, these results are relevant as far as human safety is concerned as the analysis technique focused on edible tissues that are consumed.

4.2.2 Interlaboratory validation

The results from the Interlaboratory validation are shown in Table 4. There are two main differences between the observations of the laboratories involved in this validation. First, there is a difference in the detection of microfibres and second, when considering particles only, it appears that both laboratories report different body burdens for mussels originating from the same hot-spot location. These two Interlaboratory differences are discussed in detail below.

Table 4: Results of the Interlaboratory validation of microplastic assessment in mussel tissue. Mean concentrations of microplastics (number of microplastics per gram of tissue (ww)) in *Mytilus* as observed by UGent and ILVO. STDEV represents the standard deviation of the mean.

Location	Fibres (items.g^{-1} tissue)				Particles (items.g^{-1} tissue)			
	Ugent		ILVO		Ugent		ILVO	
	Conc.	STDEV	Conc.	STDEV	Conc.	STDEV	Conc.	STDEV
Po Estuary	0.00	0.00	0.00	0.00	0.16	0.12	0.05	0.11
Tagus Estuary	0.00	0.00	0.52	0.44	0.08	0.09	0.06	0.12
Ebro Estuary	0.00	0.00	0.59	0.21	0.11	0.12	0.00	0.00

Fibres

Contrary to UGent, ILVO observed fibres in mussels originating from the Tagus and Ebro estuary, at average concentrations of 0.52 ± 0.44 fibres.g^{-1} tissue (ww) and 0.59 ± 0.21 fibres.g^{-1} tissue (ww), respectively. As was mentioned before, the use of concentrated HNO_3 has a detrimental effect on fibre detection as nylon fibres will be completely destructed (Claessens et al., 2013). Still, other types of synthetic fibres, for instance fibres originating from fishing nets (polyethylene), are

available in the marine environment and should withstand the destruction. The fibres detected by ILVO should hence be originating from plastic types that withstand the chemical digestion.

Particles

As can be seen from Table 4 and the accompanying Figure 2, ILVO reports lower particle concentrations than UGent for mussel samples originating from the same hot-spot locations. As ILVO used qualitative filters with a 20µm pore size, while UGent used filters with a 5µm pore size, differences in microplastic concentrations detected in these organisms can be attributed to the discrepancy in lower size limits. However, as can be seen from the STDEV in Table 4 and the error bars in Figure 2, average concentrations reported by both laboratories show a high variation within locations. As a result, there are no significant differences among the concentrations reported by both laboratories involved in this validation. This could be an indication that, even though two different extraction techniques were used, both extraction and detection techniques are equal in assessing the microplastic body burden of biota.

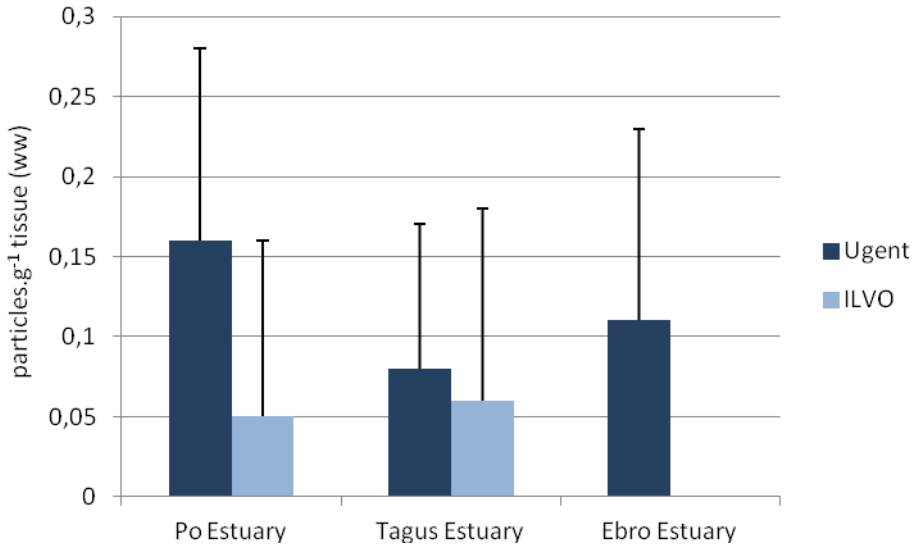

Figure 2: Average microplastic concentration (particles.g^{-1} (ww)) in the tissues of acid digested organisms, as observed by UGent and ILVO in the Interlaboratory validation.

5. Assessment of plastic associated contaminants in biota and sediment from hot-spot locations

There are two types of plastic associated contaminants. First, those that are an inherent part of plastics. These are substances added to the plastic during the production process to improve the properties of the plastic (e.g. plasticisers, flame retardants,....). These chemicals are also called additives, as they are intentionally added to the plastic. The second type of contaminants that can be found in association with (micro)plastics in the marine environment are those that are taken up from the environment by the plastic. These are typically lipophillic (and often very persistent) contaminants such as PAHs (polycyclic aromatic hydrocarbons) and PCBs (polychlorinated biphenyl).

Due to their lipophilicity, these contaminants are 'attracted' to the plastic and adsorb to it in very high concentrations (Teuten et al., 2009).

UGent analysed both types of plastic associated chemicals only in biota (and not in sediment) from the hot-spot locations, since the variation in microplastic concentrations observed in sediment samples required the analysis of all sediment available. As a result, there was no more sediment left to perform contaminant measurements. Since the assessment of microplastic concentrations in biota and sediments already took up the vast majority of time allocated to UGent in this WP, to avoid duplication of effort, the flame retardant and bisphenol A results were obtained by ICRA and ICETA, respectively.

5.1 Sampling

Mussels (*Mytilus edulis* and *Mytilus galloprovincialis*) were sampled at three European hot-spot locations (Table 1). Mussels were prepared for transport (frozen or freeze-dried, in plastic or aluminum recipients) according to ECsafeSEAFOOD partners' requests in the Sampling Plan (D2.1).

From the Western Scheldt, European flounder (*Platichthys flesus*) and sprat (*Sprattus sprattus*) were sampled. Flounder and sprat were caught and sampled according to the Sampling plan (D2.1). 25 fish were filleted and homogenized. This homogenate was then treated according to the Sampling plan (D2.1) and the demands of the analysing partners (freeze-dried, glass recipients).

5.2 Methods

5.2.1 Bisphenol A

A GC-MS method based on Cunha et al (2012) was used for quantification of the analytes. The sample preparation comprises a QuEChERS extraction followed by DLLME cleanup and a silylation of the analytes.

5.2.2 Flame retardants

Sample preparation of mussel and fish samples included a pressurized liquid extraction (PLE), followed by gravimetric determination of lipid content. Then, fat content was dissolved again and purified under an acid treatment. Finally, a SPE cleanup was carried out with Alumina cartridges. Instrumental determination of polybrominated flame retardants (PBDEs) and emerging halogenated flame retardants (HFRs) was carried out by GC-MS-MS working in electron ionization mode. Halogenated norbornenes, on the other hand, were determined by GC-MS-MS working in negative chemical ionization mode.

5.2.3 Polycyclic aromatic hydrocarbons (PAHs)

Homogenized mussel and sprat samples were fortified with internal standards of perdeuterated PAHs and saponified with methanolic potassium hydroxide in an ultrasonic bath at 60°C for 30 minutes. After saponification, a repeated (twice) extraction into 50mL hexane was performed. The extracted fraction was washed twice with a MeOH/water mixture (25mL). Following extraction and washing, further clean-up was carried out on a silica SPE cartridge conditioned with hexane (3mL).

The sample was concentrated to 1 – 3 mL via evaporation under vacuum (220mbar/50°C) and applied to the cartridge. Elution of the sample from the SPE cartridge was performed with 5mL hexane. The eluted sample was then evaporated under a nitrogen stream. After reconstitution in cyclohexane, the aromatic fraction was analyzed by GC-MS (injector at 270°C; temperature programme was from 60°C to 210°C at 12°C/min, then 8°C/min to 340°C; positive ion ionization mode).

5.3 Results and Discussion

5.3.1 Bisphenol A

Concentrations of Bisphenol A (BPA), an endocrine disruptor, are presented in Table 5. Mussels originating from Tagus Estuary had the highest levels on BPA in their tissue (12.5 ± 1.1 µg.kg^{-1} (ww)), while mussels from the Ebro estuary showed the lowest levels of contamination as the BPA levels were below the level of quantification.

Table 5: Levels of the endocrine disruptor Bisphenol A (BPA) in biota from hot-spot locations, in µg.kg^{-1} dry weight (average ± standard deviation). LOD: Limit of detection; LOQ: Limit of quantification; MQL: Minimum quantification limit.

Location	Species	Bisphenol A
Po Estuary	**Mussel**	6.30 ± 1.3
Tagus Estuary	**Mussel**	12.49 ± 1.1
Ebro Estuary	**Mussel**	< MQL
Western Scheldt	**Flounder**	8.73 ± 2.0
	LOD	0.003 – 0.01
	LOQ	0.008 – 0.04

Even though BPA is a contaminant that is inherently linked with plastic (as it is used in the production of certain types of plastics and epoxy resins) there doesn't appear to be a relation between the levels of BPA measured in mussel tissue and the microplastic burden of these animals (Table 1). Here, mussels originating from the Tagus estuary showed the highest levels of BPA contamination, while they also exhibited the lowest microplastic body burden (i.e. 0.08 ± 0.09 microplastics.g^{-1} tissue (ww)), and vice versa for mussels from the Ebro and Po estuaries. Indeed, organisms from Ebro and Po estuaries showed the highest microplastic body burdens, but the lowest BPA tissue concentrations.

Although the results presented here seem to suggest that microplastic body burden is not indicative of BPA tissue concentrations, it has been shown that plastic debris can contain high levels of BPA. Hirai et al. (2011) detected BPA in small plastics at concentrations ranging from 0 to 730 ng.g^{-1}. Leaching of BPA from plastics has been reported (Teuten et al., 2009), contributing to BPA in sewage, river and coastal waters. Yet, due to its lower hydrophobicity (log K_{ow}= 3.40), adsorption of significant concentrations of BPA to marine plastics from seawater is unlikely. Therefore, Hirai et al. (2011) suggests that the high concentrations of BPA detected in plastic fragments are most likely a result of

BPA being a constituent of the plastic. So, organisms are exposed to BPA either through plastics containing PBA as an additive, or through contaminated seawater (through PBA-leaching). Therefore, the high concentrations of BPA detected in mussels from the Tagus estuary might be attributed to higher PBA concentrations in the water, rather than microplastic exposure.

5.3.2 Flame retardants

The concentrations of flame retardants detected in mussel and flounder tissue are presented in Table 6 and The overall trend in flame retardant contamination of biota originating from hot-spot locations that can be observed here is the same as for the other plastic associated contaminant, bisphenol A: mussels originating from the Tagus estuary appear to be more polluted than organisms collected at the other hot-spot locations. And, as mussels from Tagus estuary had the lowest levels of microplastics, there is no positive trend between the presence of microplastics and the levels of plastic associated contaminants in these organisms. Yet, several laboratory and field studies indicate that the ingestion of (micro)plastic can increase the chemical body burden of organisms (Browne et al., 2013; Chua et al., 2014; Tanaka et al., 2013). For instance, Chua et al. (2014) demonstrated through feeding trials that the ingestion of microplastics will increase the uptake of higher-brominated PBDE congeners, such as BDE154 and BDE153. Analysis of PBDEs in abdominal adipose of oceanic seabirds suggested that the detected PBDEs are originated from plastics present in the animals stomach, as the congeners under investigation were not detected in natural prey (Tanaka et al., 2013). These studies suggest the transfer of plastic associated chemicals from ingested plastics to the tissues of marine-based organisms. This is, however, not reflected in the results presented here.

Table 7. Classical PBDEs (Table 6) were detected in all analysed samples with levels ranging from not quantified to 23.1 µg.kg^{-1} dry weight (dw). In contrast, emerging HFRs (The overall trend in flame retardant contamination of biota originating from hot-spot locations that can be observed here is the same as for the other plastic associated contaminant, bisphenol A: mussels originating from the Tagus estuary appear to be more polluted than organisms collected at the other hot-spot locations. And, as mussels from Tagus estuary had the lowest levels of microplastics, there is no positive trend between the presence of microplastics and the levels of plastic associated contaminants in these organisms. Yet, several laboratory and field studies indicate that the ingestion of (micro)plastic can increase the chemical body burden of organisms (Browne et al., 2013; Chua et al., 2014; Tanaka et al., 2013). For instance, Chua et al. (2014) demonstrated through feeding trials that the ingestion of microplastics will increase the uptake of higher-brominated PBDE congeners, such as BDE154 and BDE153. Analysis of PBDEs in abdominal adipose of oceanic seabirds suggested that the detected PBDEs are originated from plastics present in the animals stomach, as the congeners under investigation were not detected in natural prey (Tanaka et al., 2013). These studies suggest the transfer of plastic associated chemicals from ingested plastics to the tissues of marine-based organisms. This is, however, not reflected in the results presented here.

Table 7) (hexabromobiphenyl (HBB), pentabromoethylbenzene (PBEB) and decabromodiphenyl ether (DBDPE)) were not detected in mussels from the Ebro estuary, but were detected in the analysed samples with levels ranging from non quantified to 4.43 µg.kg^{-1} dw. The halogenated norbornenes were detected in the range of non quantified to 0.027 µg.kg^{-1} dw.

As for the endocrine disruptor Bisphenol A, tissue levels of PBDEs (Table 6) are highest in mussels originating from the Tagus estuary, and considerably lower in mussels from the Ebro estuary (23.1

µg.kg^{-1} dw and not quantified, respectively). Emerging HFRs and halogenated norbornenes (The overall trend in flame retardant contamination of biota originating from hot-spot locations that can be observed here is the same as for the other plastic associated contaminant, bisphenol A: mussels originating from the Tagus estuary appear to be more polluted than organisms collected at the other hot-spot locations. And, as mussels from Tagus estuary had the lowest levels of microplastics, there is no positive trend between the presence of microplastics and the levels of plastic associated contaminants in these organisms. Yet, several laboratory and field studies indicate that the ingestion of (micro)plastic can increase the chemical body burden of organisms (Browne et al., 2013; Chua et al., 2014; Tanaka et al., 2013). For instance, Chua et al. (2014) demonstrated through feeding trials that the ingestion of microplastics will increase the uptake of higher-brominated PBDE congeners, such as BDE154 and BDE153. Analysis of PBDEs in abdominal adipose of oceanic seabirds suggested that the detected PBDEs are originated from plastics present in the animals stomach, as the congeners under investigation were not detected in natural prey (Tanaka et al., 2013). These studies suggest the transfer of plastic associated chemicals from ingested plastics to the tissues of marine-based organisms. This is, however, not reflected in the results presented here.

Table 7) appear to be present in mussel tissue in much lower concentrations than classical PBDEs. While HBB and PBEB were only detected in Tagus estuary mussels (4.43 µg.kg^{-1} dw and not quantified, respectively), DBDPE was not detected in any of the mussel tissue analysed. In fish tissue, all of the emerging HFRs were detected, but in non-quantifiable levels. The halogenated norbornene dechlorane 602 (Dec 602) was detected in all samples analysed ranging in levels from 0.004 to 0.027 µg.kg^{-1} dw. The three other types of halogenated norbornenes, dechlorane 603 (Dec 603), and Dechlorane Plus (DP, both *syn* and *anti*) were either not detected or only in very low concentrations (not quantified).

Table 6: Levels of flame retardants (more specifically PBDEs) in biota from hot-spot locations, in µg.kg^{-1} dry weight. LOD: Limit of detection; LOQ: Limit of quantification; n.a.: Not analysed; n.q.: Not quantified.

Location	Species	PBDEs							
		BDE28	BDE47	BDE100	BDE99	BDE154	BDE153	BDE209	∑PBDEs
Po Estuary	**Mussel**	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
Tagus Estuary	**Mussel**	0.43	5.05	4.58	5.99	3.19	1.65	2.19	23.1
Ebro Estuary	**Mussel**	n.q.	n.q.	n.q.	n.q.	n.q.	n.q.	n.q.	n.q.
W. Scheldt	**Flounder**	n.q.	2.10	1.34	n.q.	n.q.	n.q.	n.q.	3.43
	LOD	0.04	0.05	0.20	0.29	0.43	0.64	10.6	
	LOQ	0.12	0.18	0.67	0.97	1.42	2.13	35.4	

The overall trend in flame retardant contamination of biota originating from hot-spot locations that can be observed here is the same as for the other plastic associated contaminant, bisphenol A: mussels originating from the Tagus estuary appear to be more polluted than organisms collected at the other hot-spot locations. And, as mussels from Tagus estuary had the lowest levels of microplastics, there is no positive trend between the presence of microplastics and the levels of plastic associated contaminants in these organisms. Yet, several laboratory and field studies indicate

that the ingestion of (micro)plastic can increase the chemical body burden of organisms (Browne et al., 2013; Chua et al., 2014; Tanaka et al., 2013). For instance, Chua et al. (2014) demonstrated through feeding trials that the ingestion of microplastics will increase the uptake of higher-brominated PBDE congeners, such as BDE154 and BDE153. Analysis of PBDEs in abdominal adipose of oceanic seabirds suggested that the detected PBDEs are originated from plastics present in the animals stomach, as the congeners under investigation were not detected in natural prey (Tanaka et al., 2013). These studies suggest the transfer of plastic associated chemicals from ingested plastics to the tissues of marine-based organisms. This is, however, not reflected in the results presented here.

Table 7: Levels of flame retardants (more specifically emerging BFRs and halogenated norbornenes) in biota from hot-spot locations, in µg.kg^{-1} dry weight. LOD: Limit of detection; LOQ: Limit of quantification; n.a.: Not analysed; n.q.: Not quantified; n.d.: Not detected.

Location	Species	Emerging HFRs			Halogenated Norbornenes			
		HBB	PBEB	DBDPE	Dec 602	Dec 603	*syn*-DP	*anti*-DP
Po Estuary	**Mussel**	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
Tagus Estuary	**Mussel**	4.43	n.q.	n.d.	0.027	n.d.	n.q.	n.d.
Ebro Estuary	**Mussel**	n.d.	n.d.	n.d.	0.013	n.q.	n.q.	n.q.
W. Scheldt	**Flounder**	n.q.	n.q.	n.q.	0.004	n.q.	n.q.	n.q.
	LOD	0.20	0.18	9.66	0.021	0.007	0.006	
	LOQ	0.67	0.61	32.2	0.070	0.024	0.018	

5.3.3 Polycyclic aromatic hydrocarbons (PAHs)

In contrast to Bisphenol A and flame retardants, PAHs are no plastic-specific contaminants. PAHs mainly enter the environment through the incomplete combustion of organic matter, mostly fossil fuels. Because of their hydrophobic nature, plastics may act as a vector for such organic contaminants (Karapanagioti & Klontza, 2008; Mato et al., 2001).

The concentrations of PAHs detected in mussel tissue are represented in Table 8 and Table 9. Only fluorene, fluoranthene and pyrene were detected in sufficiently high concentrations at all locations. Concentrations of PAHs ranged from levels below LOQ to 5.08 µg.kg^{-1} ww. Organisms originating from the Po estuary appeared the most contaminated, as they exhibited the highest concentrations. However, only mussels originating from Tagus estuary contained benzo[a]anthracene and benzo[b]fluoranthene at sufficiently high concentrations. *Mytilus* from the Ebro estuary are the least contaminated. Again, these results do not completely reflect the microplastic body burdens detected in these organisms.

Due to the ubiquitous sources of PAHs, they are present throughout the environment. They are present in the water and air, before they adsorb to hydrophobic compounds such as organic matter and plastics. Therefore, the presence of PAHs in organisms is not only linked to the ingestion of microplastics and associated PAHs, but also through the exposure of these organisms to water and organic matter concentrations of PAHs. As there are different routes of exposure to PAHs for marine organisms such as mussels, it is quite difficult to assess the contribution of microplastic ingestion to their overall body burden. Especially as it is still not clear whether microplastics actually play an

important role in the transport of contaminants to invertebrates. It has been established that microplastics, more specifically pellets, contain persistent organic contaminants (POPs): ∑PAH concentrations on pellets can vary from 53 to over 44 000 ng.g^{-1} (Antunes et al., 2013; Fisner et al., 2013; Hirai et al., 2011). It has also been demonstrated that, under physiological conditions, the desorption of such POPs from plastic is enhanced (Bakir et al., 2014). This suggests that organisms ingesting microplastics and their associated contaminants should have a higher contaminant body burden. However, both laboratory and modelling studies indicated that bioaccumulation can be reduced, due to a cleaning mechanism (Besseling et al., 2013; Koelmans et al., 2013). So, in these cases, microplastic ingestion actually decreases the body burden of exposed organisms. As it is still not completely elucidated how and whether microplastics act as a vector of associated contaminants to the tissues of exposed organisms, it is extremely hard to estimate and assess the contribution of microplastic ingestion to tissue concentrations of contaminants in exposed organisms.

Table 8: Levels of polycyclic aromatic hydrocarbons (ANTL: acenaphthylene; ANA: acenaphthene; FL: fluorene; PH: phenanthrene; ANTH: anthracene; FLAN: fluoranthene; PY: pyrene; B[a]ANTH: benzo[a]anthracene) in biota from hot-spot locations, in µg.kg^{-1} wet weight. LOQ: Limit of quantification; n.a.: Not analysed; n.d.: Not detected; <LOQ: Below Limit of Quantification.

Location	Species	PAHs							
		ANTL	ANA	FL	PH	ANTH	FLAN	PY	B[a]ANTH
Po Estuary	**Mussel**	<LOQ	<LOQ	3.67	<LOQ	<LOQ	5.08	3.84	<LOQ
Tagus Estuary	**Mussel**	<LOQ	<LOQ	2.42	<LOQ	<LOQ	4.83	3.99	1.84
Ebro Estuary	**Mussel**	<LOQ	<LOQ	2.48	<LOQ	<LOQ	2.98	1.95	<LOQ
W. Scheldt	**Sprat**	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
	LOQ	1.5	1.5	1.5	5	2.5	1.5	1.5	1.5

Table 9: Levels of polycyclic aromatic hydrocarbons (CH: chrysene B[b]FLAN: benzo[b]fluoranthene; B[k]FLAN: benzo[k]fluoranthene; B[a]P: benzo[a]pyrene; I[123-cd]PY: indeno[1,2,3-c,d]pyrene; D[ah]AN: dibenzo[a,h]anthracene; B[ghi]PERY: benzo[g,h,i]perylene) in biota from hot-spot locations, in µg.kg^{-1} wet weight. LOQ: Limit of quantification; n.a.: Not analysed; n.d.: Not detected; <LOQ: Below Limit of Quantification.

Location	Species	PAHs						
		CH	B[b]FLAN	B[k]FLAN	B[a]P	I[123-cd]PY	D[ah]AN	B[ghi]PERY
Po Estuary	**Mussel**	1.77	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
Tagus Estuary	**Mussel**	1.68	3.26	<LOQ	<LOQ	<LOQ	n.d.	<LOQ
Ebro Estuary	**Mussel**	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	n.d.	<LOQ
W. Scheldt	**Sprat**	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
	LOQ	1.5	2.5	2.5	2.5	2.5	2.5	2.5

6. Conclusions

Microplastics are ubiquitously present in the marine environment. Quite some literature has reported microplastic concentrations in sediments worldwide. The literature on the presence of microplastics in field organisms is much scarcer. The research performed in the framework of the ECsafeSEAFOOD project delivers important and new insights in the distribution of microplastics in marine biota that can reach the human food chain throughout Europe.

Again, it was demonstrated that marine bivalves, which are an important food source for humans, contains microplastics. From the analyses it appears that local sediment concentrations can be used as proxy for water column concentrations, as mussels originating from locations with lower microplastic pollution in sediments also showed a smaller body burden, and vice versa. The analysis of plastic associated contaminants (or additives) in the tissue of these organisms seem to suggest that the levels of plastic-specific contaminants (i.e. additives such as brominated flame retardants and bisphenol A) are not directly related to the microplastic body burdens of these animals. Interpretation of the results on plastics acting as vectors for plastic-associated contaminants, such as PAHs adsorbed to the plastic from the environment, are complicated by the fact that these contaminants are also present at high concentrations in the surrounding environment.

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ANNEX 1: Sampling Locations

Species or Matrix	Location	Sample ID	Latitude	Longitude
Sediment	Po Estuary	AEIFsed01 - A	44°48'54.72"N	12°17'20.94"E
		AEIFsed01 - B	44°48'53.42"N	12°17'49.34"E
		AEIFsed01 - C	44°48'53.42"N	12°17'49.34"E
		AEIFsed01 - D	44°49'25.28"N	12°20'2.98"E
		AEIFsed01 - E	44°50'29.65"N	12°17'55.54"E
	Tagus Estuary	IPMAsed01 - A	38°45'6.42"N	8°58'2.67"W
		IPMAsed01 - B	38°42'2.94"N	9°0'16.89"W
		IPMAsed01 - C	38°39'8.04"N	9°4'27.60"W
		IPMAsed01 - D	38°38'44.78"N	9°6'0.80"W
		IPMAsed01 - E	38°41'44.83"N	9°13'57.18"W
	Ebro Estuary	IRTAsed01 - A	40°36'57.48"N	0°36'35.58"E
		IRTAsed01 - F	40°36'57.48"N	0°36'35.58"E
		IRTAsed02 - A	40°37'3.42"N	0°38'22.80"E
		IRTAsed02 - F	40°37'3.42"N	0°38'22.80"E
		IRTAsed03 - A	40°37'17.94"N	0°40'8.76"E
Mussel	Po Estuary	AEIF0002	44°48'54.72"N	12°17'20.94"E
	Tagus Estuary	IPMA0002	38°41'44.83"N	9°13'57.18"W
	Ebro Estuary	IRTA0001	40°46'32.12"N	0°44'15.35"E