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Characterization of microplastics ingested by marine benthos: a methodological and field-experimental study

Tesi di laurea in

Ecosistemi marini: struttura e processi

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Abstract

Microplastics have become ubiquitous pollutants in the marine environment. Ingestion of microplastics by a wide range of marine organisms has been recorded both in laboratory and field studies. Despite growing concern for microplastics, few studies have evaluated their concentrations and distribution in wild populations. Further, there is a need to identify cost-effective standardized methodologies for microplastics extraction and analysis in organisms.

In this thesis I present: (i) the results of a multi-scale field sampling to quantify and characterize microplastics occurrence and distribution in 4 benthic marine invertebrates from saltmarshes along the North Adriatic Italian coastal lagoons; (ii) a comparison of the effects and cost-effectiveness of two extraction protocols for microplastics isolation on microfibers and on wild collected organisms; (iii) the development of a novel field-based technique to quantify and characterize the microplastic uptake rates of wild and farmed populations of mussels (*Mytilus galloprovincialis*) through the analysis of their biodeposits.

I found very low and patchy amounts of microplastics in the gastrointestinal tracts of sampled organisms. The omnivorous crab *Carcinus aestuarii* was the species with the highest amounts of microplastics, but there was a notable variation among individuals. There were no substantial differences between enzymatic and alkaline extraction methods. However, the alkaline extraction was quicker and cheaper. Biodeposit traps proved to be an effective method to estimate mussel ingestion rates. However their performance differed significantly among sites, suggesting that the method, as currently designed, is sensible to local environmental conditions. There were no differences in the ingestion rates of microplastics between farmed and wild mussels. The estimates of microplastic ingestion and the validated procedures for their extraction provide a strong basis for future work on microplastic pollution.

Summary

1. Introduction

1.1. General overview

Plastic production has grown continuously from the 1.5 million tonnes in the 1950s with an increment of mass production that is estimated to be 322 million tonnes in 2015 and that is estimated to continue to grow at 4% per year (PlasticEurope 2016).

This incredible success particularly involved some specific synthetic compounds: Polyamides/Nylons (PA), Polycarbonate (PC), Polyester (PES), Polyethylene (PE), Polyethylene terephthalate (PET), Polypropylene (PP), Polystyrene (PS), Polyurethanes (PU) and Polyvinylchloride (PVC). Because of their unique properties, including their use at a wide range of temperatures, easy manipulation, waterproofness, good isolation capacity, resistance to corrosion and low production costs, plastics have become deeply integrated in our everyday life (Andrady and Neal, 2009).

These qualities and their versatility make plastics the perfect materials for a wide range of manufacturing and packaging applications. At the same time, their so appreciated durability represents a treat for the environment when plastics become waste.

Plastic items can persist in nature for many years and their degradation is temperature-dependent (Andrady, 2015). This, accompanied with their facility to be transported by currents for thousands of kilometers (Ryan *et al*., 2009) make them ubiquitous pollutants in even the most remote areas of our planet, from the equator to the poles (Barnes *et al*., 2009; Zarfl and Matthies, 2010). It is estimated that in 2010 4.8 to 12.7 million tonnes of plastic waste entered the ocean (Jambeck *et* al., 2015), becoming the major component of litter stranded on beaches, where plastic bags, fishing equipment, food and beverage containers represented more than 80% of the garbage (Thiel *et al*., 2013). This situation can also be seen on the seafloor, where 90% of litter caught with benthic trawls is constituted by plastic (Ramirez-Llodra *et al*., 2013).

Nowadays plastic pollution in the marine environment has become a matter of increasing concern (Rochman *et al*., 2013) particularly regarding the negative physical effects on marine wildlife including birds (Kenyon and Kridler, 1969; Wallace, 1985), turtles (Balazs, 1985), seals (Fowler, 1985) and cetaceans (Cawthorn, 1985). In the early 2000s the concern and focus of researchers also started including smaller microplastic debris, whose presence had been detected and reported since the 70ies (Carpenter *et al*. 1972 and Colton *et al*. 1974).

The term “microplastic” was first mentioned by Thompson *et al*. (2004) to describe very small pieces of plastic accumulating in marine sediments and in the water column in the European

waters and has since been widely used to describe plastic particles from 20 µm to 5 mm in diameter (Arthur *et al*., 2009). Microplastics (MPs) comprise synthetic particles with a wide variety of shape, size, colour, density, chemical composition and other characteristics, and can be distinguished by usage and source as "primary" microplastics and "secondary" microplastics. "Primary" microplastics include precursors for the production of polymer consumer products (indirect use) or micro-particles present in cosmetics, scrubs and abrasives (direct use). Although most of the plastics are extremely persistent, they are not immune to degradation. Big plastic items have been reported to break down in smaller pieces due to a combination of mechanical forces as waves action, photodegradation and oxidation (Cooper and Corcoran 2010; Andrady, 2015): those particles are usually referred as "secondary" microplastics.

These tiny plastic particles enter the sea from a variety of sources (Browne, 2015): land represents the source of approximately 80% of the plastic found in marine litter (Andrady, 2011), which enter in the marine environment via rivers, wastewaters and as macroplastic garbage produced by coastal tourism or industrial production (Rillig, 2012; Koelmans, 2014; Wright *et al*., 2013). Other sources are represented by fishing gears resulted from fishing activities, which is one of the most common plastic debris found at sea or from the increasing use of geotextiles for example in coastal defence and restoration (Vianello *et al.,* 2013; Wiewel and Lamoree, 2016)

In order to understand the environmental impacts of plastic debris, many studies are trying to describe and quantify the spatial distribution of microplastics in the marine environment. This kind of researches started to be significantly abundant since 2008, with the entry into force of the Marine Strategy Framework Directive (MSFD-indicator 10.1.3), in which the EU specifies a mandatory monitoring action for microplastics pollution (Zarfl *et al*., 2011).

The spatial and temporal distributions of microplastics in the water column and associated sediment are still poorly understood, and are probably influenced by a variety of factors, including particles' properties, polymers' density, weathering and biofouling coverage, hydrodynamics and other environmental conditions (Vianello *et al.,* 2013). Microplastics can be abundant not only in the water surface, but also in the deeper water column, in marine sediments, in beaches and in the sea ice (Collignon *et al*., 2012; Van Cawenberghen *et al*., 2013; Law and Thompson, 2014; Obbard *et al*., 2014). Few studies have estimated the abundance and distribution of microplastics in the ocean via global surveys (Eriksen *et al*., 2014; Lusher, 2015). Most works focused singularly on specific oceanic regions, such as coastal areas, regional seas, gyres or the poles (Thompson *et* al., 2004; Collignon *et al*., 2012; Cincinelli *et al*., 2017).

Concentrations ranged from thousands to hundreds of thousands of particles km^{-2} (Noren, 2007; Desforges *et al.* 2014; Van Cawenbergen *et* al., 2014) and seem to be particularly high in the arctic sea (Obbard *et al*., 2014). Recently it was hypothesized a transfer of microplastics from the water column down to the deep sea sediments, which may represent an important plastic sink (Law *et* al., 2010; Van Cawenbergen *et* al., 2013; Eriksen *et al.*, 2014; Woodall *et* al., 2014).

Regarding the temporal distribution of microplastics, while some papers reported no clear temporal trends in abundance (Law *et al*., 2010), others revealed an increase over time (Thompson *et al.*, 2004; Goldstein *et al*., 2012). However, it is important to note that abundance and distribution data may be affected by different sampling methods used in each survey due to the lack of standardized methodologies that may create difficulties in producing comparable results among studies (Avio *et al*., 2015; Lusher *et al*., 2017). As for this matter, the Technical Subgroup on Marine Litter (TSG-ML) proposed a standardized monitoring strategy for microplastics in the EU (Hanke *et al*., 2013).

One of the main concern regarding microplastics is their potential to absorb (mostly from the sea water) and release persistent organic pollutants (POPs), together with plastic associated chemicals (also known as "plasticisers"), introducing them into the food web. POPs include polychlorinated biphenyls (PCBs), PAHs and organochlorine pesticides (e.g. DDT, DDE) which can be absorbed onto plastic items (Teuten *et al*., 2009) and desorbed in marine organisms tissue when plastic items are ingested by marine biota (Rochman et al., 2013; Lithner et al., 2011; Lithner et al., 2012), while plasticisers (e.g. phthalates), which are additives incorporated during manufacture to modify plastic's properties, are biologically active and easily released in organisms tissues of many marine species (Hermabessiere *et al*., 2017). These mechanisms have raised concern because, due to their small size and their ubiquitous presence, microplastics are available to a wide range of organisms (Betts, 2008), from phytoplankton (Cole *et al*., 2013) to the big marine mammals (Fossi *et al*., 2012) and are reported to cause both physical (Wright *et* al., 2013) and physiological stress (Bhattacharya *et al*., 2010; Rocham *et al*., 2013). Moreover, microplastics ingestion from low trophic levels can let them enter the marine food web, also involving species used for commercial scopes and thus representing a potential treat for human health (Galloway, 2015).

Organisms can ingest microplastics preying on plastic contaminated individuals or directly ingesting particles from the environment. Some species ingest plastics items from sediments or water column because their feeding system does not allow them to discriminate between prey

and anthropogenic elements (Moore *et al*., 2001) or because they confuse them with their habitual food (Moore, 2008). Laboratory experiments reported some organisms (holothurians in particular) selectively preferring microplastics over sediment grains (Graham and Thompson, 2009). It is important to note that most of these studies used artificial and marked (e.g. fluorescence) plastic items and exposed organisms to concentrations up to 5000 times the environmental ones (Van Cauwenberghe *et al*., 2015). While such an approach is often necessary to identify the potential toxicological effects of pollutants (especially with regards to emerging pollutants such as microplastics), it may be difficult to transpose such laboratory results to real conditions in nature (Van Cauwenberghe *et al*., 2015).

Microplastics can also act as vectors to alien invasion, providing floating habitats for the colonization of bacterial communities (McCormick *et al*., 2014) and micro-algae (Carson *et al*., 2013), possibly altering their distribution and dispersion potential.

1.2. Aims of the thesis

Despite the growing concerns for microplastics, few studies have evaluated their concentrations and distribution in wild populations sampled from their natural environment. Even less attention has been devoted to the analysis of variations in the distribution of microplastics at multiple spatial and/or temporal scales. Thus, this permits to identify characteristics scales of variation that can hint at the relevant processes that control both the dynamics of microplastics and their subsequent interactions with the marine organisms and resulting impacts. The lack of field studies can be in part attributed to the lack of standardized methods for the microplastics detection and characterization from field collected samples, associated with the difficulties to carry out field measurements of microplastics ingestion rates by organisms.

In this thesis I present the results of a multi-scale field sampling of microplastic spatial distribution in benthic invertebrates in North Adriatic salt marshes. Saltmarshes provide habitat and nursery areas for many species, including species of commercial interest, support productive food webs and play a fundamental role in sediment control and carbon storage (Duarte *et al*., 2013). At the same time they are threatened by a growing but largely overlooked accumulation of MPs in sediments from a variety of sources (Vianello et al 2013). The sampling was integrated with field experimental measurements of ingestion rates by mussels and with a methodological study aiming to identify cost-effective methods for the analysis of microplastics

in marine species. All the work was done in the context of the international Project "Plastox - Direct and indirect ecotoxicological impacts of microplastics on marine organisms", a JPI Ocean project aiming to "investigate the ingestion, foodweb transfer, and ecotoxicological impact of microplastics, together with persistent organic pollutants (POPs), metals and plastic additive chemicals associated with them, on key European marine species and ecosystems" (https://www.sintef.no/projectweb/plastox/).

This thesis is organised in three parts, focusing on quantifying the potential of microplastics to be present at certain trophic levels, plastics properties influencing their ingestion by aquatic organisms, together with the selection of cost-effective techniques for microplastics quantification and characterisation. Subsequent sections specifically present each part of the work.

1.3. Microplastics occurrence at different trophic levels

While ingestion of plastic debris has been mostly reported in vertebrates species (Laist, 1997), the discovery of the presence of tiny plastic particles implied an extension of the number of species studied, including organisms that occupy the lower levels of the trophic chain (Wright *et al*., 2013). Laboratory and field researches reported the ingestion of microplastics in a variety of animals, including fish, turtles, birds, mammals and also invertebrates (Lusher, 2015), most frequently analysing their gastrointestinal tract. Thompson *et al*. (2004) demonstrated that benthic invertebrates with different feeding strategies (detritivores, deposit feeders and filter feeders), including lugworms (*Arenicola marina*), amphipods (*Orchestia gammarellus*) and blue mussels (*Mytilus edulis*), have the potential to ingest microplastics under laboratory controlled conditions. Researchers then continued to investigate the presence of plastic items in marine organisms, using invertebrates as model species (Wesch *et al*., 2016) and focusing mainly on ecotoxicological aspects (von Moos *et al*., 2012; Wright *et al*., 2013; Li *et al*., 2016) and trophic transfer (Farrel and Nelson, 2013; Setala *et al*., 2014), where individuals were generally exposed to controlled concentrations of plastic particles. However, there is still limited knowledge and few studies about microplastics uptake, trophic transfer and effects on marine wildlife under natural environmental conditions.

Various field studies (Murray and Cowie, 2011; De Witte *et al*., 2014; Van Cawenberghe and Janssen, 2014; Van Cawenberghe *et al*., 2015) found microplastics in the digestive system of crustaceans (*Nephrops norvegicus*), bivalves (*Mytilus edulis*, *Crassostrea gigas*) and polychaetes

(*Arenicola marina*) in concentrations that were tens or hundreds times lower than those reported in laboratory researches, underling the need to better understand the ecological and biological implications of microplastics at a population, community and ecosystem levels. The characterization of the microplastics ingested by wild key species has become a research priority to fully understand the microplastic behaviour in natural coastal ecosystems, representing a necessary step to develop a more integrated scenario of ecological risk assessment (Avio *et al*., 2015).

This study investigated the presence of microplastics in the gastrointestinal tract of different wild benthic marine invertebrates. North Adriatic saltmarshes along the Italian coastal lagoons were chosen as target habitat due to their regional relevance, ecological importance and ability to trap debris (Vermerein *et al*., 2016; Weinstein *et al*., 2016). Representative key species of this area were selected for their specific feeding strategies and trophic role: the crab *Carcinus aestuarii* (omnivore) as a predator with a variety of prey choices, which were here represented by the bivalves *Mytilus galloprovincialis* (suspension feeder), *Cerastoderma glaucum* (detritivore) and the polychaete worm *Hediste diversicolor* (detritivore) (Mistri *et al*., 2001b).

The specific aims of this study were:

- To quantify and characterize MPs occurrence and distribution in benthic marine invertebrates at different trophic levels and with different feeding strategies

- To analyze whether the distribution of MPs was consistent at a hierarchy of spatial scales, ranging from meters (distance among replicated organisms within sites) to 100s of meters (distance among replicated sampling sites) to tens of kilometres (distance among replicated sampling lagoons).

1.4. Comparison of different extraction procedures from marine organisms

Environmental field studies are limited by the lack of standardized protocols and the presence of many operational difficulties associated with the extraction and characterization of microplastics from marine organisms. In order to be analysed, the particles have to be separated from the animal tissue, and this is usually done by digesting all the organic components of the digestive tract or of the whole animal (Lusher et al., 2017). Currently there

are no standard methodologies to isolate microplastics from the organisms' tissue, and different techniques need to be compared to choose the most cost-effective method.

There are various methods for digestion that use different types of chemicals: acid (De Witte *et al*., 2014; Claessens *et al*., 2013), alkaline (Rochman *et al*., 2015; Dehaut *et al*., 2016; Claessens *et al*., 2013) and enzymatic (Cole *et al*., 2014). While acidic and alkaline methods have been demonstrated to be efficient in breaking down soft tissues, some pH sensitive polymers (e.g. polyamide, polyoxymethylene, polycarbonate) may be damaged and/or destroyed by these aggressive solutions (Claessens *et al*., 2013; Van Cauwenberghe and Janssen, 2014; Vadermeersch *et al*., 2015). Of concern are particularly plastic microfibres, which are one of the most abundant class of plastics particles found in marine organism gastrointestinal tracts (Taylor et al., 2016) and that may be more easily degraded compared to other types of microplastics by overly strong digestion methods. An ideal digestion protocol would be one that has a high digestion efficiency, works for the highest number of polymer types and causes the least visual and chemical damage to polymers while having low costs and being less time consuming.

I compared the SDS-enzymatic method, which has been described to be gentler and likely not affecting the physico-chemical properties of polymers (Cole *et al*., 2013), and the 1 M Potassium hydroxide (KOH), which represent an efficient digestion method causing no evident damages on plastic particles (Kühn *et al*., 2017). I specifically compared the methods for their effects on a range of different microplastic types including fibres of different polymers. I used the crab *Carcinus aestuarii* as a target species to compare pros and cons of the two protocols when applied on field collected organisms. In fact crabs, due to their capability to accumulate contaminants from preys, often reach levels of pollution higher than those measured in other invertebrates (Micheletti *et al*., 2007).

The specific aims were:

- To compare the effects of the Enzymatic and Alkaline digestion methods on the characteristics of plastic microfibers and their recognition by microscopy and FTIR spectroscopy analysis.
- To compare the cost-effectiveness of the Enzymatic and Alkaline digestion methods on the extraction and isolation of MPs from field collected marine invertebrates.

1.5. Estimate of ingestion rates through the analysis of biodeposits

So far, the quantification of microplastics ingested by organisms has been mostly performed by analysing the particles found in their gastrointestinal tract (Lusher *et al*., 2017). Most microplastics, however, pass quickly through the organisms and are easily excreted. This fast dynamic is very important, but cannot be captured by snapshot samplings. POPs and plasticisers associated to the particles might be desorbed from the plastics during their passage, and then transferred into tissues and cells with potential physiological damage (Teuten *et al*., 2009; Lithner et al., 2011; Lithner et al., 2012; Rochman et al., 2013; Hermabessiere *et al*., 2017).

An alternative approach could be to evaluate the particles uptake rates by collecting and analysing animals' boluses, casts or faeces (Eriksson and Burton, 2003; Gil-Delgado *et al*., 2017). This approach is generally rarely used, but has been applied to some species: for example, Van Cauwenberghe *et al*., (2015) performed a study showing the presence of plastic particles in blue mussels (*Mytilus edulis*) and lugworms (*Arenicola marina*) faeces. In fact, filter feeders, such as bivalves and gastropods molluscs, can expel particles that do not constitute a food source through egestion via specialized mucus structures (pseudofaeces), without them passing through the digestive tract (Wright *et al*., 2013). Both faeces and pseudofaeces have the potential to transport microplastics from the water column to deeper waters and to the seafloor sediments (Cole *et al*., 2013), representing a potential microplastics source for benthic organisms.

Bivalve molluscs shellfish such as mussels have been extensively used in marine monitoring programmes (De Witte *et al*., 2014). Due to their sessile lifestyle, they accurately reflect local environmental conditions and, because of their wide geographic distribution, easy sampling, tolerance to a considerable range of salinity, resistance to stress and high accumulation of a wide range of pollutants, they are the ideal target organisms for environmental monitoring (Tanabe et al., 2000). Mussels are benthic filter feeders with a selective mechanism of suspension feeding: they process relatively large amounts of water during filtering, maximizing their exposure to any harmful material within the water column (De Witte et al., 2014). This can result in a wide range of microplastic particles filtered as well as in the accumulation of a wide range of chemical pollutants and microorganisms (Barker Jørgensen, 1990). While many studies on mussels demonstrated the potential for nanoparticles (particles < 20 µm) to be metabolized and in some cases also accumulated at tissue and cell levels (Browne et al. 2008), mussels have

been recorded to expel larger microplastics after approximately one hour (Farrel and Nelson, 2013).

The aim of this work was to test a novel field-based technique to quantify the microplastics uptake rates through the analysis of biodeposits using mussels as target organism. Specific aims included:

- To develop a new, low-cost protocol to characterize and measure MPs uptake rates by *Mytilus galloprovincialis* via biodeposit sampling.
- To test whether the methods performed consistently across different study sites with different environmental conditions
- To analyse potential differences in MPs uptake rates between commercial and wild type mussels.

2. Materials and methods

2.1. Microplastics occurrence at different trophic levels

2.1.1. Sampling and study area

Three lagoons of the north-eastern Italian coast were selected to include a variety of potential impacts from microplastics: Piallassa Baiona (44°28'26.6''N; 12°14'52.5"E), Bellocchio Lagoon (44°38'01.97''N; 12°15'48.78"E) and Chioggia Lagoon ((45°13'11.52''N; 12°16'44.45"E) (Fig 1).

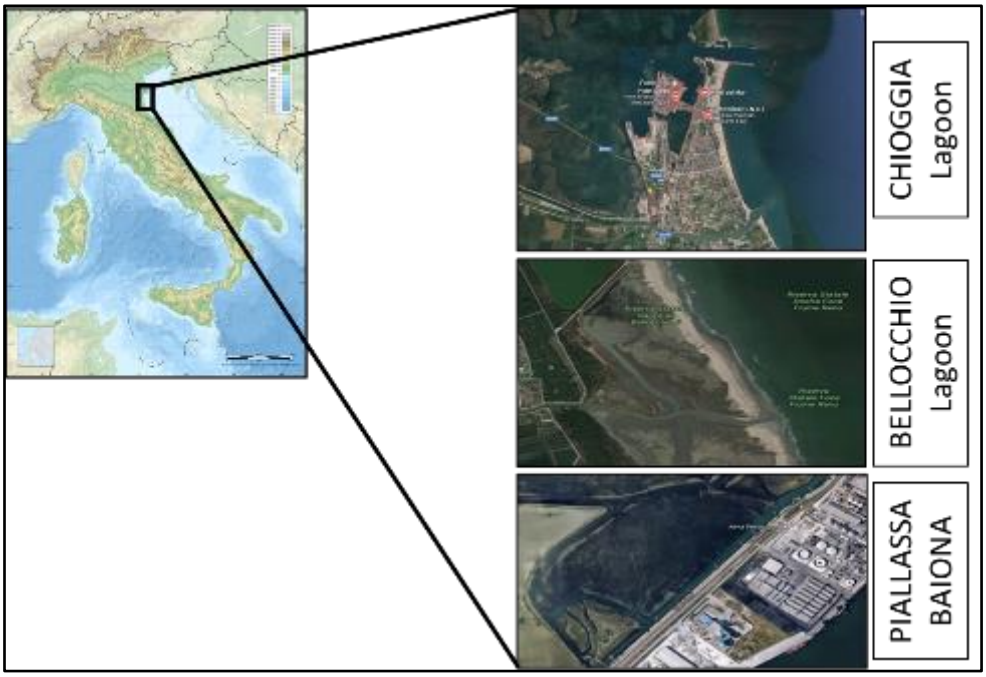

Figure 1: The three study lagoons: Chioggia Lagoon, Bellocchio Lagoon and Piallassa Baiona.

Piallassa Baiona was expected to be the most polluted lagoon due to its proximity to a high density urban area and to the presence of urban and industrial drainage and the discharge of cooling waters from two power plants (Airoldi *et al.*, 2016; Browne *et al*., 2011). Bellocchio Lagoon was considered the least polluted area, with relatively low human impacts and far from industrial zones (Strain *et al*., 2017). Chioggia Lagoon was chosen as an intermediate situation (Wong *et al*., 2015), due to aquaculture plants that may release microplastics, but with no direct discharge of urban or industrial products in the proximity of the sites where the sampling was performed.

Sampling was performed from May to August 2016. Each lagoon was sampled at three replicate sites, about 100-200 m apart. At each site 40 speciments were collected haphazardly for each of the four species.

Specimens of the crab *Carcinus aestuarii* were collected using seven standard fish traps with fresh fish baits per site. The traps were left partially submerged at regular intervals (10 meters) for two hours. *Cerastoderma glaucum*, *Mytilus galloprovincialis* and *Hediste diversicolor* were collected from five cylindrical soil samples (15 cm height, 15 cm diameter) using a stainless-steel corer (Fig. 2). These four species were defined as the most representative and abundant organisms per trophic level across all the three selected areas, although not all species were found in all lagoons and in all sampling sites. Particularly *Mytilus galloprovincialis* occurred only in the first site of Baiona lagoon and in the first two sites of Bellocchio lagoon, while *Cerastoderma glaucum* was occurrent in all lagoons and sites except for the first two sites of Piallassa Baiona. *Carcinus aestuarii* and *Hediste diversicolor* were found in all areas and sites. 10 individuals of bivalves and polychaetes (when available) and 20 individuals of *Carcinus aestuarii* (see section 2.2.) per site were chosen, stored at -20 °C and then processed for microplastics analysis.

Figure 2: Stainless steel corer. Tube lenght: 40 cm. Handle lenght: 65 cm. Tube diameter 12,5 cm.

2.1.2. Practices used to avoid contamination

An important part of the design and execution of the project consisted in developing field and laboratory practices to prevent any source of microplastics contamination. All the sampling equipment was cleaned prior to use, and every plastic component was annotated together with

its specific colour, in order to be able to trace any potential source of plastic contamination in the samples during the subsequent analysis.

Sample processing (digestion and filtering) was carried out in a “microplastic-clean” laboratory (Fig.3). All the lab surfaces and the floor were cleaned every time before starting the work, and external contamination was prevented by minimizing air circulation from the doors and using cotton curtains to isolate the processing area from the main entrance. For the same reason, a restricted number of personnel was allowed to enter in the clean lab.

Figure 3: The clean laboratory for samples processing.

Because of their ability to hover in air, fibres have a high contamination potential, and can cause overestimation problems during microplastic quantification (Hidalgo-Ruz *et al.*, 2012; Nuelle *et al*., 2014; Norén 2007). Therefore, we avoided wearing synthetic clothes in the lab and we opted for 100% cotton clothes and lab coats. Synthetic clothes were also avoided during the field sampling.

Prior to the organisms processing, tin foils were placed on the lab surfaces used for the experiment. Every instrument, including filters, were cleaned using bio-detergent and accurately washed with Milli-Q water, which was also used for rinsing the equipment every time before passing to a different sample during processing. Control blank filters with a 20µm nylon mesh (PLASTOK®) were positioned at various places in the lab to monitor potential air contamination in the biota, keeping them open approximately for the same time the samples

were exposed to air. To validate the effectiveness of our preventive practices and to test the potential of different digestion methods to expose the samples to possible external contamination, 4 procedural blanks containing SDS + enzymes and KOH were performed for each batch of organisms processed.

Samples and instruments were covered in tin foils whenever possible to minimize the time of exposure to air. Coloured nitrile gloves were used during all processing in order to allow the recognition of their potential contribute to samples' contamination during the visual analysis.

Visual analyses were performed using a stereomicroscope (50X magnification). Methods to avoid samples' contamination in this phase included the cleaning of the work surfaces, the standardization of the inspection time, the use of thin foil to cover the samples when not analysed and the use of non synthetic clothes only.

2.1.3. Digestion

Ten samples per site were defrosted and the organisms were rinsed with Milli-Q water prior to dissection to reduce potential contamination from attached particles. Bivalves' (*C. glaucum* and *M. galloprovincialis*) shells lengths were measured with a gauge, and the soft tissues were transferred to a glass beaker for the digestion procedures. Crabs' carapaces were measured and the animals were dissected using scissors and a metal scalpel in order to extract all the gastrointestinal tract from mouth to anus (Fig.4 A-B), being careful to avoid the gills and, when possible, the hepatopancreas. For *Hediste diversicolor*, the body length was measured and the entire organism was used for digestion. The soft tissue removed from each animal was transferred in the respective clean beakers and the wet weight was measured with the electronic scale (KERN 440-445).

70 *C. glaucum*, 30 *M. galloprovincialis*, 90 *C. aestuarii* were processed following the enzymatic digestion protocol. Other 90 *C. aestuarii* were processed with the KOH method (Fig.4 C) (see section 2.2.). Given that, the comparison between the two methods on *C. aestuarii* indicated no substantial differences between the two extraction methods but a significant lower cost of KOH (see next session 2.2 and results) the remaining last 90 *Hediste diversicolor* were analysed with KOH.

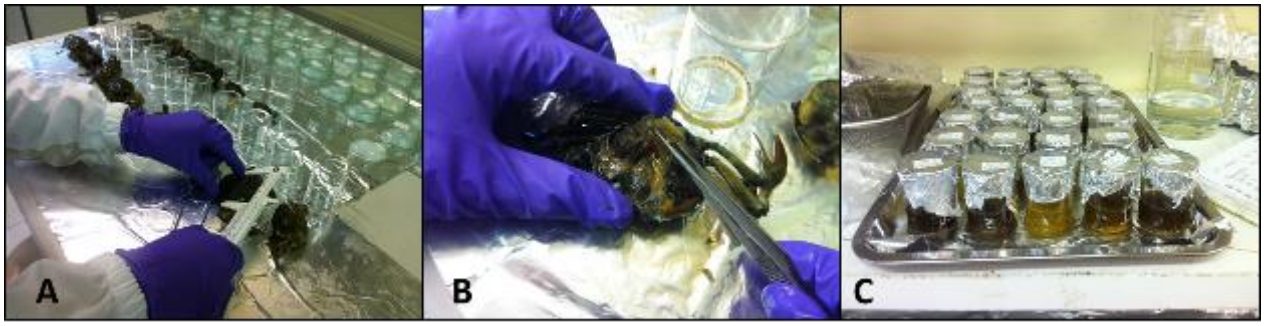

Figure 4: Organism measurement (A), dissection (B) and digestion (C).

For the enzymatic digestion, 10 ml of a 320g/l solution of Sodium Dodecyl Sulfate (SDS) (pre-filtered twice through Whatman Grade-1 filter paper) were added to each sample and stirred with a glass rod. Samples were then incubated for 24h at 70˚C. An enzymatic mixture of lipase, protease and amylase (5 ml each of commercially available detergent Biozym F and SE, filtered twice through Whatman Grade-1 filter paper) was added to the samples, that were then left two days at room temperature.

For the KOH method, 1M potassium hydroxide (KOH) water solution was added to each sample. Quantity of the KOH solution varied depending on species, to approximate three times the organisms tissue volume: 20 ml were added to each crab sample and 5 ml to each *H. diversicolor* sample. Crabs and Polychaetes samples were then stirred and left at room temperature from 1 to 3 days, depending on the tissue properties.

For each batch of animal processed (both for the enzymatic and the KOH digestion) four air filters and four procedural controls were prepared to monitor potential environmental and procedural contamination and for subsequent methods comparison (see section 2.2.2.).

2.1.4. Filtering

After the incubation period, each sample digested with enzymatic or KOH method was filtered with a vacuum filtering apparatus over a 20 µm nylon mesh filter (PLASTOK®). Every filter was previously cut to size (5 cm diameter), and then carefully cleaned, washed with Milli-Q water and visually checked for fibres or particles contamination before use. Each beaker containing samples or procedural controls was first emptied in the vacuum filtering apparatus and then rinsed three times with Milli-Q water to collect all possible microplastics. When passing to a new sample, the filtration funnel was always washed and rinsed with Milli-Q water and covered

on top with a tin foil to avoid contamination from other samples and from the environment. Each filtered sample was then positioned on a clean petri dish using metal tweezers, being sure to maintain it in a horizontal position to avoid possible particles loss. Each petri dish was covered with its cap and labelled with the respective sample code. Samples were left to dry at room temperature and then sealed for subsequent analysis.

2.1.5. Visual analysis and selection

Dried sample filters, procedural control filters and air control filters (Fig.5) were first analysed visually under a stereomicroscope (Leica microsistems) at 50 X magnification to isolate particles suspected to be microplastics for further analysis using the micro-FTIR spectroscopy (see section 2.1.6.).

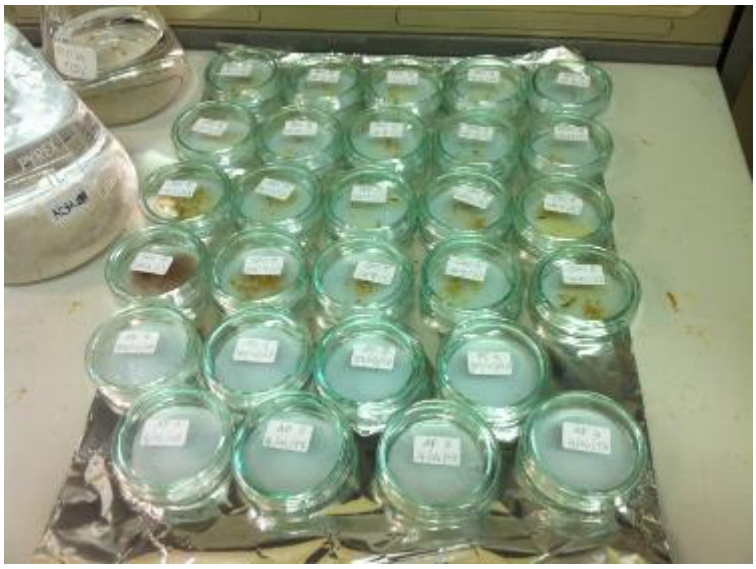

Figure 5: Dried sample filters, procedural control filters and air control filters in their clean petri dish.

The particles selection was based on the Guide to Microplastic Identification (Marine & Environmental Research Institute, online at http://stjohns.ifas.ufl.edu/sea/documents/MERI_Guide%20to%20Microplastic%20Identification.pdf) and following the identification rules by Hidalgo-Ruz *et al*., (2012): particles have to be less than 5 mm, showing no cellular or organic structure, fibers should be equally thick throughout their entire length, no twisted structure and, in the end, particles should exhibit clear (sometimes bright) and homogeneous colour throughout. However, some exceptions to

the previous rules due to weathering or biofouling actions have to be taken in consideration (Turner *et al*., 2011).

Visual analysis was performed for a standardised amount of time: the first 5 minutes (up to 10 minutes for filters rich in residuals and/or sediment) were dedicated to count all the anthropogenic fibres and to identify and isolate those suspected to be synthetic. Fibres were counted and isolated as a first step in order to reduce the risk of overestimation due to airborne contamination. Then the analysis continues for other 5 up to 10 minutes looking for fragments and other microplastic categories.

Particles were manipulated using needles or tweezers to identify and thus exclude non-plastic elements like glass, sand, minerals or shells, which tend to break when touched while plastic particles do not. All particles suspected to be plastic were isolated on a marked region of the filter and then counted and categorised based on colour and morphology (Hanke *et al.*, 2013). Fibers and fragments were finally photographed and measured digitally (length for the former, length and width for the latter) using the MotiConnect 1.5.9 software installed on a camera (moticam) connected to the stereomicroscope. Photos were labelled and stored for future reference.

2.1.6. Polymers characterization and particles quantification

Selected particles were then transferred onto a gold slide and spectroscopically characterized in terms of polymer typology using the micro-FTIR (Thermo Fisher-Scientific) in attenuated total reflectance mode (ATR) (Harrison *et al*., 2012) (Fig.6).

Figure 6: A model of an FTIR spectroscopy.

This technique, based on infrared radiation, enables to obtain a characteristic absorbance spectrum of an unknown material that can be compared to customised libraries, leading to the polymers identification. To be sure to get reliable spectra, at least two measurements were taken from each sample. The resulting ATR spectra of our selected particles were therefore compared with the ones collected in databases (http://www.ftir-polymers.com/soon.htm, http://lisa.chem.ut.ee/IR_spectra/textile-fibres/) using the Spectragryph software 1.0.6. All particles identified as microplastics were annotated with the relative polymer type and counted. Also all non-synthetic anthropogenic fibers were counted for subsequent analysis.

2.1.7. Statistical analysis on microplastics quantifications

The proportion of organisms with microplastics in their digestive tract was calculated for: (i) all species individually, (ii) all organisms and (iii) spatial distribution of all individuals containing plastics. The average of microplastics ingested was calculated per organism. The frequency of ingestion for species and for organisms caught in different areas was compared with the non-parametric Kruskal-Wallis test as data were not normally distributed even after log(x+1) data transformation. As such, non-parametric analyses were conducted. All analysis were performed using the R software (R Core Team, 2014).

2.2. Comparison of different extraction procedures from marine organisms

2.2.1. Comparison of SDS/KOH extraction procedures on fibers

The effects of SDS-enzymatic and KOH digestion methods were tested on different types of plastic fibers: Polyester terephtalate (PET 3,3 dtex), Polyester of different colours and Polypropylene. Moreover, the effects of the two methods were tested at increasing times of exposure.

Plastic fibers (5 mm) were removed from known fabrics or plastic materials and placed in individual 20 ml glass scintillation vials. Fibers were exposed to either methods in duplicates with a total exposure time of one week. For the temporal analysis, fibers were picked up from vials after 1, 2, 3, 4 and 6 days of exposure. Untreated samples for each typology were used as controls.

Samples were inspected and photographed under a stereomicroscope before and after treatment. The visual impact of the degradation method was evaluated in accordance to the protocol developed by Enders *et al*. (2017) (Fig.7).

Level of impact	Description
L1	Initial visually recognizable changes (colour, surface morphology)
L2	Morphological changes and early stages of dissolution
L3	Strong morphological disintegration and change of bulk structure
L4	Complete dissolution or disintegration

Figure 7: Classification of the level of impact (LOI) of the degradation method on the plastic fibers from Enders et al. (2017). L0 corresponds to no change or unaffected.

All samples were then analysed using the ATR micro-FTIR spectroscopy (see section 2.1.6.) and the obtained spectra compared between time scales and with the no treated reference material.

2.2.2. Comparison in the amount of plastic and anthropogenic fibers occurent in SDS/KOH crabs samples, procedural controls and air filters

Both enzymatic and KOH digestion methods were used to break down the gastrointestinal tract of 90 crabs (see section 2.1.3.), giving a total of 180 soft tissue samples. All samples, including the respective procedural controls and air filters, were filtered (see section 2.1.4) and analysed under a stereomicroscope (see section 2.1.5.). After the ATR micro-FTIR analysis (see section 2.1.6.), all fibers present on filters were counted and annotated for each sample, discerning plastic fibers from non-plastic anthropogenic fibers.

2.2.3. Statistical analysis on the amount of plastic and anthropogenic fibers

The proportion and average of non-synthetic anthropogenic fibres resulted from the two digestion methods were calculated for: (i) organisms samples (ii) air filters (iii) procedural blanks. The frequency of airborne contamination for each group of filters from the two digestion methods was compared with the one-way ANOVA as data resulted normally distributed after log(x+1) transformation and exhibited homogenous variance (Cochran test P-value < 0.01) . As such, the parametric analyses were conducted.

To compare plastics levels in crabs samples processed with the two digestion methods, the average number of microplastics extracted per organisms was calculated (Lusher *et al*., 2017). As these data were not normally distributed even after log(x+1) transformation non-parametric Kruskal-Wallis test was applied. All analysis were performed with the software R (R Core Team, 2014).

2.3. Estimate of ingestion rates through the analysis of biodeposits

2.3.1. Sampling and study area

Sampling was performed in July 2017 at three sites located in the Ravenna harbour: Darsena (44°25'21.9"N; 12°12'49.8"E), the Yacht Club (44°29'32.6"N; 12°17'15.2"E) and the port entrance (44°29'33.7"N; 12°18'23.0"E) (Fig.8). Sites were chosen for their different exposure and hydrodynamic conditions. The Yatch club was the most protected area from currents and waves action with less water circulation. The port entrance was chosen as the most exposed site to hydrodynamic force while the Darsena site represents the in-between exposed site with no strong currents dynamic but located in one of the main channels of the Ravenna harbour. These different levels of exposition accompanied by urban drainage near the Darsena site, fuels and garbage coming from boats in the sheltered waters of the Yacht Club and the consistent amount of fuels and macro plastic debris from boats and port activities in the port entrance site, may expose the sites to different level of potential plastic pollution.

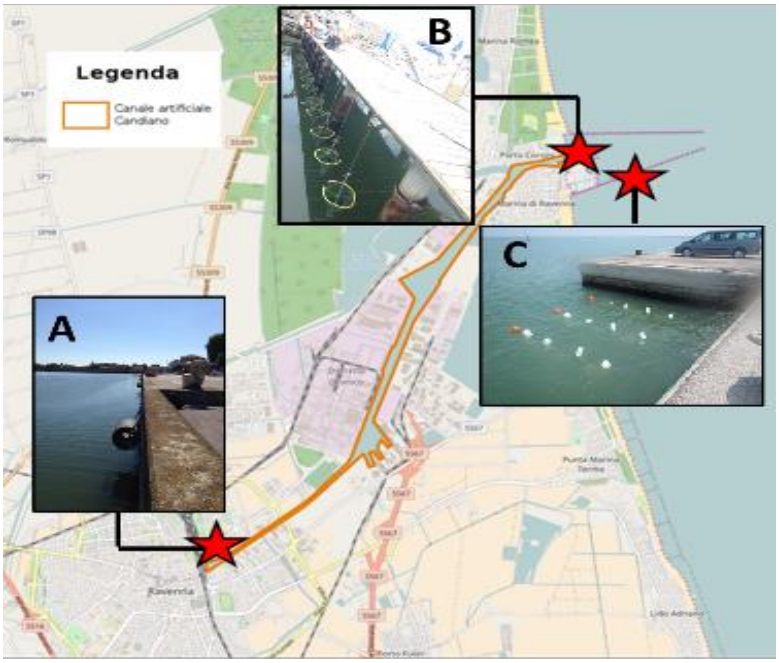

Figure 8: The three selected sampling site. Darsena (A), the Yacht Club (B) and the port entrance (C),

Wild mussels (*Mytilus galloprovincialis*) were collected from Marina di Ravenna using a metal scraper, while farmed mussels were taken from the Cesenatico aquaculture centre (Cooperativa Casa Del Pescatore A.R.L.).

Sampling was performed in one day per each site. All mussels needed for one site were left to acclimatize in situ for 24 hours before the start of the experiment (mussels for the port entrance sampling were left to acclimatize in the Yacht Club site to avoid potential damage or theft).

Before the experiment, mussels (wild and aquaculture) were weighted, cleaned, washed and divided to form eight different net bags, 1.5 Kg each. Other four net bags contained 1.5 Kg of empty mussel shells used as controls.

To collect biodeposit a specific made conical traps were used (referred in the text as "sample units" or "biodeposit traps"), inspired and modified on the model by McKindsey *et al*. (2009) (Fig.9).

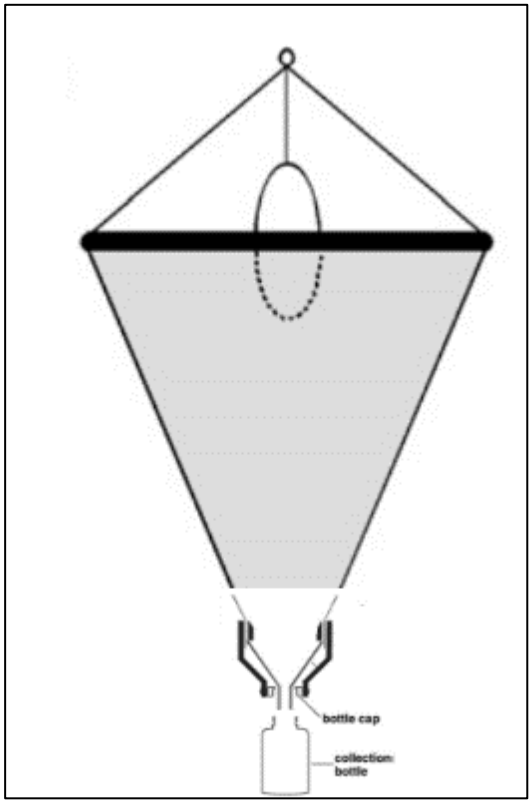

Figure 9: The cone trap model to collect mussels biodeposit developed by McKindsey *et al*. (2009).

Cones were approximately 70 cm high and 40 cm wide at the large opening and were made in polyethylene, fixed on a pipe ring (the large opening of the cone) using cable ties. 250 ml polyethylene containers were used to collect biodeposits and their tops were cut and attached at the narrow end of the cones to permit the passage of the fecal pellets and the screwing/unscrewing of the containers. Two strings of nylon rope were tied to the pipe ring to form a cross in the middle, in order to hook the net bags with the mussels, and four strings were bound to one side at the pipe ring and the other tied together with the other three to a hook. An additional nylon rope was bound to the hook and cut to be long enough to permit a 30 cm immersion of the cone under the water surface and to fasten the other end to a support. Three series of four equidistant holes (diameter 2.5 cm) were made along the cone height to allow the entrance and the exit of the water flow.

A total of 12 cones (4 with wild mussels type, 4 with aquaculture type and 4 controls) were placed randomly at each site to form a line (3 lines composed of 4 cones in the port entrance site). At least 1.5 m distance occurred between cones from their pipe ring border. Cones were always washed with fresh water before sampling to avoid contamination from environment and between sites.

Cones were left under water for an exposure time of 4 hours in each site and then recovered on land, being careful to avoid samples lost during the water emptying. The containers were cautiously unscrewed from the cones, closed with a cap and labelled.

Biodeposit, controls and water samples were stored at 4˚C and then filtered (see section 2.1.4.) and the average weight of clean filters was annotated in order to obtain the dry weight from all biodeposit and control samples, using the electronic scale (KERN-770). All samples were then visually examined at the stereomicroscope (50X magnification) (see section 2.1.5.) and the isolated particles were analysed with the ATR micro-FTIR spectroscopy (see section 2.1.6). No digestion method was applied to these samples.

2.3.2. Statistical analysis on microplastics found in biodeposit

The number of microplastics was calculated for all sample units across sites and treatments (mussels and controls). In order to provide additional information about the microplastics concentration in the water column the number of microplastics per liter was calculated in each site.

The differences in the number of microplastics and quantity of biodeposit in sample units across sites and treatments (mussels types and controls) were compared with permutation non-parametric univariate analysis of variance. The resemblance matrix between observation was constructed based on Euclidean distances.

Spatial distribution of the microplastics size classes (since other microplastics properties resulted homogeneous among the particles majority) across sample unit containing mussels and controls were analysed using principal coordinate analysis (PCO) based on Bray-Curties dissimilarities. Differences in microplastics size composition between sites and sample units containing mussels and controls were assessed by permutational non-parametric multivariate analysis of variance (PERMANOVA). Differences in microplastics size composition between different levels (wild, farmed and controls) of the factor “mussel type” within sites were assessed with the PERMANOVA analysis and represented with principal coordinate analysis (PCO) ordination method. All the analysis were run at $\alpha=0.01$ (with the exception of microplastics and biodeposit quantification, in which the analyses were run at $\alpha=0.05$) significance test and performed with package PERMANOVA+ with PRIMER 6.0 software (Clarke and Green, 1988; Clarke, 1993).

3. Results

The rigorous precautions adopted while handling and processing the samples in all the phases of the work effectively prevented any airborne contamination with microplastics since no plastic particles were found in air filters and procedural blanks.

3.1. Microplastics occurrence at different trophic levels

3.1.1. Microplastics characterization and quantification

Crabs (*Carcinus aestuarii*) and polychaete worms (*Hediste diversicolor*) were common species at all the selected sites, whereas the bivalves *C. glaucum* and *M. galloprovincialis* were patchily distributed across sites. This heterogeneous distribution resulted in a different number of sampled specimens per lagoon: 110 organisms were sampled from Piallassa Baiona, comprised of 60 crabs, 30 *Hediste diversicolor*, 10 *C. glaucum* and 10 *M. galloprovincialis*; 140 organisms were sampled from the Bellocchio Lagoon, comprised of 60 crabs, 30 polychaete worms, 30 *C. glaucum* and 20 *M. galloprovincialis* and 120 organisms were sampled from the Chioggia Lagoon, constituted of 60 crabs, 30 polychaete worms and 30 *C. glaucum.* No *M. galloprovincialis* was found in this lagoon.

After the microscopic analysis on the total amount of digested individuals sampled from the 4 target species at the 9 sites (3 sites per lagoon), many isolated particles (n=203) were confirmed to be microplastics by the FTIR analysis. The FTIR spectra of some plastic particles with different polymer types and the relative particles pictures from the stereomicroscope analysis are shown in Fig. 10, 11 and 12. The other particles analysed comprised natural materials, such as cotton, wool, viscose or carbonate material.

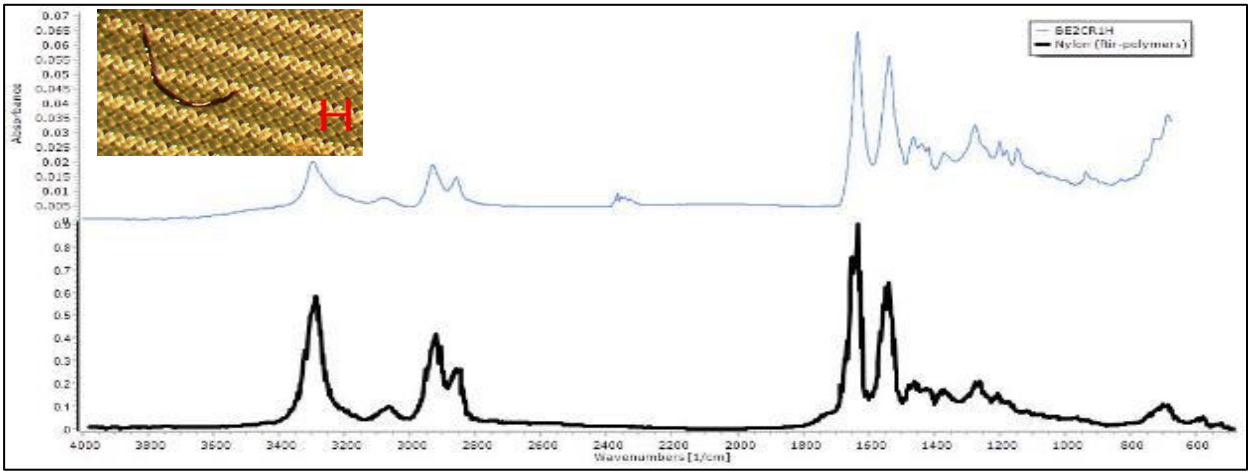

Figure 10: Spectra comparison. Sample fiber BE2CR1H (blue line) and Nylon reference spectra (black line).

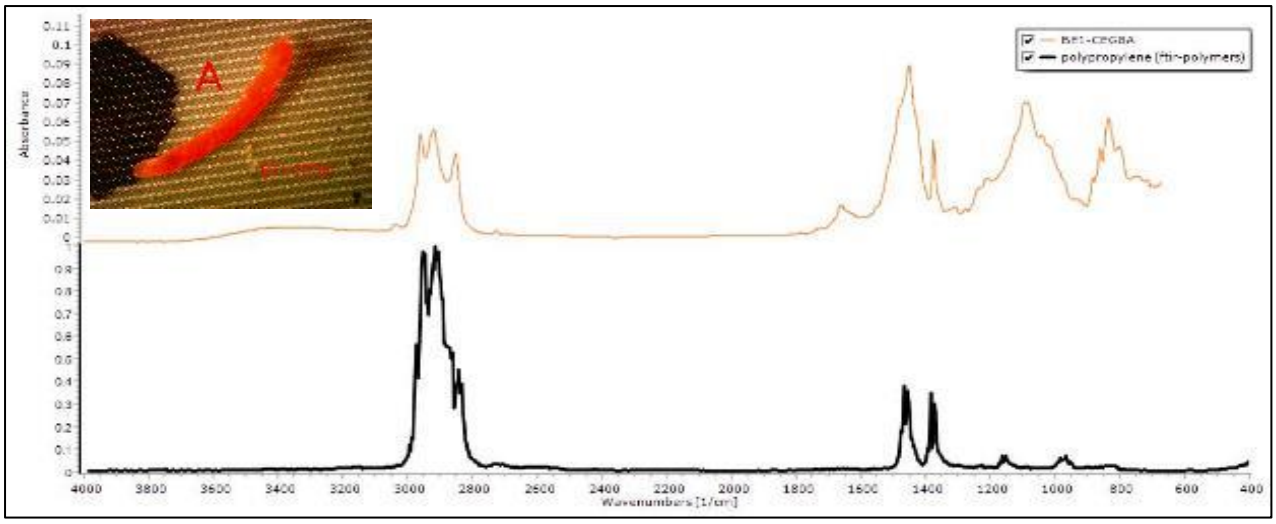

Figure 11: Spectra comparison. Sample fiber BE1CEG8A (beige line) and Polypropylene reference spectra (black line).

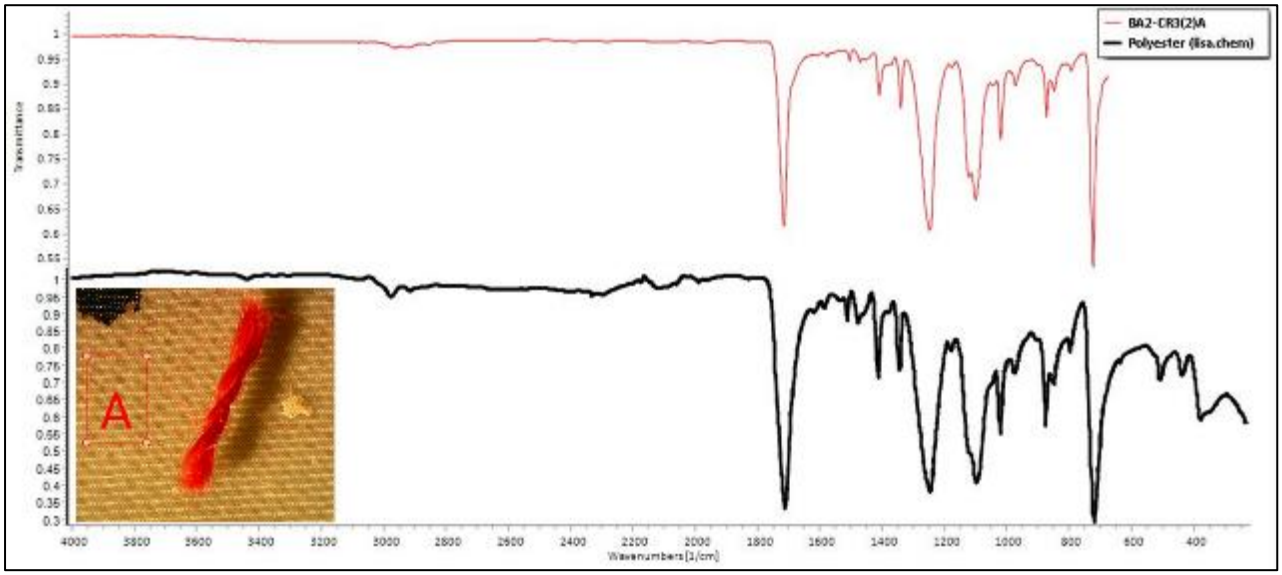

Figure12: Spectra comparison. Sample fiber BA2-CR3(2)A (red line) and Polyester reference spectra (black line).

All microplastics found were ascribable to 4 different polymer types: Polyester (n=200), Polypropylene (n=1), Polyamide (industrial name: Nylon) (n=1) and Acrylonitrile (industrial name: Orlon) (n=1). The results are represented in Fig.13.

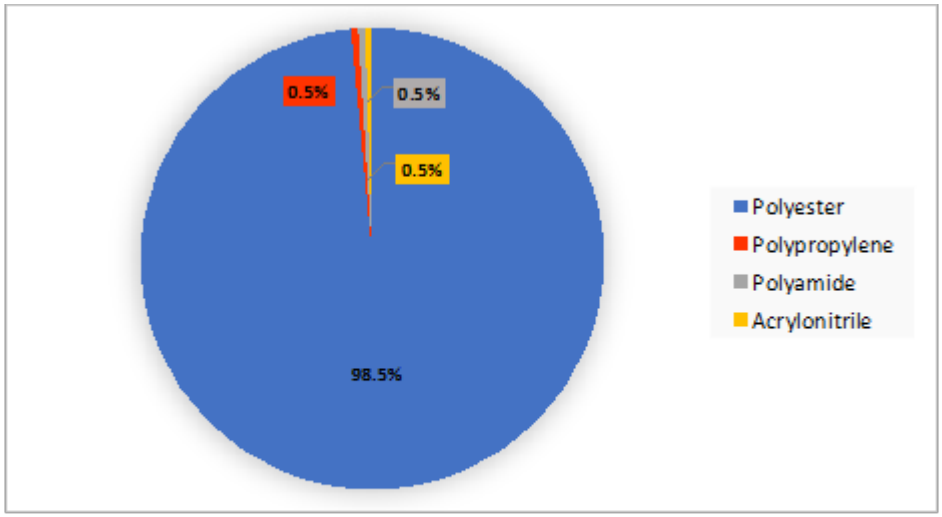

Figure13: Percentage of polymer types found across all the specimens.

All particles confirmed to be microplastics were measured and divided into 4 size classes (0-100µm, 100-500µm, 500-1000µm and 1000-5000µm) following the model developed by Avio *et al*. (2015). Most of the plastic particles (n=124) were between 1000 and 5000 µm long, followed by microplastics (n=76) ranging between 500 and 1000 µm. Only few particles (n=3) were between 100 and 500 µm long. No particles were less than 100µm long. Results are represented in Fig.14.

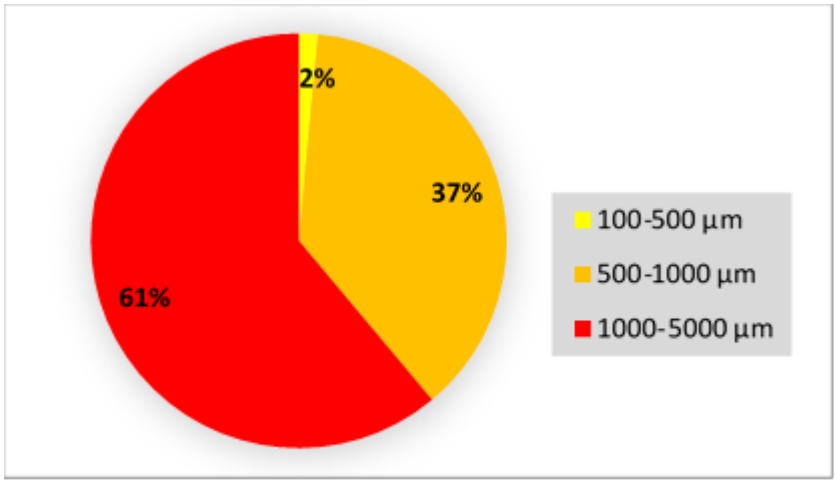

Figure 14: Percentages of size classes of microplastics found in organisms.

Amount and characteristic of microplastics are represented in Table 1.

Table 1: Number and shape of microplastics per species					
Species	**N organisms analysed**	**N organisms containing MPs**	**Plastic particles**	**Fragments**	**Syntethic fibre**
C. galucum	70	1	1	1	0
M. galloprovincialis	30	1	1	0	1
C. aestuarii	180	11	201	0	201
H. diversicolor	90	0	0	0	0

Except for two crabs individuals, all organisms containing microplastics contained a maximum of one particle each and the majority of organisms did not contain any microplastics (Fig.15).

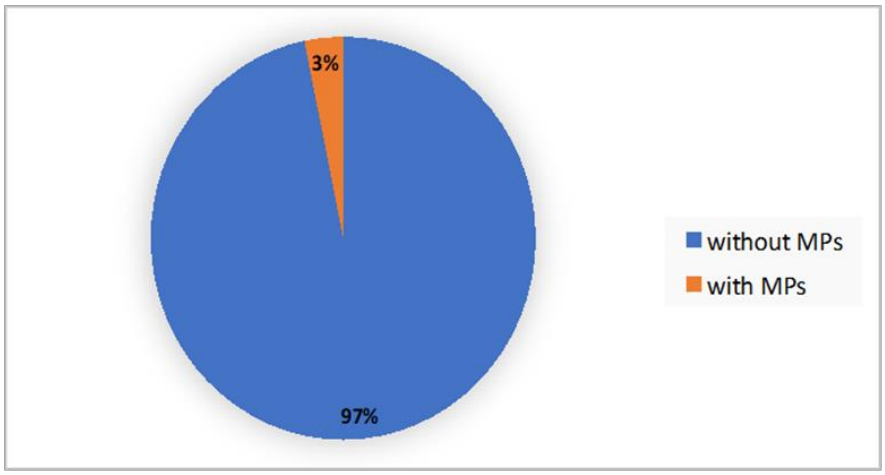

Figure 15: Percentages of organisms with and without microplastics.

Only one particle was a fragment while the others were all plastic microfibers. Only 1 out of 70 *C. glaucum* specimens contained 1 plastic fragment (Polypropylene), resulting in an average of 0.01 (SE=±0.01) plastic particles per organism and 1.1% of individuals containing microplastics. Only 1 out of 30 of *M. galloprovincialis* contained 1 plastic microfibre (Polyester) with an average density of 0.03 (SE=±0.03) plastic particles per organism and 3.3% of animals containing microplastics. Seven out of 180 individuals of *C. aestuarii* contained 1 plastic microfiber each (1 in Polyamide "Nylon", 1 in Acrylonitrile "Orlon" and 5 in Polyester material) while 3 individuals contained an agglomerate of plastic microfibers, 76 and 117 Polyester plastic microfibres respectively. This led to an average of 1.12 (SE=±0.77) particles per organism and 5.6% of individuals containing microplastics. All characteristics of microplastics found in organisms are summarised in Table 2.

Table 2: Characteristics of microplastics particles found in each individual in four benthic species from salt marsh habitats. (* mean of a subsample). BA: Baiona, BE: Bellocchio, CHI: Chioggia

Species and site	Carapace/ Shell/ body length (cm)	Gut wet weight (g)	Polymer type	Characteristics	Length (µm)	Width (µm)
C. glaucum (BE1)	1.5	1.9	Polypropylene	Orange fragment	2971	409
M. galloprovincialis (BE3)	3.9	2.3	Polyester	Blue fibre	1192	
C. aestuarii (BE2-1st batch)	4.1	1.1	Polyamide	Black fibre	202	
C. aestuarii (BE3-1st batch)	2.3	0.2	Acrylonitrile	Black fibre	193	
C. aestuarii (BE3-1st batch)	2.4	0.2	Polyester	Red fibre	825	
C. aestuarii (CHI2-1st batch)	4.0	1.8	Polyester	Black fibre	2556	
C. aestuarii (BA2-2nd batch)	4.7	1.1	Polyester	Red braid	3421	
C. aestuarii (BA2-2nd batch)	4.9	2.1	Polyester Polyester	Black fibre 75 red fibres	1512 551 *	
C. aestuarii (BA3-2nd batch)	3.2	0.6	Polyester	117 red fibres	2973 *	
C. aestuarii (CHI1-2nd batch)	4.8	3.0	Polyester	Transparent fibre	1799	
C. aestuarii (CHI1-2nd batch)	4.9	1.2	Polyester	Black fibre	2040	
C. aestuarii (CHI3-2nd batch)	5.1	0.5	Polyester	Grey fibre	323	

No plastic particles were found in any of the 90 *Hediste diversicolor* organisms. Results are represented in Fig.16 and Fig.17. Overall, the amount of microplastics in the gastrointestinal tract did not differ significantly between species, due to the large variability between individuals (Kruskal wallis non-parametric test, P-value > 0.01).

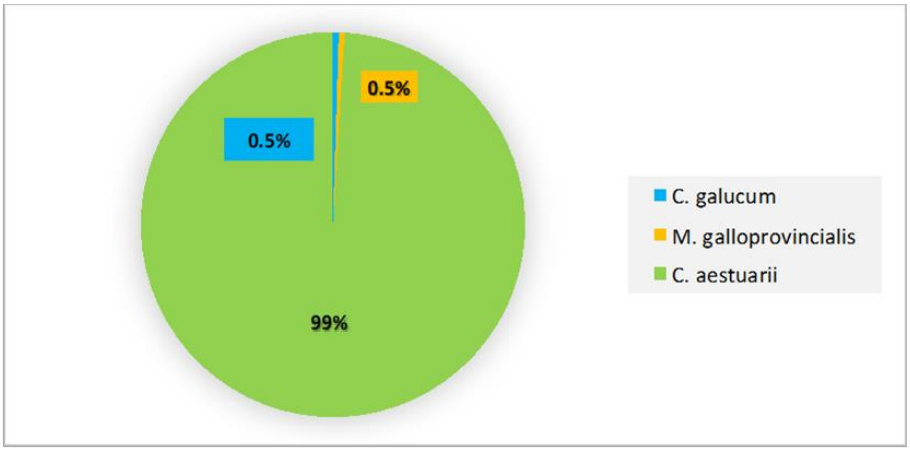

Figure 16: Percentages of microplastics found in the target species species. *H. diversicolor* is not indicated as no microplastics were found.

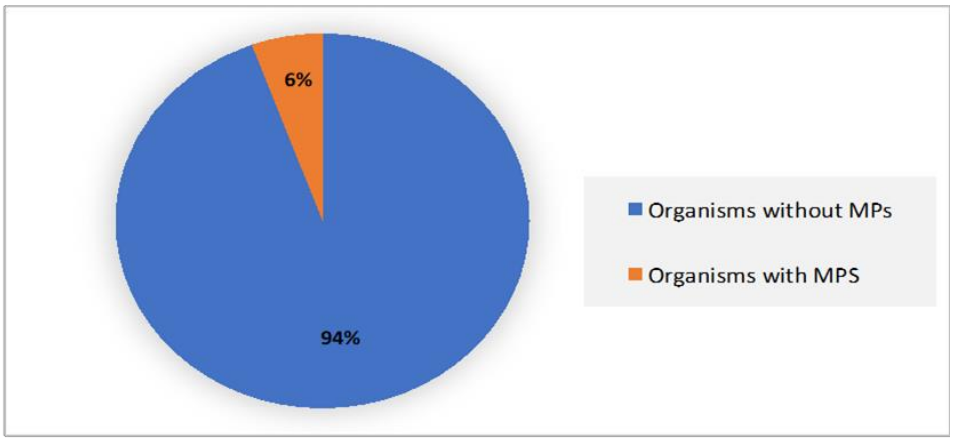

Figure 17: Percentages of crabs *(C. aestuarii) (N=180)* containing microplastics.

3.1.2. Microplastics spatial distribution

Some microplastics were found in each species with the exception of *H. diversicolor,* and in organisms coming from all the three selected areas. However, the number of individuals with microplastics was so low that it was not possible to make a formal statistical analysis of spatial distribution patterns for any of the target species. Overall, Piallassa Baiona was the lagoon with the highest number of microplastics (n=194) ingested by the organisms with an average density of 1.76 (SE=±1.26) microplastics per organism and 2.7% of animals containing microplastics, followed by Bellocchio (n=5), 0.04 ± 0.02 (SE) microplastics per organism and 3.6 % animals containing microplastic and Chioggia (n=4), 0.03 ±0.02 (SE) microplastics per organism and 3.3% of animals containing microplastic (Fig. 18).

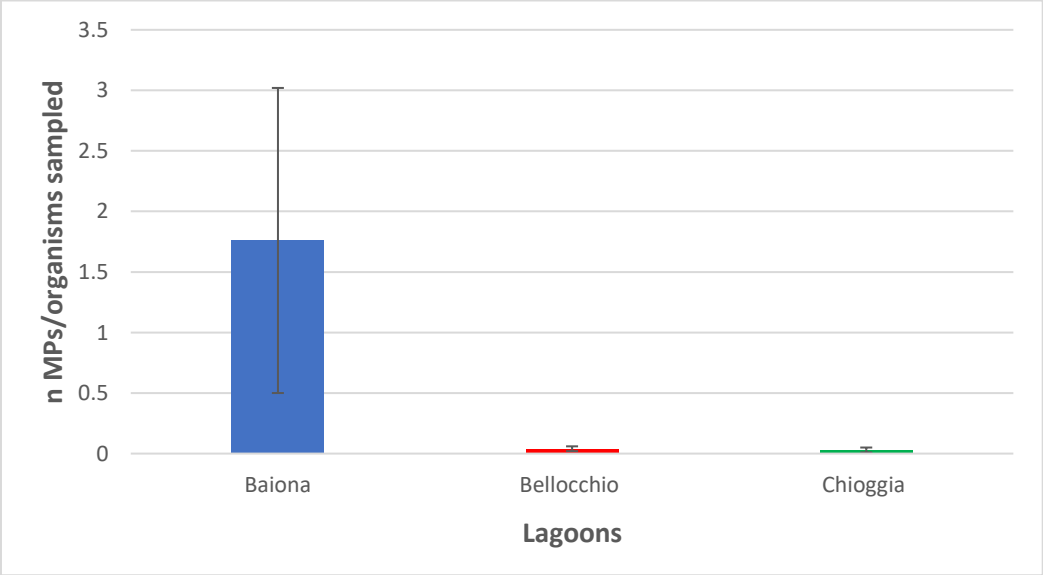

Figure 18: Average (±SE) abundance of microplastics found in organisms in the three study lagoons (merged across 3 sites for each lagoon).

The largest number of microplastics at Baiona was due to two individuals crabs from the Baiona 2 and Baiona 3 sites in which were found 76 and 117 plastic fibres respectively. This suggests an extremely patchy distribution of microplastics, where the majority of individuals do both present any microplastics while very few individuals present very high numbers.

3.2. Comparison of different extraction procedures from marine organisms

3.2.1. Effects of SDS/KOH method and exposure time on fibers

In the test with the microfibre polymer types, visual inspection of fibres extracted using enzymatic digestion in combination with SDS showed no visible damage or change in any of the fibers (level of impact L0, corresponding to no change or unaffected.). The KOH digestion method had a slight negative effect resulting in little changes in the fibre structure and discoloration (level of impact L3) only on PET fibres exposed to the treatment for the longest exposure of 6 days in comparison with no treated material, while no visual change was observed in Polyester (PS) and Polypropylene (PP) fibres (Fig.19) or on PET fibers exposed to shortest periods.

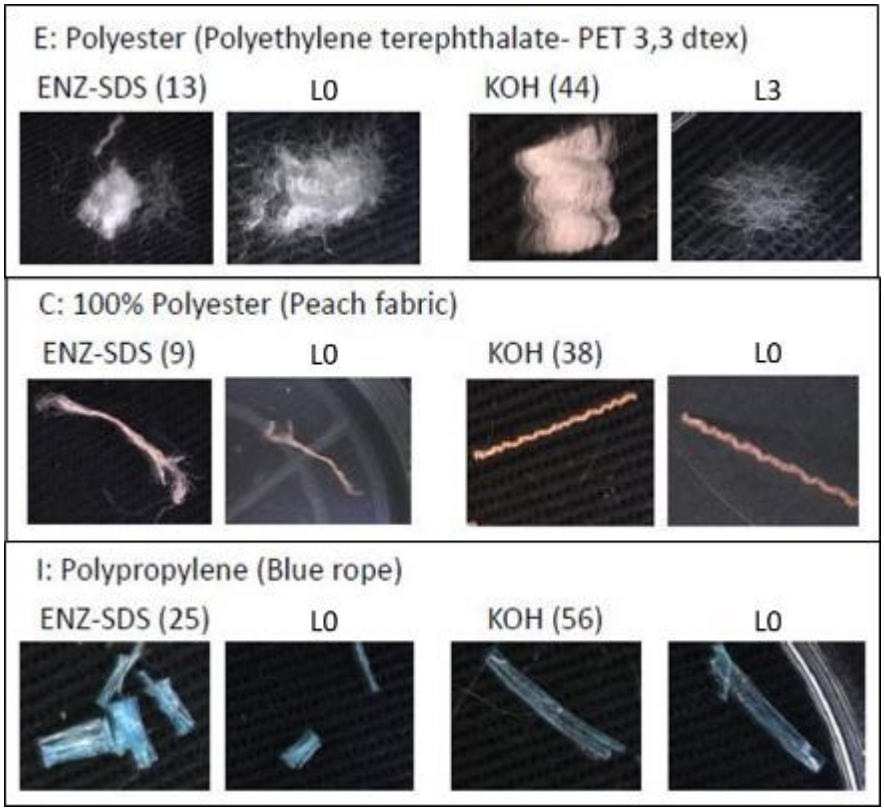

Figure 19: Polyester PET, Polyester and Polypropylene fibers (5 mm in length) before and after treatment with the SDS-enzymatic method and the KOH (1M) method.

The FTIR spectra showed no visible difference between fibers treated separately with the SDS-enzymatic method and the KOH method or any evidence of chemical alteration that may act on the FTIR analysis and on the subsequent polymer characterisation (Fig.20). No visible differences emerged between spectra from KOH treated materials at different time scale (Fig.21).

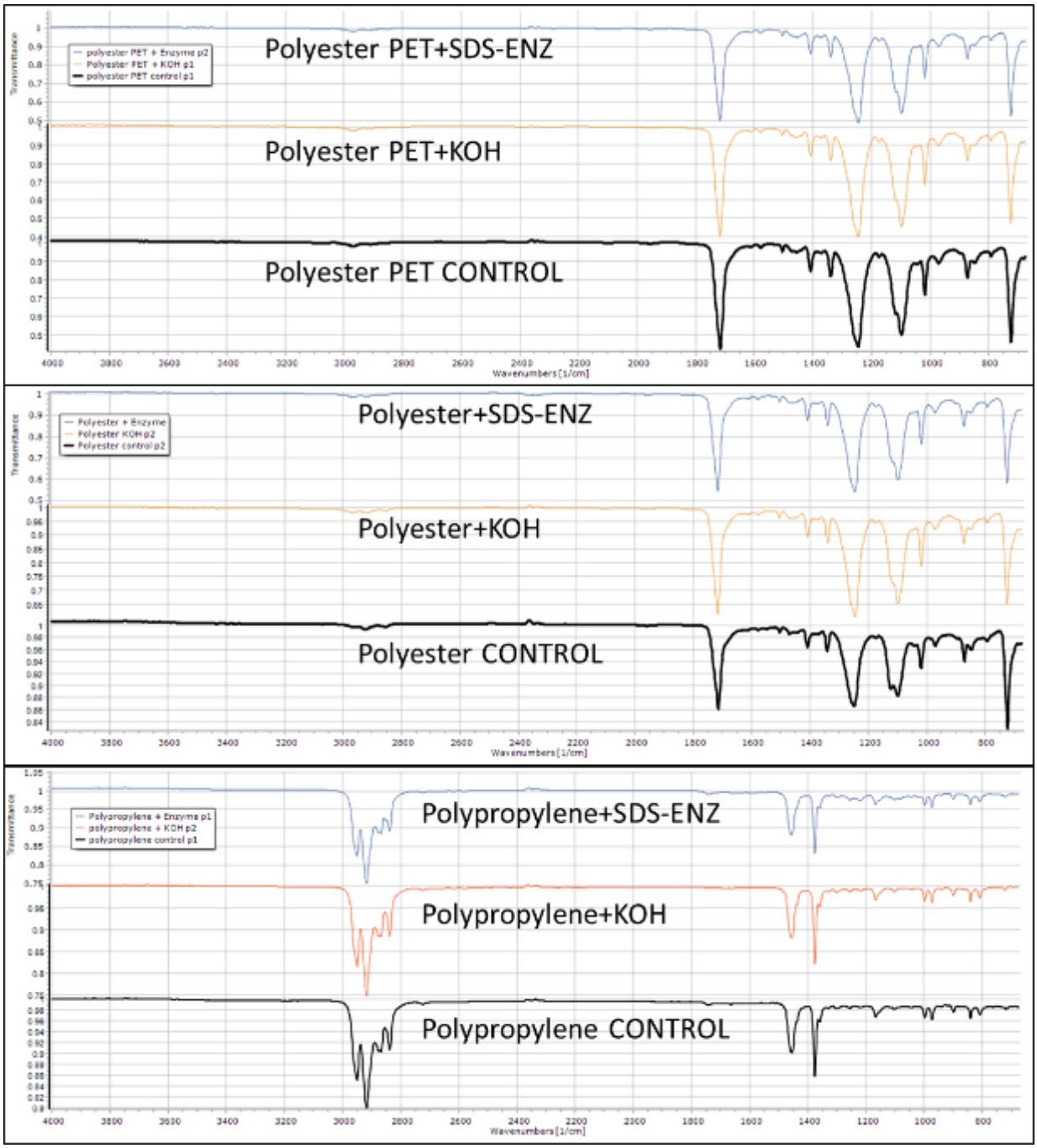

Figure 20: Polyester PET, Polyester and Polypropylene fibers spectra after 6 days treatment with the SDS-enzymatic method (blue lines) and the KOH (1M) method (beige lines) compared with the spectra of the respective no treated material (black lines).

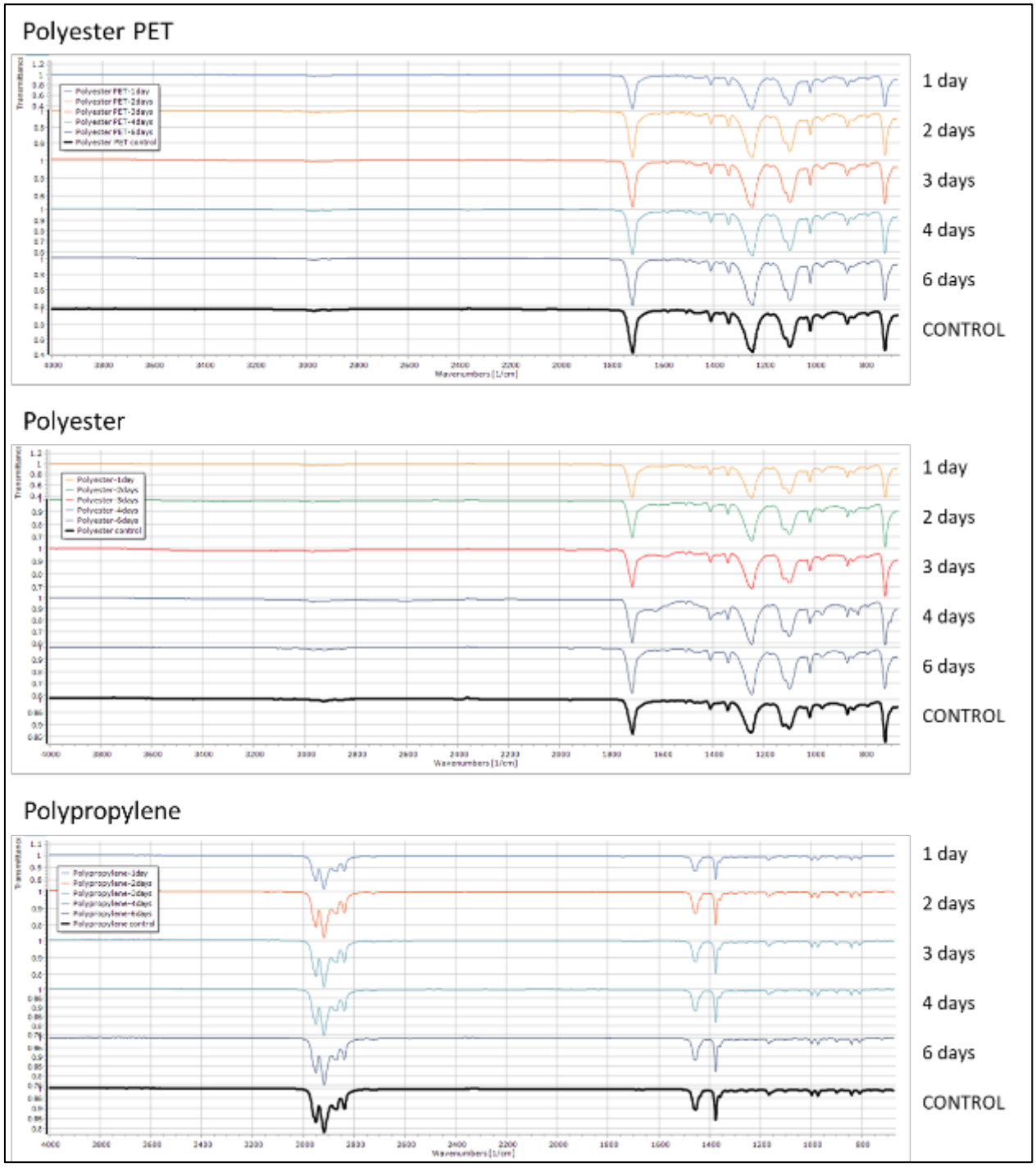

Figure 21: Polyester PET, Polyester and Polypropylene fibers spectra after different time scales (1-6 days) of KOH (1M) exposure.

3.2.2. Comparison in the amount of plastic and non-synthetic anthropogenic fibres found in SDS/KOH treated crabs samples, procedural controls and air filters

The comparison between SDS-enzymes and KOH methods in the amount of microplastics found in crabs (*C. aestuarii*) reported an average number of 0.04 (SE=±0.02) plastic particles per organism digested with the SDS enzymatic method and 2.19 (SE=±1.54) plastic particles per organism digested with the KOH method (Fig.22).

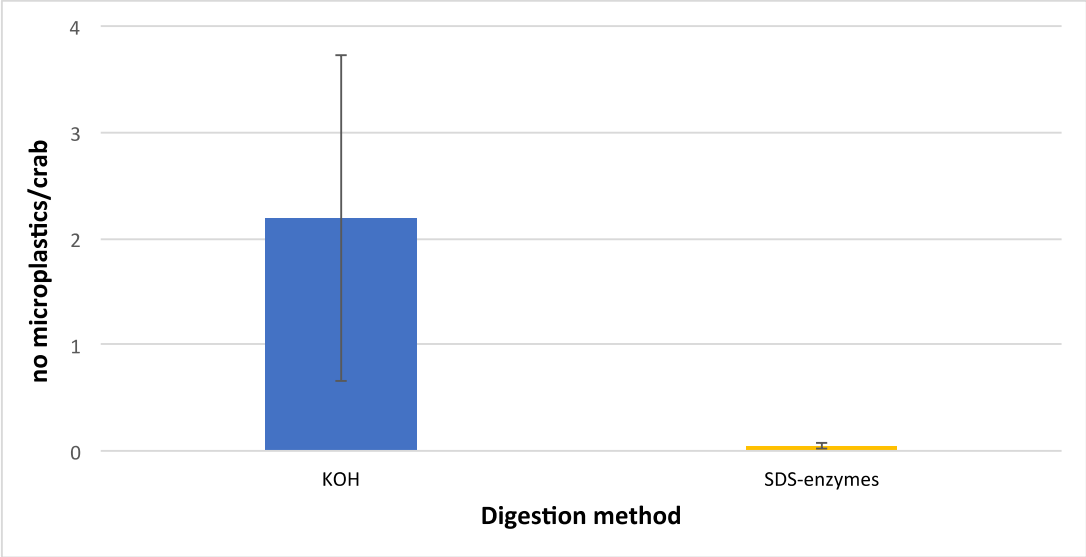

Figure 22: Comparison between SDS-enzymes and KOH methods in the amount of microplastics found in crabs (*C. aestuarii*) digested with the respective solution.

Although the average number of KOH-extracted microplastics particles per crab was much larger than the average number of SDS-enzymes extracted particles (Fig.22), the difference was not statistically significant according to Kruskal-Wallis non-parametric test due to the extremely large variability among individual crabs (P-value > 0.01).

Comparison between lengths divided per size classes of microplastics found in crabs with the SDS-enzymatic method and the KOH method resulted in 2 particles belonging to the 100-500 µm size class, 1 to the 500-1000 µm size class and 1 to the 1000-5000 µm size class for the former and 1 particle belonging to the 100-500 µm size class, 75 to the 500-1000 µm size class and 121 to the 1000-5000 µm size class for the latter (Fig.23).

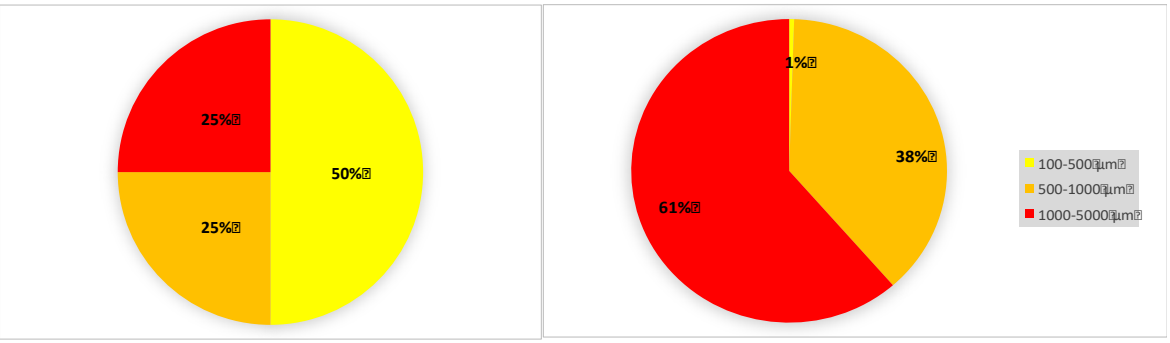

Figure 23: Percentages of size classes of microplastics measured in samples of crabs digested with the SDS-enzymatic (left) and the KOH (right) methods.

The average number of anthropogenic non-synthetic fibers in SDS enzymatic-treated crabs, procedural control and air control filters was 4.56 (SE=±0.38), 3.46 (SE=±0.39) and 4.33 (SE=±0.37) respectively, and 6.33 (SE=±0.60), 3.1 (SE=±0.35) and 1.56 (SE=±0.26) respectively in the KOH treated crabs (Fig.24). No statistically significant differences were registered between the two methods (one-way ANOVA p-value > 0.01).

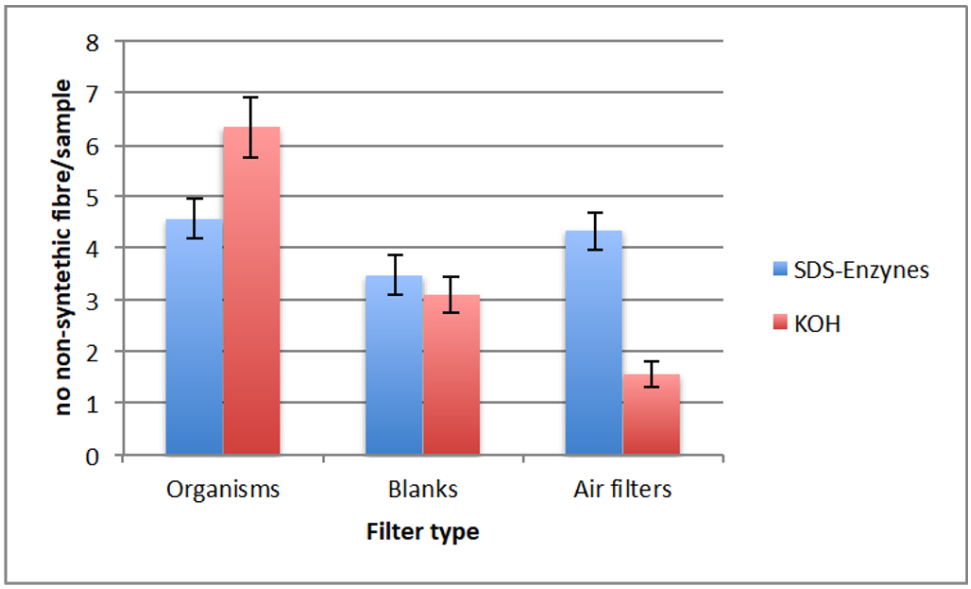

Figure 24: Comparison between SDS-enzymes and KOH methods in the amount of non-synthetic anthropogenic fibers present in crabs (*C. aestuarii*) organisms, procedural control and air control filters.

SDS-Enzymatic digestion takes approximately 5 days (120 hours) to digest crab gastrointestinal tract samples while the KOH digestion takes 3-4 days depending on the amount of organic tissue to digest. For the enzymatic digestion there are more restrictions for the incubation temperature, since samples need to be incubated at 60°C after the SDS addition, then again at room temperature before the addition of enzymes. Incubation following enzyme addition for 48h should not be lower than 20°C as low temperatures cause solidification of the mixture, which presents difficulties during the filtering and particle isolation steps. Enzymatic digestion requires three steps in the addition of reagents (SDS + 2 Enzymes), while KOH can be added in just one step and the samples do not need to be opened again until the filtration step. Moreover during the filtration process the SDS-Enzymes solution may creates foam that need repetitive Milli-Q water rinsing to pass through the filter. Thus, the KOH method is more straightforward and less time-consuming.

Finally, the KOH method is less expensive than the SDS-Enzymatic method, costing €1.50 vs €42.28 for the digestion of 90 crabs.

3.3. Estimate of ingestion rates through the analysis of biodeposits

3.3.1. Microplastics characterisation and quantification in biodeposit samples

Most of the particles analysed with the FTIR spectroscopy were made of synthetic materials, leading to a total of 76 microplastics found in all the biodeposit and controls containers and 14 in all water samples (90L). Microplastics from biodeposit included only 3.3% of fibers (2 fibers found in wild mussel biodeposit containers), while all the other 96.7% were plastic fragments (n=60). All microplastics (n=14) from controls were fragments. Microplastics from water samples, comprised 86% of fragments (n=12) and 14% of fibres (n=2). Among all plastics items found in biodeposit, controls and water samples (n=90) 88.9% were comprised of blue items.

The FTIR spectra of two fragments from the biodeposit samples and the respective pictures from the stereomicroscope analysis are shown in Fig.25 and Fig.26.

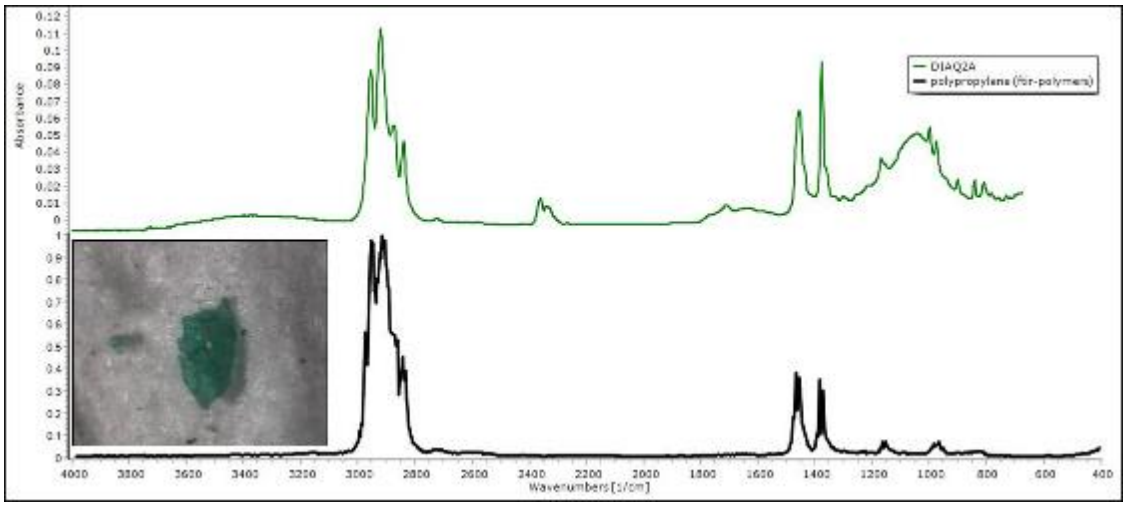

Figure 25: FTIR Spectra comparison. Sample DIAQ2A (green line) and Polypropylene (black line).

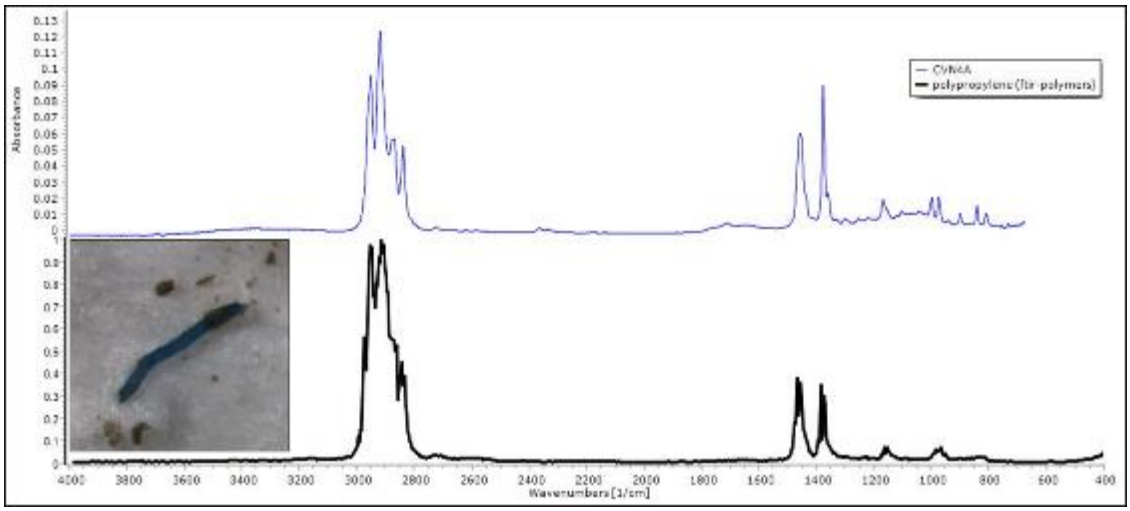

Figure 26: FTIR Spectra comparison. Sample CVN4A (blue line) and Polypropylene (black line).

All microplastics were ascribable to five different polymers: Polypropylene (n=83), Polyester (n=4), Polyacrylonitrile (n=1), Polyamide (n=1) and Polyacrylic (n=1). 58 microplastics found in biodeposits were Polypropylene, followed by 2 particles of Polyester, 1 particle of Polyacrylonitrile and 1 particle of Polyamide (Fig.27). The last three polymer types were only found in wild mussels biodeposits, while microplastics from farmed mussels biodeposits were all Polypropylene, as well as all 14 microplastics found in controls.

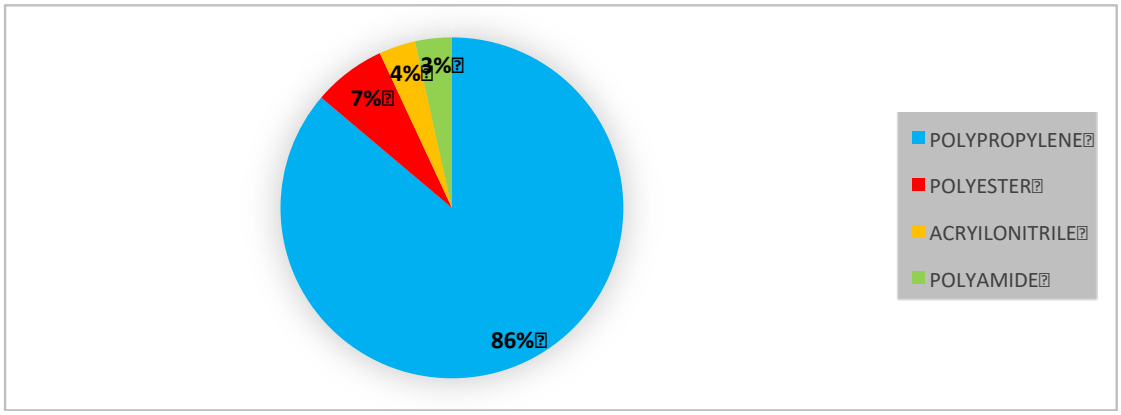

Figure 27: Percentages of polymer types of all microplastics found in wild mussels biodeposit containers.

Water samples from all three sites (90L) contained a total of 14 microplastics, 10 of which were Polypropylene, 2 Polyester and 2 Polyacrylic (Fig.28).

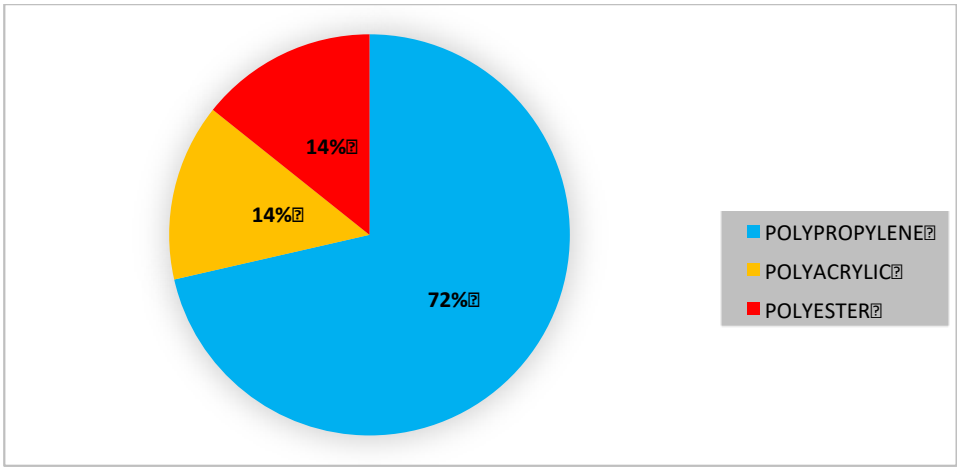

Figure 28: Percentages of polymer types of microplastics found in water samples from the three sites.

Particles found in wild mussels biodeposits were ascribable to the 0-100 µm size class (n=10), the 100-500 µm size class (n=11) and the 500-1000 µm size class (n=7), followed by just 1 microplastic that was between 1000-5000 µm long. Particles found in farmed mussels biodeposits were mostly ascribable to the 0-100 µm size class (n=12) and the 100-500 µm size class (n=18), while only 2 particles were between 500-1000 µm long and 1 particle was between 1000-5000 µm long (Fig.29).

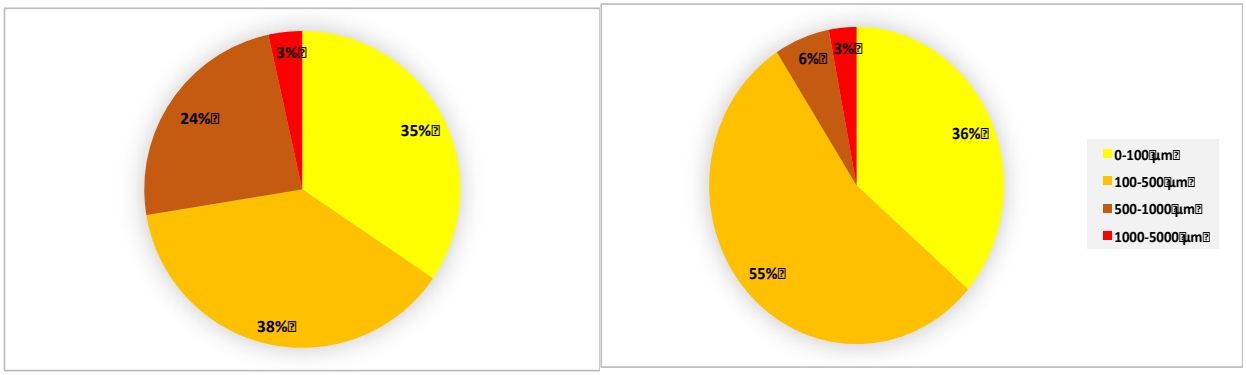

Figure 29: Percentages of size classes of microplastics measured in wild mussels (left) and farmed mussels (right) biodeposit samples.

Microplastics found in controls were mostly between 100-500 µm long (n=7), followed by particles (n=4) ascribable to the 1000-5000 size class. Only 2 particles were between 500-1000 µm long and just one particle was ascribable to the smallest size class 0-100 µm (Fig.30).

Plastic particles measured in water samples were equally distributed among size classes: 3 particles belonged to the 0-100 µm size class, 4 to the 100-500 size class, 4 to the 500-1000 size class and 3 to the 1000-5000 size class.

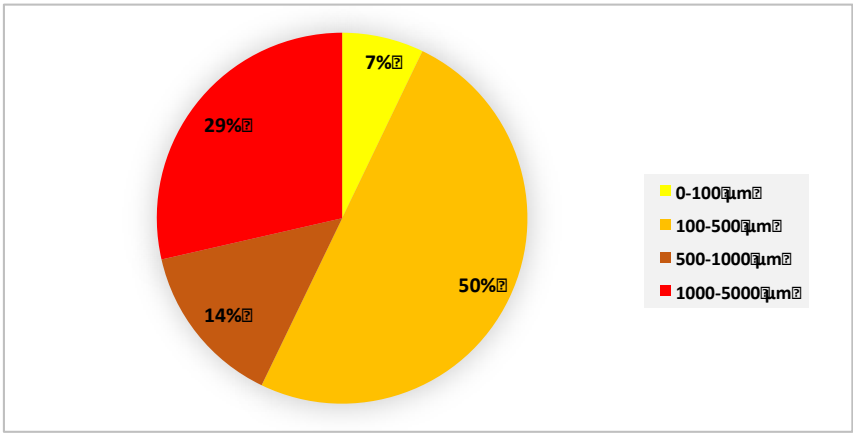

Figure 30: Percentages of size classes of microplastics measured in controls samples.

Concerning biodeposit containers, we found 29 and 33 of microplastic particles for wild mussels and farmed mussels, respectively.

By considering the microplastics quantities in sample units, PERMANOVA test showed significant differences among sites (Table S1).

Yatch club (CV) was the site with significantly higher amounts of microplastics compared to Darsena and Port entrance sites. In the Yatch Club comparing the number of plastics found in

biodeposit sample units with controls PERMANOVA test showed significant differences, while no significant differences were found between farmed and wild mussel biodeposits sample units (Table S2). A very low number of plastic particles was registered in biodeposit samples from Darsena (AP) and port (DI) sites, with 0.5 ± 0.26 and 1.37 ± 0.75 plastic per biodeposit containers respectively.

Comparison among plastics quantification in all samples are represented in Fig. 31.

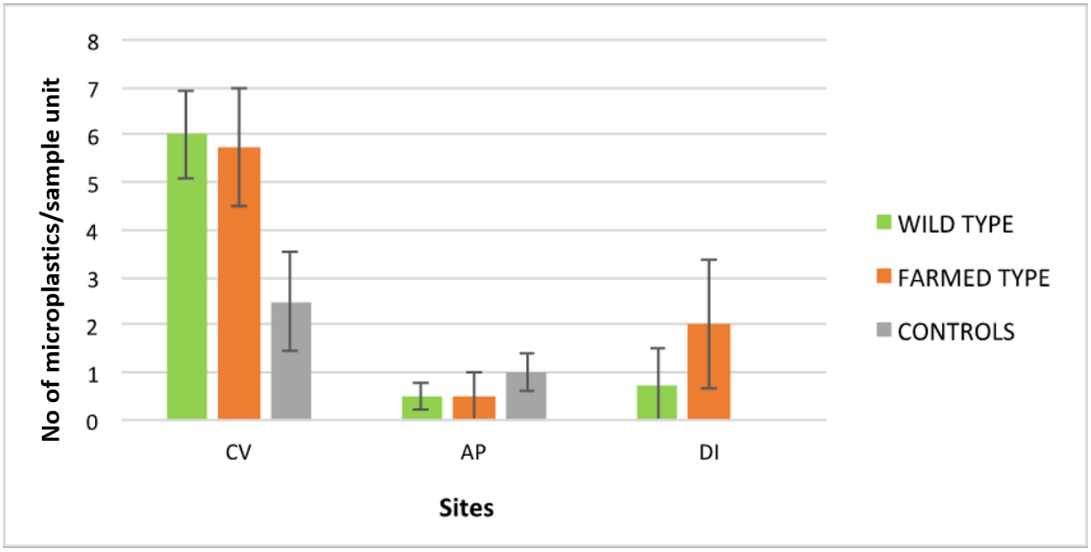

Figure 31: Comparison of the number of microplastics (mean±SE, n=4) between treatments (wild type, farmed type and controls) and sites (CV=Yacht Club, AP=Darsena, DI=port entrance).

The Fig.32 shows the biodeposit quantification expressed in mean of dry weight (DW) per sample unit. The highest amount of biodeposit was found in the samples from the port site (DI) in both wild and farmed mussels experimental. However, PERMANOVA analysis did not reveal any significant differences neither in the amount of biodeposit produced by the two types of mussels neither among sites, with the exception of the Yatch club (CV) where the biodeposit production was statistically significant higher in farmed mussels (P-value < 0.05) (Table S3).

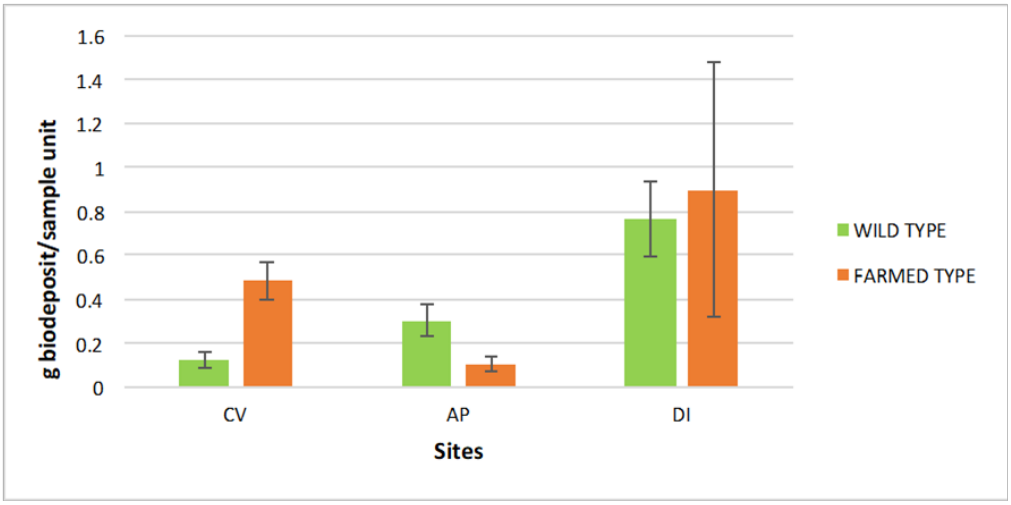

Figure 32: Comparison of biodeposit quantities (grams) (Mean ±SE, n=4) between treatments (wild and farmed mussels) and sites(CV=Yacht Club, AP=Darsena, DI=port entrance).

Microplastics quantification in water samples (Fig. 33) showed a higher amount of particles per liter from the water samples from the Yatch Club (CV) than in the other two sites.

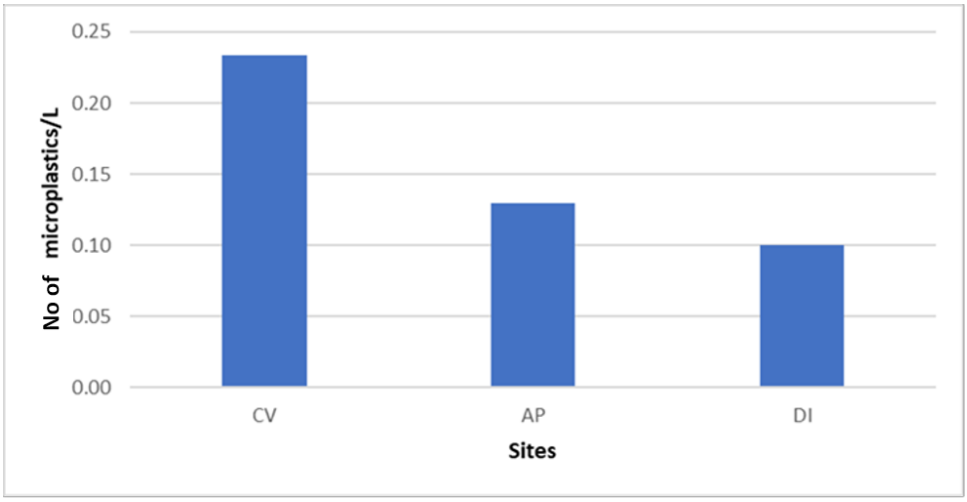

Figure 33: Microplastics quantification in water samples across sites

3.3.2. Microplastics dimensional patterns

The Principal Coordinates analysis (Fig.34) suggests differences in the size distribution of microplastics both among sites, with the Yatch Club clustering separately from the DI and AP, and at the yacht club also between biodeposits and controls. The two PCO axes explained 83% of the cumulative variation, thus the two dimensional projection is likely to capture the salient patterns in the full data cloud. The PCO1 and PCO2 axes respectively were strongly associated with site and treatment and explained 66.1% and 16.9% of the total variation. The results were confirmed by the PERMANOVA analysis revealing significant differences (P-value < 0.01) in the

microplastics size composition between mussels samples across sites. At the Yatch club there were also significant differences in plastic size composition between biodeposits and controls but no significant differences in plastic composition between the wild and farmed mussels. The Principal Coordinates analysis (Fig.35) shows two main clusters: one constituted by all the wild and farmed mussels, and the other one comprising only controls.

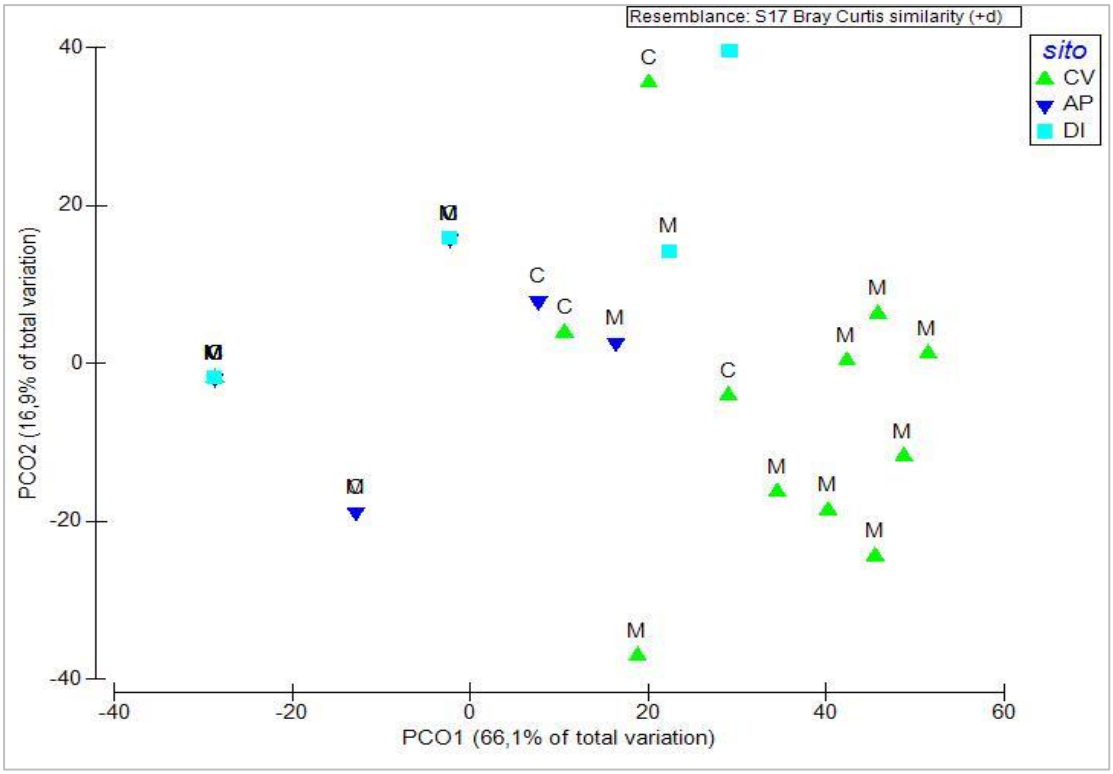

Figure 34: Principal coordinate analysis (PCO) plot of microplastics characteristics (frequency of 4 dimensional classes) of particles found in mussels biodeposits (M) and controls (C) in the three sites (CV=Yacht Club, AP=Darsena, DI=port entrance).

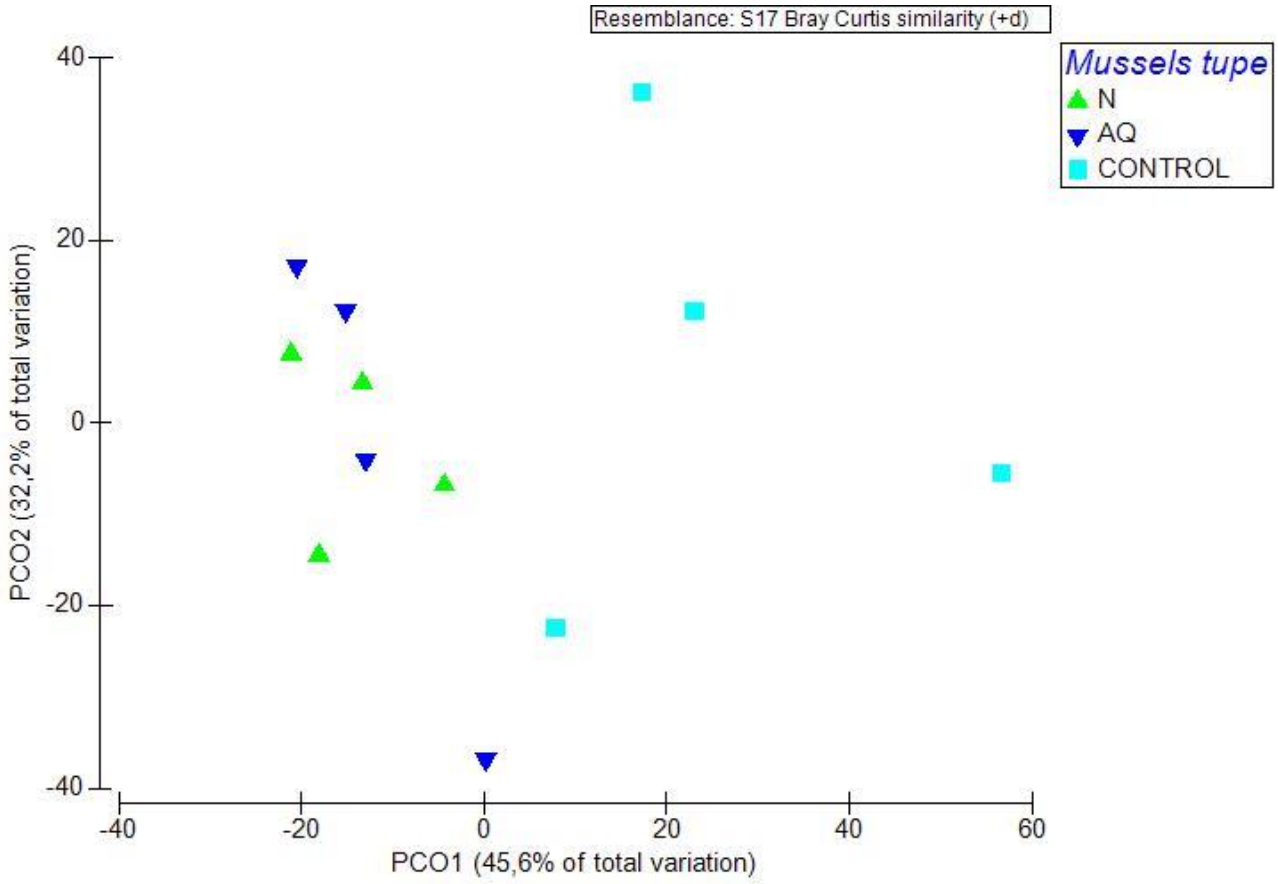

Figure 35: Principal coordinate analysis (PCO) plot of microplastics characteristics (frequency of 4 dimensional classes) within treatments (wild, farmed mussels and controls) for particles found in the Yacht Club.

4. Discussion

4.1. Microplastics occurrence at different trophic levels

The presence of microplastics has been documented in the water-column and in sediment samples, and their presence has been worldwide reported for a high number of different taxa, including planktonic species, invertebrates, fishes and cetaceans, occupying different positions in the trophic chain (Browne *et al*., 2011; Hidalgo-Ruz *et al*., 2012; Foekema *et al*., 2013; Lusher *et al*., 2013; Fossi *et al*., 2014; Van Cauwenberghe *et al*., 2014, 2015a; Avio *et al*., 2015). Laboratory studies further proved the capability of microplastics to be ingested by a wide range of marine biota including bivalves, crustaceans and fishes including those used for human consumption, with a potential impact at cellular, metabolic and physiological level (Browne *et al*., 2008; Von Moos *et al*., 2012; Wegner *et al*., 2012; Avio *et al*., 2015). Considering the growing interest on the impact of microplastics in the marine environment (Galgani *et al*., 2013), understanding the environmental relevance of these contaminants needs to be considered as a research priority (Avio *et al*., 2015).

There are still few studies focusing on the microplastics occurrence in coastal Adriatic wild species. The majority of the studies have described the distribution of these particles in the gastrointestinal tract of fish or bivalves species while there are no available data about microplastic ingestion and retention by crustaceans or polychaetes. This study quantifying provides the first insight on microplastics occurrence and distribution in benthic marine invertebrates from North Adriatic saltmarshes, including species at different trophic levels and with different feeding strategies,

The results revealed the presence of microplastics, particularly microfibers, in the digestive tract of North Adriatic benthic species. The overall quantities and types of microplastics in our organisms corroborated the major part of the last years researches focusing on this topic (Van Cawenberghe and Janssen, 2014; De Witte *et al*., 2014; Van Cawenberghe *et al*., 2015).

The crab *Carcinus aestuarii* (top predator of the considered trophic chain) presented the highest microplastics occurrence, while no to very little microplastics were found in *Cerastoderma glaucum, Mytilus galloprovincialis* and *Hediste diversicolor*. These results support the hypotheses that microplastic occurrence differs among species with different feeding habits (Murray *et al*., 2011; Lusher *et al*., 2013; Avio *et al*., 2015; Devriese *et al*., 2015; Trevail *et al*., 2015; Van Cauwenberghe *et al*.,2015), being generally greater at higher levels of the trophic chain (such as in crustaceans, fishes and birds).

Although *C. aestuarii* was the species with the higher number of microplastics, these were found only in 5.55% of the individuals analysed, suggesting that, as for the other three species investigated, the majority of the organisms did not contain any plastic. These data highlighted a very high variability in terms of plastic occurrence in the digestive tract, not only among species but also among nearby individuals, corroborating similar observations from other studies (Foekema *et al*., 2013; Lusher *et al*., 2013 Avio *et al*., 2015; Bessa and Sobràl personal communication). Microplastics were comprised mainly of fibres, and showed a predominance of the 1-5 mm size (61%), followed by 37% of 0.5-1 mm particles, and only by 2% of smaller particles 0.1-0.5 mm large. The observed size classes distribution is consistent with results from Lusher *et al*., (2013) and Lusher *et al*., (2015). The main colours showed by extracted microplastics were red (95.56%) and black (2.95%).

The FT-IR characterisation revealed that the most abundant polymer type was polyester (98.5%) while Polypropylene, Polyamide and Acrylonitrile accounting for approximately 0.5% each. These are the main polymer classes found in the gastrointestinal tract of invertebrate marine species (Taylor *et al*., 2016), and their occurrence is generally reported to be higher in surface waters (Thomson *et al*., 2004). Most of the microplastics were found in two organisms from the same area who have probably ingested plastics from the same source.

Although the very low number of particles found prevented a formal analysis of spatial distribution patters, it was clear that microplastic distribution is extremely variable among individuals. as also found in other systems (Foekema *et al*., 2013; Lusher *et al*., 2016). Baiona was the lagoon with the highest amount of microplastics detected, as expected from the greater exposure to anthropogenic activities and subsequently pollution sources (Airoldi *et al.*, 2016).

The consistent lack of plastic items in most organisms does not imply that the organisms did not ingest any particles. Rather it could be due to the animals' ability to quickly expel particles that don't represent a food source without them passing through the digestive system or to the rapid transit of the particles through the digestive tract (Farrel and Nelson, 2013). The microplastics quantification in the gastrointestinal tract of organisms describes only a snapshot of the materials ingested by the organisms and consequently the method does not allow to estimate ingestion rates. Moreover, fecal output may lead to the loss of plastic particles during the interval between field collection and storage. Preserving animals in ethanol on site just after the sampling could limit in part particles loss (Karlsson *et* al., 2017).

Another explanation for the limited number of particles found could be represented by our operational particle size detection limit. Marine invertebrates may ingest very small plastic particles (< 50 μm), which are too tiny to be detectable at the stereomicroscope, leading to a possible underestimation of the real plastics ingestion by these organisms. The particles most often ingested by marine invertebrates are "nanoplastics" (< 20 μm) that for their very low dimensions can pass through the tissue and be then accumulated at tissue and cellular level (Wegner *et al*., 2012; Rossi *et al*., 2014). By contrast, microplastics detected in the gastrointestinal tract of our sampled organisms ranged between 100 and 5000 μm in length, and particles of this size are probably excreted very fast, thus possibly contributing to explain the generally low amount of plastics found (Lusher 2013; Van Cawenberghe and Janssen, 2014; De Witte *et al*., 2014; Avio *et al*., 2015; Van Cawenberghe *et al*., 2015; Lusher et al., 2016).

Fibres were the most abundant class of plastics particles found in marine organisms' gastrointestinal tracts (Lusher *et al*., 2015; Rochman *et al*., 2015; Taylor *et al*., 2016). We followed very rigid and effective protocols, which excluded any environmental contamination during sample processing. We also analysed all particles found by micro-FTIR spectroscopical analysis, which revealed that many fibres optically isolated were in fact non-synthetic materials, underlying the importance of the adoption of this technique for particles characterization (Löder and Gerdts, 2015) with a special attention to microfibers (Wesch *et al*., 2016). Micro-FTIR might result as a long analytical procedure but at the moment it represents the only method to prevent plastic overestimation and to produce comparable data across studies (Lusher *et al*., 2017) as other more rapid and available methods have not yet been developed. Studies showing high concentration of microplastics in the organisms (Mathalon and Hill, 2014; Wójcik-Fudalewska *et* al., 2016) might be affected by biases caused by the absence of visual sorting validation and polymer characterization via spectroscopical techniques (Lusher *et a*l., 2017).

In conclusion the work provides the first description of microplastics occurrence and distribution in the gastrointestinal tract of benthic marine invertebrates from the north Adriatic saltmarshes, and suggests that the highest amount of plastic particles are found in organisms at the higher levels of the trophic chain (*Carcinus aestuarii*) and at the most urbanised sites, and that there is high variability in plastic occurrence among species and individuals Further complementary information about the microplastics distribution in sediment and water column is in progress to clarify the microplastics distribution in the marine environment.

The work also suggests that the analysis of gastrointestinal contents may not be an effective approach to measure microplastic uptake by marine species, stimulating work towards developing costs-effective techniques to directly measure in the field microplastics uptake by aquatic organisms. Methodological issues and the lack of standardized protocols in the microplastics field research remain the most important factors influencing data reliability and comparison among studies (Lusher *et al*., 2017), rising the need of further efforts towards a resolution to this matter to better understand this emerging threat.

4.2. Comparison of different extraction procedures from marine organisms

Current research on the impacts of microplastics is limited by the lack of standardized protocols and the presence of many operational difficulties associated with the extraction and characterization of microplastics from marine organisms. In order to be identified and characterised the particles need to be separated from the animal tissue and this is usually done by digesting all the organic components of the digestive tract or digesting the whole animal (Lusher et al., 2017). While there are many studies analysing how plastics fragments or pellets are affected by Acidic (Avio et al., 2015; Yang et al., 2015; Dehaut et al., 2016), Alkaline (Foekema et al., 2013; Cole et al., 2014; Dehaut et al., 2015) or Enzymatic (Cole et al., 2014; Nuelle et al., 2014) tissue digestion procedures, the effects of different digestion methods have rarely been properly compared.

Here two extraction protocols were compared for their effects on different polymers types of plastic microfibers, and to test whether these methods can affect the subsequent microplastics quantification and characterisation via optical and spectroscopical analyses. We also carried out the first field validation assessment to provide a reliable and standardized technique testing two digestion methods for the extraction and isolation of microplastics from wild marine invertebrates' gastrointestinal tract.

The visual analysis confirmed that the SDS-enzymatic digestion is the gentlest method on plastic particles, causing no physical damages (Cole *et al*., 2014, Catarino et al., 2017) and preserving the original colour and shape of all microfibres polymers even after 6 days of digestion. Microfibres treated with KOH (1M) digestion showed no physical damages for Polyester and Polypropylene, while Polyester PET samples showed some slight structural changes and discoloration of fibers but only after 6 days of treatment. No visible changes in the chemical polymer structure emerged from the FTIR analysis of different microfibres polymers

treated with either the enzymatic (SDS) or the alkaline (KOH) method compared to no treated materials.

SDS-enzymes and KOH methods were applied to separately digest the gastrointestinal tract of two batches of wild collected crabs (*C. aestuarii*). The SDS-enzymatic digestion took approximately two days to process samples and other two days for an acceptable digestion of tissues, while (once easily prepared) the KOH solution is ready to be added to tissue samples and took approximately one to three days to completely digest organic components. The sample solution obtained with the SDS-enzymatic method was very viscous and with the tendency to become jelly with a room temperature lower than 20 °C, leading to a double amount of filtering time if compared with samples treated with KOH. This latter method allowed a good digestion of the tissues with no remaining organic particles in the digestate (Dehaut *et al*., 2016) and a very liquid solution which allowed a good filtration through the 20 µm filters mesh.

The different amount of effort in the tissue digestion could have led to a different amount of environmental contamination between the two procedures, also revealed from the slightly higher mean quantity of non-synthetic fibres found in controls and air filters from the enzymatic processing in comparison to those found in the same type of samples from the KOH digestion. The higher amount of non-synthetic fibres found in organisms treated with KOH solution in comparison to those found in samples treated with SDS-enzymes could be explained by the higher amount of debris and the presence of a thin film formed during drying on SDS treated samples that made it difficult to inspect and identify fibres, leading to a possible underestimation of these particles (including those made of plastic) which was mostly avoided in the residual clear samples obtained with the KOH treatment.

Comparisons between estimates of microplastics from *Carcinus aestuarii* digested with the two methods showed on average a lower number of plastic particles in individuals treated with SDS-Enzymes and a greater abundance of microplastics in specimens digested with KOH, irrespective of the organisms dimensions. However, 98% of the plastics was extracted from only 2 individuals, and the difference between the two methods was not significant.

All the plastic particles isolated were fibres and, apart from one Polyamide (Nylon) and one Polyacrylonitrile (Orlon) fiber, all were identified as Polyester. No evident sign of degradation or weathering was observed for microfibers extracted with the KOH method and no residuals were present on these isolated particles which was not the case of fibres extracted with enzymes, that showed some organic film on their surface. Anyway, this condition did not seem

to affect polymer identification via FTIR analysis as also confirmed by Cole *et al*., (2014), Catarino et al., (2017), and Courtene-Jones *et al*., (2017).

The most important difference between the two methods was in terms of cost and time. Digesting 90 gastrointestinal tracts of crabs with the enzymatic protocol implied 120 processing hours and a purchase of approximately 42 euros, while for the digestion of the same amount of samples, the KOH method required 72 processing hours and a purchase of 1.50 euros.

In conclusion this study suggested no evident negative effects on microfibers for either methods. However, the greatest number of procedural steps, the longest filtering time and higher amount of operator manipulations and the higher costs suggest that the KOH (1M) treatment represents a more convenient method for microplastics extraction, isolation and characterization from marine invertebrates' soft tissues.

4.3. Estimate of ingestion rates through the analysis of biodeposits

Microplastics uptake by marine organisms has been mostly evaluated by analysing their gastrointestinal tract (Lusher *et al*., 2017) but, as many species (and especially invertebrates) can easily expel non edible particles (Beecham, 2008; Wegner *et al*., 2012; Wright *et al*., 2013) this approach may not provide reliable data to quantify the actual amount of microplastics ingested by aquatic marine organisms.

This work aimed to test a novel field-based technique to quantify and characterize the microplastics uptake rates through the analysis of biodeposits using mussels (*Mytilus galloprovincialis*) as model organisms. Moreover, a comparison between wild and farmed mussels has been performed to evaluate potential differences on microplastics uptake.

The presence of microplastics was registered in biodeposit samples in all sites and from both wild and farmed mussels, with a significantly higher amount of particles in samples from the Yacht Club site compared to the other two sites: at the former site a higher microplastics concentration was also found in water samples. No differences were found between farmed and wild mussels, despite slightly different mean body sizes (5-6 cm the farmed type, 3.5-4 cm the wild type) and dimensional composition (homogeneous for the farmed type, variable for the wild type). This is consistent with similar observations from De Witte et al. (2014). Except for few exceptions, microplastics found in the biodeposits reflected the shape, colour and polymer of the majority of microplastics found in water samples, being mostly polypropylene blue fragments. This is expectable, due to the fact that Polypropylene and Polyamyde are the

main materials used for fish and boats equipments (i.e ropes and nets), highly employed the Ravenna harbour areas. Polypropylene, which is the lowest density polymer among plastic materials, also probably constitutes the most common plastic type in the surface water layers, where the sample units where located during the experiment. It could be worth in the future to explore whether mussel ingestion of microplastics differs among different depths, reflecting different distributions of microplastics in the water column.

Comparisons about the different sites allowed us to infer about the most suitable sites conditions to carry out these measurements. The Darsena site showed almost the same number of plastics particles in control samples compared to biodeposits. This condition is probably imputable to the considerable presence of natural assemblages of mussels in this area and to their immediate proximity to where the biodeposit traps were positioned, leading to a possible contamination of our blanks from externally-produced biodeposit. Natural assemblages of mussels were also abundant at the port entrance site, but the sampling units where few meters apart from the seawall where mussels lived. Further the higher hydrodynamism in this area, may have limited the effectiveness of the cones at collecting the biodeposits, thereby explaining the very low rates of microplastic accumulation in the traps.

Microplastics quantities in control samples were significantly lower than those present in mussels biodeposit only at the Yacht Club site. This site was the most sheltered from water turbulence and currents, and with no mussel assemblages nearby, preserving samples from external disturbance and potential contamination and also facilitating sample recovery. Results from this site suggest that mussels have in fact taken up microplastics. Specifically, 1.5 Kg of mussels can ingest (and then egest) microplastics at a rate of approximately 4 particles in 4 hours. Mussels were exposed to a concentration of 0.23 plastic particles L^{-1} at this site and, based on previous studies their microplastics uptake was expected to be higher (Clausen and Riisgard, 1996; Cusson *et al*., 2005, Van Cauwenberghe *et al.,* 2015). The lower concentrations found in our biodeposit samples suggest that the animals might have ingested less microplastics possibly due to a lower filtration rate caused by physiological and environmental conditions (Cusson *et al*., 2005). It is also possible to hypothesize that during the short exposure time (4 hours) some particles may have been retained in organisms' digestive tract, since mussels gut depuration differs depending on species, temperature and food quantity (Hawkins and Bayne, 1984). Also the structure of the biodeposit traps might constitute an obstruction to the water flow, limiting the entrance of the particles through the holes and thus further reducing microplastics exposure level.

At the Yacht Club site there were also differences in the size of particles from biodeposit samples (irrespective of mussel type) and control samples. Microplastics biodeposits were on average smaller (mostly < 500 μm) than those found in controls, suggesting a size selection of particles operated by mussels (Van Cawenberghe and Janssen, 2014). This is an additional information confirming that a "mussel effect" was registered for the deployment carried out in the Yacht Club site, where environmental conditions, such as low hydrodynamic and the absence of mussels natural assemblages, probably facilitated all the sampling steps providing more accurate measurements.

The protocol, pending some improvements in the selection of the characteristics of the deployment sites, in the length of deployment as well as in the design of the traps, seems to be a promising method to directly measure the microplastics uptake by sessile invertebrates species in the marine environment. Further field tests are needed to understand the physiological and environmental mechanisms, together with microplastics properties, that influence microplastics uptake and egestion by mussels. The method can be applied both using aquaculture or wild specimens, as there do not seem to be differences in uptakes between these two groups.

5. Conclusions

Microplastics have been a recently explored phenomenon and have been discovered to be now ubiquitously present in seas and oceans worldwide, potentially affecting a wide range of marine organisms and ecosystems. This situation has raised concerns and has led to mobilize researchers from all over the world looking for solutions to better comprehend and contain the problem. Nowadays we are just starting to understand microplastics dynamics and their potential impact on wildlife and human beings, and many gaps are still limiting our knowledge of the real extent of this new type of threat. In this thesis I quantified microplastics uptake and occurrence in marine invertebrates from coastal lagoons. I also filled in some knowledge gaps about methodological extraction procedures. As research progresses, it becomes fundamental to define standardized protocols and provide reliable data on the distribution of microplastics in the environment and biota. There is still a long way to go and obstacles to overcame, since the issue is really complex and variegated.

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Supplementary Materials

Table S1: PERMANOVA table of plastic concentration. ***si*** *= site,* ***Mu*** *= mussels and controls.*

PERMANOVA table of results

Source	df	SS	MS	Pseudo-F	P(perm)	Unique perms
si	2	85,583	42,792	16,097	0,0002	9960
Mu	1	16,056	16,056	1,6033	0,3516	59
sixMu	2	20,028	10,014	3,767	0,0376	9947
Res	30	79,75	2,6583			
Total	35	241,56				

Table S2: PERMANOVA table of plastic dimensions. ***si*** *= site,* ***Mu*** *= mussels and controls.*

PERMANOVA table of results

Source	df	SS	MS	Pseudo-F	P(perm)	Unique perms
si	2	11794	5897,1	8,1148	0,0001	9950
Mu	1	2246,1	2246,1	1,0391	0,4466	59
sixMu	2	4323,3	2161,7	2,9746	0,0141	9956
Res	30	21801	726,7			
Total	35	45201				

Table S3: PERMANOVA table of plastic dimensions. ***Mu*** *= wild mussels, farmed mussels and controls*

PERMANOVA table of results

Source	df	SS	MS	Pseudo-F	P(perm)	Unique perms
Mu	2	3159,1	1579,6	2,5116	0,031	4744
Res	9	5660,2	628,92			
Total	11	8819,4				

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