SCUOLA DI DOTTORATO
UNIVERSITÀ DEGLI STUDI DI MILANO-BICOCCA

Department of Earth and Environmental Sciences (DISAT)

PhD program Chemical Geological and Environmental Sciences — Cycle XXXIV

Curriculum in Terrestrial and Marine Environmental Sciences

MICROPLASTICS IN FRESHWATER SYSTEMS: CHARACTERIZATION, QUANTIFICATION AND INTERACTION WITH AQUATIC ORGANISMS

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ACADEMIC YEAR 2020/2021

Contents

Abstract

Among the multiple stressors that affect aquatic ecosystems, plastic pollution is deemed a widespread and pervasive environmental issue. The majority of the research has been conducted in marine environments and information about the occurrence and effects of these pollutants in freshwater systems is scattered. Moreover, there is a lack of consensus on sampling and analytical procedures for their characterization and quantification, which makes comparison among studies difficult. Besides, more research is needed to assess the influences of plastics and microplastics on ecosystem functions and aquatic organisms, especially focusing on lower trophic levels. Given these gaps, the present project describes four pieces of work that contribute to enhancing our knowledge about plastics and microplastics in freshwater ecosystems. Firstly, since polymer identification constitutes a fundamental step in plastic analysis, the suitability of Raman spectroscopy for polymeric characterization was examined, and a free database with Raman spectra of plastics complemented by a new R package with tools for their processing were developed and described. Advantages and drawbacks of this technique were discussed, with a particular emphasis on plastic additives, which are contained in the majority of polymers but are still poorly investigated, and a catalog with detailed information about peaks of most common plastic polymers was reported to provide guidance for further studies. Secondly, microplastic occurrence in surface water of different freshwater systems was assessed. Indeed, water samples of 38 lakes from 28 different countries covering an assortment of limnologically diverse freshwater ecosystems under varying levels of anthropogenic stress were collected, following a common protocol. This global investigation allowed obtaining comparable data about plastic concentration and features. Moreover, the results

suggested the existence of a relationship between urban-related attributes of lakes/watersheds and the plastic concentration but also highlighted as large and deep lakes with high retention times accumulated plastic debris at higher concentrations. Lastly, the relationship between microplastics and microalgae was investigated. This was pursued by combining a critical review of the literature with an experimental approach aimed at analyzing the phytobenthos establishment on two different plastic polymers using a multi-site mesocosm system. This experiment highlighted that microplastics supported the growth of a rich and diversified community of microalgae, showing that many species could coexist on the surface of relatively small plastic items. Species-specificity in the colonization of the different plastic polymers was not observed. Indeed, local species pool and nutrient concentration rather than polymeric composition seemed to be the determinant factors defying the community diversity.

Chapter 1

General introduction

1.1. Overview

Of all the water on Earth (approximately 1.4 billion km^3), only 2.5% is freshwater. Of this fraction, almost 70% is stored in the ice caps and as soil moisture or is present as groundwater in deep aquifers not accessible for human use. Less than 1% of global fresh water (which corresponds to roughly 0.007% of all water on Earth) is accessible for sustainable use (Petersen *et al.* 2021). Although freshwaters account for only a small amount of water resources, they are of great importance and play a crucial role in natural ecosystems and human activities, providing a variety of ecosystem services. Indeed, freshwaters are a 'provisioning' service, that supply water for domestic use, irrigation, power generation, and transportation, but also provide cultural, regulating, and supporting services that contribute directly and indirectly to human well-being (Jenny *et al.* 2020). From a resource perspective, water scarcity, emerging from water quantity and quality issues, increased substantially in the last decades in many parts of the world, and it is expected to further exacerbate in the future driven by socio-economic and climatic changes (Greve *et al.* 2018). From a conservation perspective, the recognition that freshwater ecosystems contribute disproportionately to global biological richness is being counterbalanced by the realization that extinction risks in freshwaters may be among the most serious of all (Ormerod *et al.* 2010). On their own, these are already major concerns that show how the exploitation and impairment of freshwaters have outpaced our best management efforts (Ormerod *et al.* 2010).

Among the multiple stressors that affect freshwater ecosystems, plastic pollution has been widely documented as a widespread and pervasive environmental issue and the role of lakes and rivers in global plastic pollution has been increasingly recognized. Indeed, these systems on one side represent a source of plastic pollution, since they contribute to transport of plastic debris from land-based sources to coastal and marine environments,

on the other they can also represent a sink and can be impaired by the presence of these contaminants (Horton *et al.* 2017). This group of pollutants is extremely diversified due to the variety of plastic sizes and characteristics (e.g., physical and chemical properties). As a result of the diversity and ubiquity of plastic particles within aquatic systems, organisms can interact with, become entangled in or ingest plastic particles in a variety of ways (Windsor *et al.* 2019).

Despite the relevance of plastic pollution in freshwater systems, the majority of the research has been conducted in marine environments and information about distribution and effects of these pollutants in freshwater systems are still limited and should receive more scientific attention (Wagner *et al.* 2014; Szymańska and Obolewski 2020).

1.2. Plastic pollution

Plastic debris has become evident as a globally ubiquitous pollutant over the last decade. While the benefits of plastics are undeniable, the widespread use of these polymers, namely in discardable forms, ultimately leads to their accumulation in the environment (da Costa *et al.* 2017). Plastic waste is now so ubiquitous in the environment that it has been suggested as a geological indicator of the proposed Anthropocene era (Geyer *et al.* 2017). Indeed, several scientists suggest that the plastic layers are indicative of the start of the Anthropocene and that, after the bronze and iron ages, the current period will be known as the 'plastics age' (Porta 2021). The issue of aquatic plastics has been also recognized in the Sustainable Development Goals (SDGs) adopted by the United Nations in 2015 under Goal 14 and, specifically, in target 14.1: "*By 2025, prevent and significantly reduce marine pollution of all kinds, in particular from land-based activities, including marine debris and nutrient pollution".* This will be measured by indicator 14.1.1: *"Index of coastal eutrophication and floating plastic debris density*" (Walker 2021).

The proliferation of plastics has been driven by rapid growth in plastic production and use combined with linear economic models that ignore the externalities of waste (Lau *et al.* 2020). Geyer *et al.* (2017) estimated that global production of plastics increased from 2 Mt in 1950 to 380 Mt in 2015, with a compound annual growth rate of 8.4%, substantially outpaced any other manufactured material. A global shift from reusable to single-use containers has accelerated the growth of application of packaging that is material designed for immediate disposal, which currently represents the largest plastics' market. As a consequence, the share of plastics in municipal solid waste by mass increased from less than 1% in 1960 to more than 10% by 2005 in middle- and high-income countries (Jambeck *et al.* 2015; Geyer *et al.* 2017).

The durability and resistance to degradation of plastics, which render these materials incredibly versatile in several applications, make these materials difficult or impossible for nature to assimilate (Geyer *et al.* 2017). Although a fraction of this plastic waste is recycled, most of it ends up in landfills, where they may take a few hundred years to decompose (Cole *et al.* 2011). For instance, in 2015, approximately 262 Mt of municipal solid waste (MSW) was generated in the United States. 13% of the 262 Mt of MSW (34.5 Mt) was made up of putatively recyclable plastic waste. However, only 9% of these 34.5 Mt of plastic waste was recycled. This compares with 16% that was incinerated and 75% that was landfilled (Thiounn and Smith 2020). Thus, near-permanent plastic waste pollution of the natural environment is becoming a growing concern (Geyer *et al.* 2017).

Thousands of different plastic polymers have been synthesized, with more to come in the future. Polymers can be grouped into two groups based upon their processing characteristics or the type of polymerization mechanism: (i) thermoplastic polymers, polymers that can be melted when heated above a specific temperature and harden upon cooling, recasting almost indefinitely; (ii) thermosetting polymers or "thermosets", whose individual chains have been chemically linked by covalent bonds during polymerization or by

subsequent chemical or thermal treatment during fabrication and, when heated, undergo a chemical change, creating a three-dimensional network, and thus cannot be re-melted and reformed (Fried 2014). Polystyrene (PS), polyolefins (e.g., polyethylene, PE, and polypropylene, PP) and polyvinyl chloride (PVC) are an important example of commercial thermoplastics. Principal examples of thermosets include epoxy, phenol–formaldehyde resins, and unsaturated polyesters (Fried 2014; Lebreton *et al.* 2017). Besides thermoplastics and thermosets, the large family of synthetic polymers also includes elastomers, polymers that are capable of high extension under ambient conditions, and synthetic fibers, suitable for textile application such as nylon and polyester (Fried 2014).

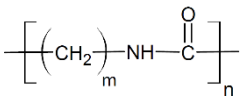
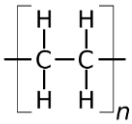
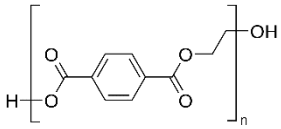
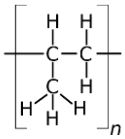
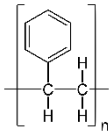
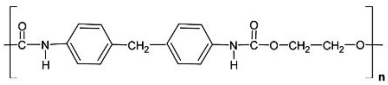
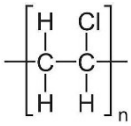
The chemical composition is the most fundamental criterion for defining plastic debris. Some disagreement exists on which polymers should be considered "plastics" (Hartmann *et al.* 2019). Strictly speaking, only thermoplastics and subsets of the other groups display thermoplastic character and should thus be referred to as "plastics". However, the environmental science community has adopted the practice of referring to all polymers as "plastics" (Lehner *et al.* 2019; Hartmann *et al.* 2019).

Accordingly to the analysis of Geyer *et al.* (2017), the largest groups in non-fiber plastics production are PE (36%), PP (21%), and PVC (12%), followed by PET (polyethylene terephthalate), PUR (polyurethane), and PS (<10% each). Polyester, most of which is PET, accounts for 70% of all polyester, polyamide, and acrylic fiber production. Together, these seven groups account for 92% of all plastics ever made. Approximately 42% of all non-fiber plastics have been used for packaging, which is predominantly composed of PE, PP, and PET. The building and construction sector, which has used 69% of all PVC, is the next largest consuming sector, using 19% of all non-fiber plastics.

These polymers have different chemical and physical properties (Table 1.1), and this will likely result in very heterogeneous fates and effects once they enter the environment (see Section 1.4). Indeed, microplastics, like other

classes of chemical contaminants, is a catch-all term for a variety of unique chemical compounds (Rochman 2015).

Table 1.1. Densities and structures of the most common plastic polymers (Hidalgo-Ruz *et al.* 2012).

Polymer	Density (g cm^{-3})	Structure
Polyamide (PA)	1.02-1.05	
Polyethylene (PE)	0.91-0.96	
Polyethylene terephthalate (PET)	1.37-1.45	
Polypropylene (PP)	0.90-0.91	
Polystyrene (PS)	1.04-1.10	
Polyurethane	1.20	
Polyvinyl chloride (PVC)	1.16-1.58	

The presence of plastic debris has been reported across worldwide oceans, from nearshore to offshore and pelagic regions, at sea surfaces, in water columns and sea bed sediments (Xu *et al.* 2020). Accumulations of plastic debris have been observed for instance in the South Pacific (Eriksen *et al.* 2013b), South Atlantic (Ryan 2014), North Pacific (Law *et al.* 2014), and North Atlantic (Law *et al.* 2010). Plastic debris are also abundant in the semi-

enclosed ocean Mediterranean, which receives large amounts of plastic litter from the surrounding land masses (Collignon *et al.* 2012; Xu *et al.* 2020).

When plastic debris enter the marine environment, buoyant plastic can be transported by surface currents and winds, recaptured by coastlines, degraded into smaller pieces by the action of sun, temperature changes, waves and marine life (see section 1.4), or lose buoyancy and sink. However, some of these buoyant plastics are transported offshore and enters oceanic gyres (Lebreton *et al.* 2018). To date, the largest mass concentrations of positively buoyant plastic waste for the surface ocean have been recorded in subtropical oceanic gyres, which are often referred to as ocean 'garbage patches' (Egger *et al.* 2020). Indeed, mass plastic concentrations in these zones reach values of hundreds of kilograms per km^2 and numerical concentrations exceed one million pieces per km^2 for particles >500 µm in size (Lebreton *et al.* 2018; Egger *et al.* 2020).

Land-based sources, rather than marine-based sources, are considered the primary input of plastics into oceans (Lebreton *et al.* 2017). A recent study by Meijer *et al.* (2021), using a modelling approach with geographically distributed data on plastic waste, land use, wind, precipitation, and rivers, calculated the probability for plastic waste to reach a river and subsequently the ocean. They calculated that 1000 rivers account for 80% of global annual macroplastic emissions, which was estimated between 0.8 million and 2.7 million MT (Fig. 1.1). This estimation is in the same order of magnitude of previous river emission assessments, which estimated 1.15 million to 2.41 million MT (Jambeck *et al.* 2015) and 0.41 million to 4 million MT (Lebreton *et al.* 2017) for global riverine plastic emissions. However, freshwater ecosystems not only transport plastics from land to sea but can also act as plastic pollution sinks. Despite the relevance of investigating microplastics in freshwater ecosystems, the majority of microplastic research has focused on seawater and comprehensive investigations on occurrence and fate of plastic in freshwaters are scarce and highly fragmented (see Section 1.3.2.).

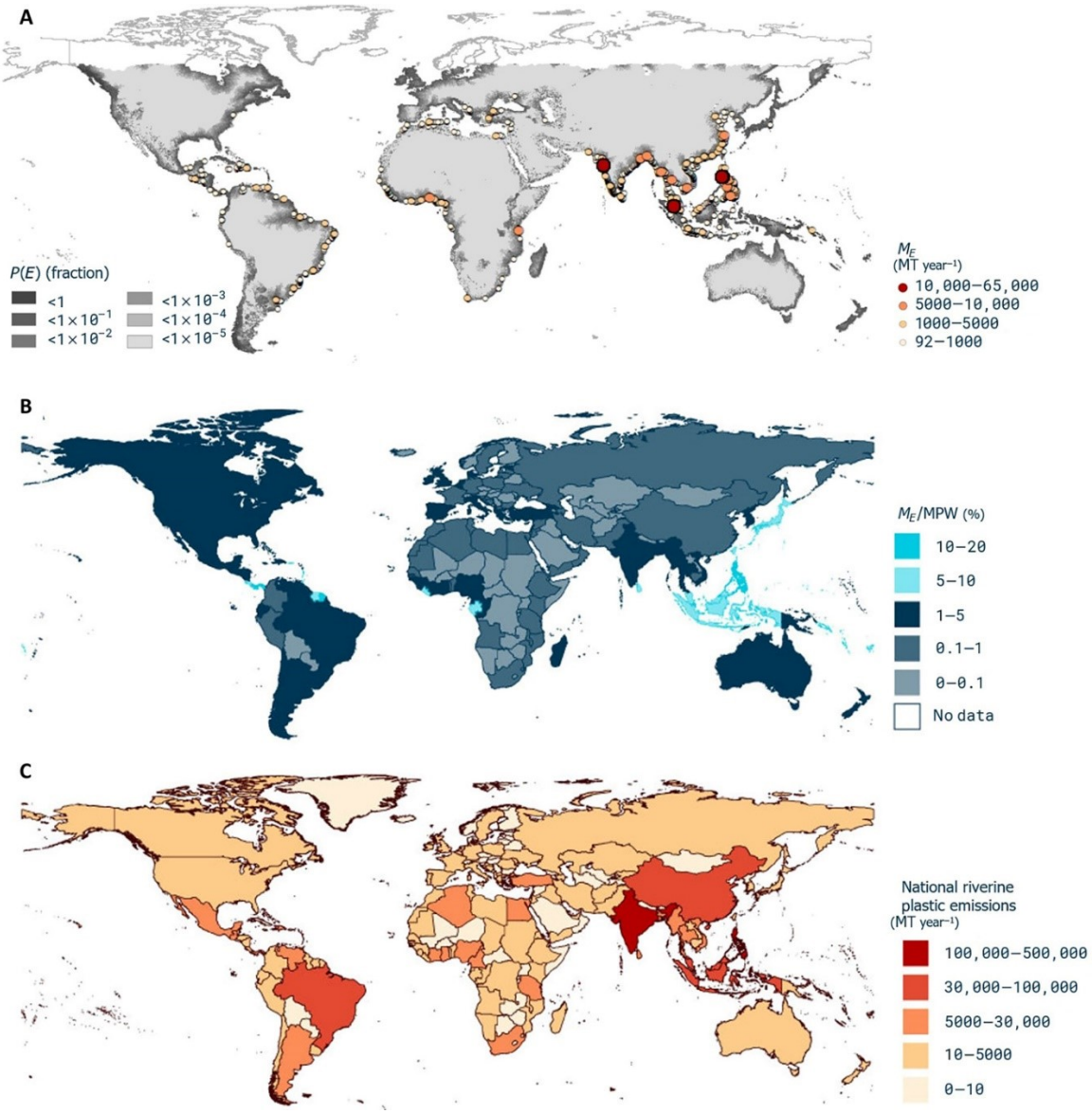


Figure 1.1. A) The geospatial distribution of plastic entering the ocean through rivers. The 1656 rivers accountable for 80% of the total influx are presented. The gray shading indicates the probability for plastic entering the ocean [P(E)] on a 10 × 10^{-km} resolution. (B) Total emitted plastic into the ocean ME per country divided by the national generation of MPW, globally ranging between 0 and 20%. (C) Total emitted plastic into the ocean ME (MT $year^{-1}$) per country. Figure originally from Meijer *et al.* (2021). "More than 1000 rivers account for 80% of global riverine plastic emissions into the ocean", Science Advances 7 (18), eaaz5803. Reprinted with permission from AAAS.

1.3. Microplastics

1.3.1. Classification and sources

Microplastics (MPs) are defined as small plastic items whose larger dimension is lower than 5 mm. This term was initially coined by Thompson *et al.* (2004) to describe the accumulation of microscopic pieces of plastic in marine sediments and in the water column of European waters. Then, Arthur *et al.* (2009) proposed an upper size limit to the initial term and microplastics where known as "plastic particles smaller than 5 mm" (Frias and Nash 2019). However, there is still no consensus on this higher limit of the microplastic range (5 mm) and some researchers have recently suggested different thresholds (e.g., 1 mm; Hartmann *et al.* 2019).

Besides categorization based on size, microplastics can be classified by their origin into two categories: primary and secondary. Primary microplastics are specifically manufactured in the micrometer size range, such as scrubbers in cosmetic products, as well as manufactured pellets used in feedstock or plastic production (Cole *et al.* 2011). Secondary sources of microplastics include fibers or fragments resulting from the breakdown of larger plastic items (Browne *et al.* 2011; Eerkes-Medrano *et al.* 2015). These are affected by UV radiation, high temperature, and wave action which can cause chemical changes making plastics brittle and more susceptible to fragmentation (Eerkes-Medrano *et al.* 2015; Horton *et al.* 2017). Sources of secondary microplastics derived from plastic litter are both numerous and diverse, ranging from releases during municipal solid waste collection, processing and landfilling, release from transportation and disposal systems to individuals creating litter either accidentally or intentionally. This includes large plastic items and sanitary waste input to rivers via combined sewage overflows (CSOs). An additional source of secondary microplastics is derived from synthetic fabrics, which can shed up to 1900 fibers per garment during washing. Although microfibers are secondary particles they will be released

to the environment along with primary microplastics through wastewater effluents and sludge application (Browne *et al.* 2011; Horton *et al.* 2017). The current-generation sewage treatments can remove large amounts of microplastics from wastewater, however, the number of particles bypassing the filtration systems remains high. Moreover, in many countries, wastewater plants are underestimated and sewages directly reach the watercourses without treatment thus increasing the number of microplastics, together with other contaminants, that can reach freshwater systems (Duis and Coors 2016; Horton *et al.* 2017). Apart from these sources, particles generated by the abrasion of tire tread against road surfaces, typically referred to as tire wear particles (TWPs), are recognized as microplastics owing to their physical and chemical properties. Modeling and chemical marker studies suggest that TWP may be substantial contributors of microplastic pollution and that rivers may facilitate the transport of TWP to the ocean (Wik and Dave 2009; Leads and Weinstein 2019). For example, a previous survey conducted in Norway suggested that TWP were the most important single source of primary microplastics to the natural environment (Sundt *et al.* 2014). These particles, together with textile fibers, can reach freshwater systems through atmospheric deposition. Indeed, atmospheric fallout enriched in microplastics, even if to date not largely explored, has been observed in urban, industrial, and remote areas (Mbachu *et al.* 2020).

Aside from size, microplastics are commonly categorized on the basis of shape and color. Frequent descriptors of shape are spheres, beads, pellets, foams, fibers, fragments, films, and flakes (Kooi and Koelmans 2019). No universal definition exists to classify particles on the basis of shape; however, Hartmann *et al.* (2019) in an attempt of proposing a definition and categorization framework for microplastics, suggested to subdivide into five types based upon their appearance: spheres, spheroid, cylindrical pellet, fragment, film, and fiber. Generally, the shapes more commonly found in aquatic samples are fibers and fragments (e.g., Dusaucy *et al.* 2021).

During sample preparation, categorizing microplastics by color can help in identifying potential origins as well as potential contaminations. However, color information can be biased since brighter colors are easier to identify during visual assessment. Dark, transparent, or translucent particles, on the other hand, may be underrepresented. Moreover, attributing colors may be subjective (Hartmann *et al.* 2019). Nevertheless, color may be relevant in the biological context, as selective feeding for different colors of microplastics has been observed previously in fish and other organisms (Santos *et al.* 2016; Xiong *et al.* 2019), since color determines whether plastic objects may be more or less likely to be mistaken as food. Thus, information about colors of microplastics detected in the environment are generally included.

Most common plastics range in density from 0.85 to 1.41 g cm^{-3}, where polypropylene and low/high-density polyethylene (LDPE, HDPE) plastics have densities lower than 1 g cm^{-3}, and polystyrene, nylon 6, polyvinyl chloride, and polyethylene terephthalate have densities higher than 1 g cm^{-3}. Since this range includes material of lower, equal, or higher density than water, microplastics can be distributed throughout the water column (Morét-Ferguson *et al.* 2010; Eerkes-Medrano *et al.* 2015). Thus, particle density can determine whether a particle occupies a pelagic versus benthic transport route; low-density plastics occupy the surface and neustonic environment, while high-density plastics are found at depth and on the benthos (Eerkes-Medrano *et al.* 2015). Degradation and biofilm formation on the surface of floating plastic particles may facilitate the attachment of organic coatings, inorganic material (sand, shells), or other plastic debris. Because of this additional weight, microplastics may also tend to settle in sediments; therefore, sediments can represent a long-term sink for these pollutants (Van Cauwenberghe *et al.* 2015).

1.3.2. Occurrence in freshwater systems

The majority of microplastics study has focused on the seawater environment and freshwaters-related studies account for less than 4% of microplastic research. However, based on the limited information available, the number of microplastics in freshwaters is comparable to that found in the marine environment, and their distribution is highly heterogeneous (Li *et al.* 2018).

Microplastic abundance in lakes and rivers varies largely between different studies (Table 1.2) as a result of differences in sampling locations, proximity to urban centers, water residence time, size of the water body, the type of waste management used, amount of sewage overflow, and sampling approaches (Eerkes-Medrano *et al.* 2015; Li *et al.* 2018).

Table 1.2. Data of microplastics concentration for rivers and lakes. Modified from Dusaucy *et al.* 2021; Li *et al.* 2018.

Country	System	Concentration	References
Argentina	Lake (La Salada)	$9.0 \cdot 10^{-1} \pm 6.0 \cdot 10^{-1}$ MP m^{-3}	(Alfonso *et al.* 2020a)
Argentina	Lakes (9)	$1.4 \cdot 10^{2} \pm 4.0 \cdot 10^{1}$ MP m^{-3}	(Alfonso *et al.* 2020b)
Austria	River (Rhine)	0.317 ± 4.665 MP m^{-3}	(Lechner and Ramler 2015)
Canada	Lake (Winnipeg)	$1.93 \cdot 10^{5}$ MP km^{-2}	(Anderson *et al.* 2017)
Canada, USA	Lakes (Superior; Huron; Erie)	$4.3 \cdot 10^{4} \pm 1.2 \cdot 10^{5}$ MP km^{-2}	(Eriksen *et al.* 2013a)
Canada, USA	Lake (Michigan)	$1.7 \cdot 10^{4}$ MP km^{-2}	(Mason *et al.* 2016)
Canada, USA	Lakes (Erie; Ontario)	$2.3 \cdot 10^{5}$ and $4.5 \cdot 10^{4}$ MP km^{-2}	(Mason *et al.* 2020)
China	Reservoir (Three Gorges)	$4.7 \cdot 10^{3} \pm 2.8 \cdot 10^{3}$ MP m^{-3}	(Di and Wang 2018)
China	Lake (Wuliangsuhai)	3.1 – 11 MP m^{-3}	(Mao *et al.* 2020)
China	Lake (Taihu)	$0.01 \cdot 10^{6}$ – $6.8 \cdot 10^{6}$ MP km^{-2}	(Su *et al.* 2016)

China	Lakes (20); rivers (Hanjiang; Yangtze)	$1.7 \cdot 10^3 \pm 6.4 \cdot 10^2$ MP m^{-3}	(Wang *et al.* 2017)
China	Lakes (Dongting; Hong)	$1.2 \cdot 10^2 \pm 2.3 \cdot 10^3$ MP m^{-3}	(Wang *et al.* 2018)
China	Lake (Poyang)	5 – 34 MP m^{-3}	(Yuan *et al.* 2019)
China	Reservoir (Three Gorges)	$8.47 \cdot 10^6$ MP km^{-2}	(Zhang *et al.* 2015)
Finland	Lake (Kallavesi)	0.27 ± 0.18; $1.6 \cdot 10^2 \pm 73$; 12 ± 17; 1.8 ± 2.3 MP m^{-3}	(Uurasjärvi *et al.* 2020)
France	River (Seine)	30 and 0.35 MP m^{-3}	(Dris *et al.* 2015)
Italy	Lakes (Bolsena; Chiusi)	2.5 and 3.0 MP m^{-3}	(Fischer *et al.* 2016)
Mongolia	Lake (Hovsgol)	$1.3 \cdot 10^4$ MP km^{-2}	(Free *et al.* 2014)
Netherlands, Germany, France, Switzerland	River (Rhine)	$8.93 \cdot 10^5$ MP km^{-2}	(Mani *et al.* 2015)
Netherlands, Germany	Rivers (Rhine, Meuse); Amsterdam canals	$1 \cdot 10^5$ MP m^{-3}	(Leslie *et al.* 2017)
Switzerland	Lake (Geneva)	$4.81 \cdot 10^4$ MP km^{-2}	(Faure *et al.* 2012)
USA	Rivers (29 Great Lakes tributaries)	4.2 MP m^{-3}	(Baldwin *et al.* 2016)

In the Great Lakes of North America, pelagic microplastic counts reached up to 466305 particles km^{-2} in the highly populated Lake Erie, while particle counts for the less populated Lakes Huron and Superior reached 6541 particles km^{-2} and 12645 particles km^{-2} respectively (Eriksen *et al.* 2013a; Eerkes-Medrano *et al.* 2015). Even in Lake Hovsgol (Mongolia), a remote area with low population densities, the estimated pelagic microplastic densities reached 44435 particles km^{-2} (Free *et al.* 2014). Thus, microplastic contamination of freshwater environments has been found even in remote regions; although studies are limited, this suggests that microplastics are distributed in freshwater systems throughout the world. Therefore, more

systems should be studied to fill the gap in our knowledge of the distribution of microplastic pollution in freshwater environments globally.

1.3.3. Sampling and pre-treatment

Despite the growing number of studies about microplastics in freshwater systems, the methodologies on sample collection, pre-treatment, quantification and identification are not yet standardized (Li *et al.* 2018). Results often vary between studies, but it is difficult to distinguish whether these dissimilarities are linked to different abundance and distribution of microplastics or to different methodological approaches. Moreover, data about microplastics are often reported using alternative reference units, as either the number (or mass) of microplastic particles per unit area (e.g., m^2) or per volume (e.g., m^3), making it difficult to compare research results (Horton *et al.* 2017). Despite this, continued method development is improving researchers' ability to identify microplastics and common practices have been established.

Microplastics in surface water can be collected through bulk, or volume-reduced samples. In bulk samples, the entire volume of the sample is taken without reducing it during the sampling process; instead, in volume-reduced samples the volume of the bulk sample is usually reduced during sampling, preserving only that portion that is of interest for further processing (Hidalgo-Ruz *et al.* 2012). Only a few studies used the water collected by the bulk sampling approach, instead the samples are usually obtained by filtering large volumes of water with nets, using neuston nets, plankton nets, drift nets or manta trawls, and the most common size used is 300/333 µm, even if nets with a variety of mesh size have been used in previous studies (Rios Mendoza and Balcer 2019; Stock *et al.* 2019). The advantage of this technique is that large volumes of water can be filtered and that particles are directly

concentrated during sampling, obtaining a more representative samples integrated over the space. In contrast, the abundance of microplastics is usually underestimated due to the loss of smaller particles (Stock *et al.* 2019). For instance, Lindeque *et al.* (2020) compared nets with mesh sizes 100, 333 and 500 µm and showed that sampling using nets with a 100 µm mesh resulted in the collection of 2.5-fold and 10-fold greater microplastic concentrations compared with using 333 and 500 µm meshes respectively ($P < 0.01$). However, most of the authors still use larger mesh sizes as nets with the smaller mesh size clog more easily and, in general, manta trawls or nets are strongly recommended for large-scale surface water sampling in lakes or seas (Mai *et al.* 2018; Stock *et al.* 2019).

Regardless of the sampling methodology adopted, microplastic samples require further processing in the laboratory. Four main steps can be distinguished during laboratory treatment of samples: density separation, filtration, sieving, and visual sorting.

In density separation, materials of various densities are placed in a liquid of intermediate density, where the less dense material floats and separates from the denser sinking material. Changing the density of the liquid permits particles of various densities to float in the solution (Quinn *et al.* 2017). Due to the low density of most common plastics (0.8–1.4 g cm^{-3}), plastics float on the surface and can be retrieved. For separating them, several high-density solutions are utilised, and this separating solution is frequently a concentrated salt solution such as NaCl of varying densities, usually 1.2 g mL^{-1}, since it is cheap and inert. Due to the low density achievable using this salt, other studies have proposed different solutions, for instance, zinc chloride ($ZnCl_2$), sodium iodide (NaI), and sodium polytungstate ($Na_2WO_4 \cdot 9\ WO_3 \cdot 2\ H_2O$). While these alternative solutions help achieving a major density and thus potentially a greater recovery rate of microplastics, problems of toxicity and/or cost arise from all of them.

However, density separation procedures are more commonly used for sediment samples rather than water samples (Prata *et al.* 2019). The majority of studies analysing microplastics in surface water of environment samples pre-treated the samples with a chemical or enzymatic digestion for destroying the organic matter. This is a pivotal step as biological material is often confused with plastics, leading to a potential overestimation of environmental concentrations and increasing the number of particles subjected to further analysis (Prata *et al.* 2019). One of the most common approach is to pre-treat the samples with using an oxidizing agent, namely hydrogen peroxide (H_2O_2) at a concentration of 30% (v/v) (e.g., Su *et al.* 2016; Blettler *et al.* 2017; Li *et al.* 2019). The National Oceanic and Atmospheric Administration (NOAA) Marine Debris Program has suggested a wet peroxide oxidation method to extract microplastics from water and sediments that uses 30% hydrogen peroxide (H_2O_2) and an iron (Fe(II)) catalyst, with the application of heat during digestion and drying of samples (Masura *et al.* 2015). However, studies have demonstrated that there is potential for wet peroxide oxidation to impact polymers, thus, it has been suggested to adopt lower concentration of H_2O_2 (e.g., 10-15%; Nuelle *et al.* 2014; Zhao *et al.* 2017; Munno *et al.* 2018).

Besides oxidizing methods, digestion can also be acidic (e.g., nitric acid - HNO_3, perchloric acid, $HClO_4$), alkaline (e.g., sodium hydroxide - NaOH, potassium hydroxide - KOH) or enzymatic; however all these methods are less common for water samples and more widely used for sediments or biota extraction (Prata *et al.* 2019).

Since microplastics are everywhere, including indoor air they can easily contaminate samples if no contamination control measures are seriously adopted, leading to a potential overestimation of environmental concentrations (Dris *et al.* 2017; Prata *et al.* 2021). Contamination cannot be eliminated but can be reduced and controlled during the sampling and treatment steps. Measures that should be adopted to manage contamination

include, for instance, using procedural blanks and replicates to control for airborne contamination; keeping samples covered as much as possible and handling them in clean rooms with controlled air circulation and/or limited access; avoiding the use of synthetic textiles during sampling or sample handling, preferring the use of 100% cotton lab coat; using glass and metal equipment instead of plastics (Prata *et al.* 2021).

1.3.4. Identification and chemical characterization

After sample pre-treatment, visual examination still remains an obligatory step, especially for samples collected in environmental matrices in which not-plastics particles can still be abundant after pre-processing procedures. Careful visual sorting of residues is necessary to separate the plastics from other materials, such as organic debris (e.g., shell fragments, animal parts, microalgae) and other items (e.g., minerals, glass). This is done by direct examination of the sample by naked eye or with the aid of a dissecting microscope (Hidalgo-Ruz *et al.* 2012).

Still, it is commonly recognized that the visual identification is still necessary but not enough, with potentially high error rates, especially for smaller particles, and the adoption of additional identification steps (e.g., vibrational spectroscopy) is needed to achieve higher accuracy, precision and certainty of MP identification by avoiding false-positive and/or false-negative (Lenz *et al.* 2015). Indeed, several studies have already highlighted the discrepancy between the visual identification and spectroscopic methods (Lenz *et al.* 2015; Löder *et al.* 2015; Cheng *et al.* 2015).

It is generally agreed that potential microplastics' chemical characterization represents an essential step in the microplastic analysis, especially for smaller fragments (Prata *et al.* 2019). Among the current techniques applied for polymer identification, the most commonly used are Raman spectroscopy and Fourier-transform infrared (FT-IR) spectroscopy, which are also recommended by the Marine Strategy Framework Directive Technical

Subgroup on Marine Litter in European Seas (Hanke *et al.* 2013). These spectroscopic techniques allow the discrimination of plastic and natural particles, minimizing the occurrence of false positives, are non-destructive, and require low sample amounts with minimal sample preparation (Silva *et al.* 2018).

FT-IR and its optimized technologies, such as micro FT-IR, attenuated total reflectance (ATR) FT-IR, and focal plane array detector-based micro FT-IR imaging, are widely used in MP studies (Mai *et al.* 2018). With this technique, the samples are irradiated with IR light with a defined wavelength range and the IR radiation absorbance collected by the equipment is structure specific. The prerequisite for the IR absorption is the change of the permanent dipole moment of a chemical bond, thus this technique is mainly used to analyse the molecules with the polar functional groups (Li *et al.* 2018).

Raman spectroscopy is rapidly gaining ground in the field of microplastics. This spectroscopic technique depends, instead, on a change in polarizability of a molecule and it is based on the inelastic scattering of light that provides information upon the molecular vibrations of a system in the form of a vibrational spectrum. The Raman spectrum is akin to a fingerprint of chemical structure allowing identification of the components present in the sample (Araujo *et al.* 2018).

These two techniques have different advantages and disadvantages. Exemplarily, Raman has a better size resolution (detects particles down to a size of 1 µm), but fluorescence often impairs the quality of Raman spectra of environmental microplastics. In contrast, FTIR has a less precise size resolution (detects particles down to a size of 10–20 µm), and its spectral quality is not influenced by fluorescence but by the presence of water (Cabernard *et al.* 2018). Despite the importance of these techniques for polymer identification, many gaps need to be filled in order to optimize their use and the results obtained.

Besides spectroscopic methods, thermoanalytical methods such as pyrolysis-gas chromatography – mass spectrometry (Py-GC/MS), and thermal extraction-desorption gas chromatography mass spectrometry (TED-GC/MS) have been used for the microplastic analysis. In Py-GC/MS, microplastics are thermally decomposed (pyrolyzed) under inert conditions and the gas formed is cryo trapped and separated on a chromatographic column, identified by mass spectrometry. Instead, TED-GC/MS combines a thermogravimetric analysis (TGA) for thermal degradation (100–600°C) and solid phase extraction of plastic degradation products, further analyzed by thermal desorption in GC-MS (Prata *et al.* 2019; Goedecke *et al.* 2020). These methods present the advantage of analyzing relatively high masses, improving representativeness, but they are destructive, and the information provided is limited to chemical composition.

1.4. Impacts on aquatic ecosystems

Microplastic poses a risk to organisms across the full spectrum of biological organization from cellular to population level effects (Fig. 1.2). Due to differences in shape and density, microplastics disperse diversely in various compartments of the aquatic environment (water surface, water column and sediment), and this influences their availability to organisms at different trophic levels and/or occupying different habitats (Galloway *et al.* 2017). So far, our knowledge about the uptake and biological effects of microplastics comes from laboratory studies that applied simplified exposure regimes (e.g., one polymer and size, spherical shape, high concentrations) often with limited environmental relevance. However, the available data reveals species- and material related interactions and highlights that microplastics represent a multifaceted stressor (Scherer *et al.* 2018).

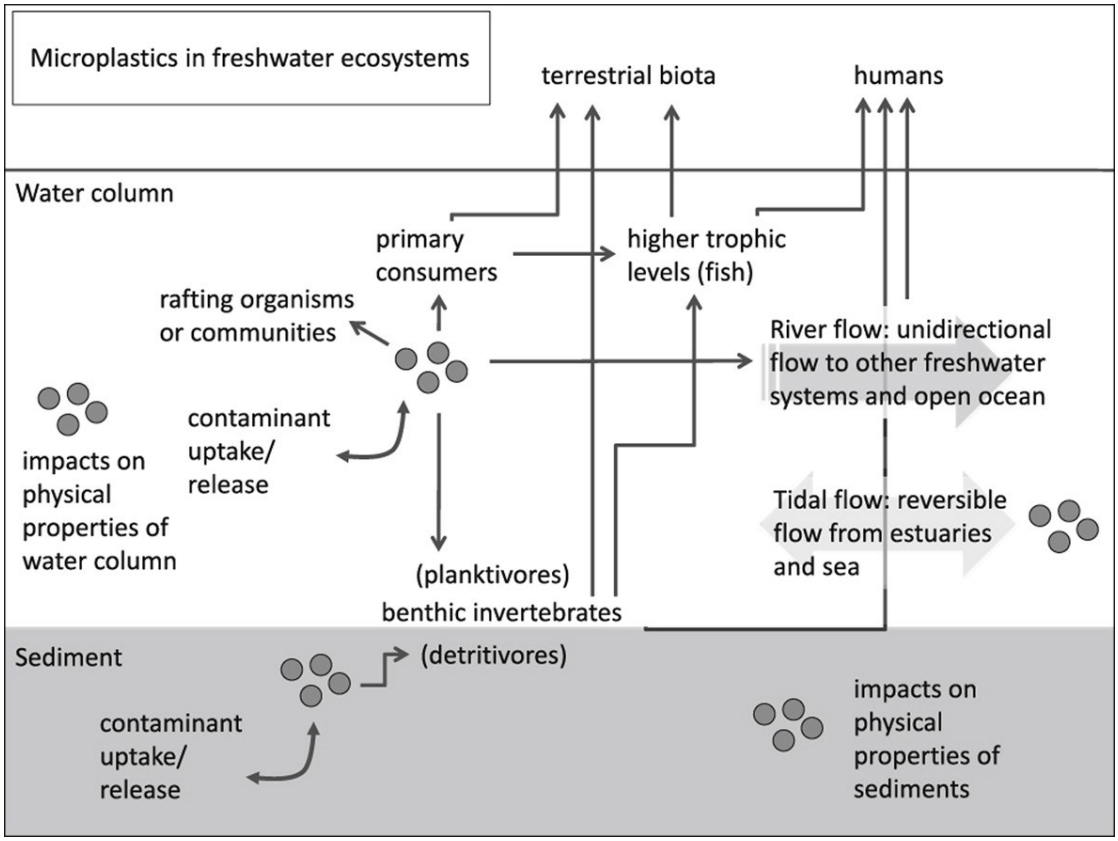


Figure 1.2. Diagram showing potential transfer pathways, and interaction with aquatic biota of microplastics in freshwater ecosystems. Figure originally from Eerkes-Medrano *et al.* (2015). "Microplastics in freshwater systems: A review of the emerging threats, identification of knowledge gaps and prioritisation of research needs", Water Research 75, 63-82. Reprinted with permission from Elsevier.

More than 690 marine species have been reported to ingest macro- or microplastics (Provencher *et al.* 2017). Field studies concerning microplastics quantification within freshwater fauna are highly limited compared to marine investigations, but the available literature suggests that freshwater organisms also ingest microplastics. Considering freshwater systems, ingestion of plastic particles has been reported for zooplanktonic organisms, like bacterivorous and herbivorous ciliates (e.g., *Halteria* sp., *Vorticella* sp.), rotifers (e.g., *Anuraeopsis fissa*), and cladocerans (*Daphnia* sp.); Bivalvia (e.g., *Sphaerium corneum, Anodonta cygnea*, C*orbicula flumine*); Oligochaeta (*Tubifex tubifex*) and several fish species (e.g., *Gobio gobio, Micropogonias furnieri*) (Scherer *et al.* 2018; Collard *et al.* 2019; Wang *et al.* 2019).

Microplastics uptake in most cases occurs accidentally since aquatic living organisms are often unable in distinguishing microplastics from the natural prey items. Previous studies on MP ingestion by freshwater species suggest that the role of the organisms in the food web (generalist vs. specialized feeders) may influence dietary MP uptake. Indeed, generalists (e.g., *Daphnia* sp.) or deposit feeders like the dipteran *Chironomus riparius* frequently ingested microplastics in laboratory experiments, while more specialized raptorial and carnivorous feeders like the cyclopoid copepod *Mesocyclops* sp., the rotifer *Asplanchna* sp. and the ciliate *Didinium* sp. did not (Scherer *et al.* 2018). However, given the potential of microplastics to enter complex aquatic food webs at low trophic levels, carnivorous predators may also consume microplastics preying on lower trophic organisms that have been contaminated by microplastics (Wesch *et al.* 2016; Wang *et al.* 2019). For instance, microplastics were transferred by prey in food chain tests with *D. magna* and *Chaoborus flavicans.* While the predator *C. flavicans* did not directly consume suspended microplastics (PS beads, 10 µm), the feeding of MP-containing daphnids (pre-fed on microplastics) resulted in an indirect uptake (Scherer *et al.* 2018).

The interaction of microplastics with aquatic biota actually start from low trophic levels. Indeed, different studies highlighted that plastic particles can interact with primary producers (microalgae) and this has impacts on their respective fates (Yokota *et al.* 2017). Existing studies on this issue have been mainly focused on the toxic effects of phytoplankton after exposure to microplastics, reporting effects on growth (e.g., Zhao *et al.* 2019; Venâncio *et al.* 2019), on photosynthetic activity (e.g., Zhang *et al.* 2017; Mao *et al.* 2018), and morphological changes (e.g., Mao *et al.* 2018). However, the interaction between microplastics and microalgae is far more complex with a wide range of consequences. Indeed, plastic debris constitute suitable substrates for the colonization by microalgae, and this may affect plastic degradation process, either having potential for biodegradation or, on the contrary, protecting plastics from ultraviolet radiation and photo-catalysis (Carson *et al.* 2013).

Furthermore, formation of a biofilm on the microplastics (biofouling) cause an increase of microplastic density and, thus, may affect the vertical fluxes of microplastics, determine their position along the water column and consequently their bioavailability (Long *et al.* 2015; Kooi *et al.* 2017). At a broader scale, it has been argued that this interaction may also have effects at the ecosystem levels, since it can affect primary productivity with consequences for aquatic ecosystem functioning (Zhang *et al.* 2020). Despite the relevance of this topic, studies addressing the interaction with organisms at the base of aquatic food webs are still extremely limited, especially in freshwater ecosystems (Wang *et al.* 2019).

In general, the detrimental effects caused by microplastics can be roughly categorized as physical (related to the shape, color and dimension of the particles) and chemical (related to the presence of additives and/or sorbed chemical contaminants).

There is a wealth of literature regarding physical impacts of macroplastic (e.g., Markic *et al.* 2018; Collard *et al.* 2018; López-Martínez *et al.* 2021), but detrimental effects are also likely to apply to ingestion of microplastics. These effects result in potentially fatal injuries such as blockages throughout the digestive system or abrasions from sharp objects. Other feasible impacts, as suggested by the *Task Group 10 Report Marine litter - Marine Strategy Framework Directive,* include blockage of enzyme production; diminished feeding stimulus; nutrient dilution; reduced growth rates; lowered steroid hormone levels; delayed ovulation and reproductive failure; and absorption of toxins (Galgani *et al.* 2010 and references therein). There is potential for microplastics to clog and block the feeding appendages of aquatic invertebrates or even to become embedded in tissues. Browne *et al.* (2008) employed the mussel *Mytilus edulis* to investigate ingestion, translocation, and accumulation of microplastics. The experiments showed that particles translocated from the gut to the circulatory system within 3 days and persisted for over 48 days, with smaller particles more abundant than larger ones.

Besides physical impacts of microplastics, chemical effects have been also reported. Indeed, plastic debris is associated with a complex mixture of chemicals. This includes those substances that are ingredients of the plastic material (e.g., residual monomers or oligomers of the component molecules of the plastics and additives such as nonylphenol (NP), bisphenol A (BPA), and phthalates), byproducts of manufacturing (e.g., chemicals composed during the combustion of the raw material petroleum) and chemical contaminants in the aquatic environments that accumulate on plastic (e.g., persistent organic pollutants (POPs) and metals) (Rochman 2015). Due to their large surface-to-volume ratio and chemical properties, microplastics can accumulate hydrophobic organic chemicals (HOCs), like polychlorobiphenyls (PCB), polycyclic aromatic hydrocarbons (PAH) or polybrominated diethyl ethers (PBDEs) (Wagner *et al.* 2014; Koelmans 2015). A study conducted by Frias *et al.* (2010) analyzing microplastics from two beaches of the Portuguese coast resulted in the contamination of POPs in all the pellet classes sampled. The predominant families of POPs found were PAHs (pyrene, fluoranthene, chrysene and phenanthrene) and PCBs (congeners: 18, 31, 138 and 187). Additional data have been published within the 'International Pellet Watch', an ongoing project to monitor POPs using plastic resin pellets as passive samplers. Pellet samples were collected from 75 locations across the world covering 26 countries and were analyzed for PAH, showing that PAH concentrations with the sum of 28 parent and methyl PAHs varied geographically, ranging from 0.035 to 24.4 μg/g-pellet (Yeo *et al.* 2017). Despite the growing number of studies about contaminants sorbed on microplastics, there is still a lack of information on other important contaminants like pharmaceuticals and endocrine-disrupting compounds (EDCs) (Wagner *et al.* 2014).

It has been demonstrated in laboratory studies that exposure to microplastic and toxic contaminants could result in bioaccumulation of the latter in aquatic animals that ingested microplastics (Rodrigues *et al.* 2019). Batel *et al.* (2018) reported transference of the hazard POP benzo[a]pyrene both in

gills of adult fish (*Danio rerio*) and fatty tissues of fish embryos not only via ingestion, but also by simple attachment to epithelia or via the water column. Avio *et al.* (2015), after exposing the mussels *Mytilus galloprovincialis* to pyrene, highlighted a marked capability of contaminated microplastics to transfer the contaminant to the exposed organisms with alterations of immunological responses, lysosomal compartment, peroxisomal proliferation, antioxidant system, neurotoxic effects, and onset of genotoxicity. However, the overall contribution of microplastics to the transport of hydrophobic organic chemicals (HOCs) is still under discussion. Indeed, Koelmans *et al*. (2016), analyzing data from previous studies, showed that on average the fraction of HOCs sorbed by plastic also is negligible compared to the fraction held by other media (e.g., DOC, colloids).

Unrealistic high microplastic exposure concentrations have been used in many studies and thus information on the current risks to aquatic ecosystems is missing. Under more realistic environmental conditions, organisms may simply ingest not enough microplastic particles compared to natural prey, rendering the effect on bioaccumulation to be even below a 10–20% difference in either direction (Koelmans *et al.* 2016). As the role of microplastics in disseminating persistent organic pollutants and biomagnification is still not clear, it is necessary to assess the presence of these chemicals on microplastics and their concentration levels.

1.5. Thesis outline

As described, the global contamination of aquatic environments with microplastics is of great concern and, despite the growing research interest, microplastic research has still many fundamental gaps, especially regarding freshwater ecosystems.

This thesis addresses some of these identified gaps by contributing to the current lack of knowledge concerning the following topics: i) methodological approaches for the identification of plastic polymers; ii) the distribution and extent of microplastic pollution in freshwater ecosystems worldwide; and iii) the interaction of this contaminants with microalgae, which represent the base of aquatic food webs.

This thesis is arranged into 6 chapters (Fig. 1.3). This introduction, which provided an overview of the literature and the aims and motivations of the thesis, is followed by a collection of four papers (three of which already published or submitted and one in preparation). A synthesis of the overall findings, outlining of future areas for research, is provided in the last chapter.

As summarized in section 1.3.4, the polymer identification of microplastics is becoming unavoidable to increase the data quality in this field. Raman spectroscopy has gaining importance in microplastic research, due to the several advantages of this technique. Therefore, in **Chapter 2**, I analyze and elaborate the current knowledge about Raman spectroscopy for plastic analysis in aquatic ecosystems and this investigation is combined with the development of an open-source database and R package for the identification of plastic polymer and additives through this spectroscopic technique. Firstly, the advantages and the drawbacks of Raman spectroscopy for plastic analysis are outlined. In particular, I discuss issues linked to fluorescence interference and the analysis of weathered polymers, which may complicate the interpretation of Raman signatures. I also provide a catalog with detailed information about peaks of most common plastic

polymers with the aim of systematically synthesized plastic Raman peaks to help future studies in polymer identification. Then, I describe and comment on a new R package 'RamanMP' that includes a database of 356 spectra, that I have created to provide a comprehensive, complete and freely available Raman spectral libraries of plastic polymers and additives.

Besides methodological gaps, data of contamination of microplastics in freshwater systems are still limited and highly fragmented. In **Chapter 3**, I present the results of the first global standardized sampling and analysis effort that I led to investigate the occurrence and distribution of microplastics in surface water of lakes and reservoirs with different geographical distribution and anthropogenic impacts. These data are the results of a scientific collaboration within the scientific network '*GLEON - Global Lake Ecological Observatory Network*', which involved more than 70 researchers across the globe to collect water samples of 38 freshwater systems with different features (e.g., area, depth, thermal regime, watershed), following a common protocol. This project has allowed obtaining comparable data about microplastic contamination in different freshwater systems around the globe. With this global dataset, the goals are to determine whether a relationship exists between the abundance of microplastics and the waterbody/watershed attributes and understand which factors influence the occurrence of microplastics in surface water of lentic systems.

Beyond the distribution of microplastics in freshwater systems, for the advancing of microplastic research is pivotal to understand the interaction of these pollutants with aquatic organisms. In particular, the relationship between microplastics and microalgae is often overlooked despite the fundamental role played by primary producers in aquatic systems. Thus, in the remaining chapters, I investigate this relationship in freshwater ecosystems.

In **Chapter 4**, a critical and wide-ranging literature review is conducted to synthesize the current state of knowledge on the microalgae-microplastic

relationship, analyzing about 80 peer-reviewed papers, from 1972 to 2020. Specifically, the main objectives are to: i) analyze whether some taxa of microalgae may preferentially colonize microplastic surfaces and which are the features and extent of the colonization process; ii) synthetize the environmental factors affecting microplastic colonization by microalgae; iii) discuss the consequences of colonization by microalgae on the microplastic fate and characteristics; iv) summarize the effects that microplastics may exert on microalgae; v) evaluate the effects at the ecosystem level of the interaction between microplastics and microalgae. Moreover, I comment on potential future questions and research directions needed to further define the implication of the relationship between microalgae and these concerning pollutants.

In **Chapter 5**, the relationship between microalgae and microplastics, previously deepened through the critical literature review, is investigated by performing a mesocosm experiment. The project is funded by "*AQUACOSM - Network of Leading European AQUAtic MesoCOSM Facilities Connecting Mountains to Oceans from the Arctic to the Mediterranean*" (EU H2020-INFRAIA-project No 731065) and the experiment has been performed at the Iberian Pond Network (IPN). In particular, periphyton growth and diversity on two different plastic polymers (i.e., high-density polyethylene - HDPE, polyethylene terephthalate - PET) is assessed in freshwater mesocosms distributed across five locations with different environmental conditions. The aim is to evaluate the biomass development and species composition of biofilms on different plastic polymers and to investigate whether plastic substrates exert a strong enough selection to drive species sorting, overcoming other niche-defining factors.

Lastly, in **Chapter 6** some general conclusions regarding the results described in the previous four chapters are drawn and future perspectives are highlighted.

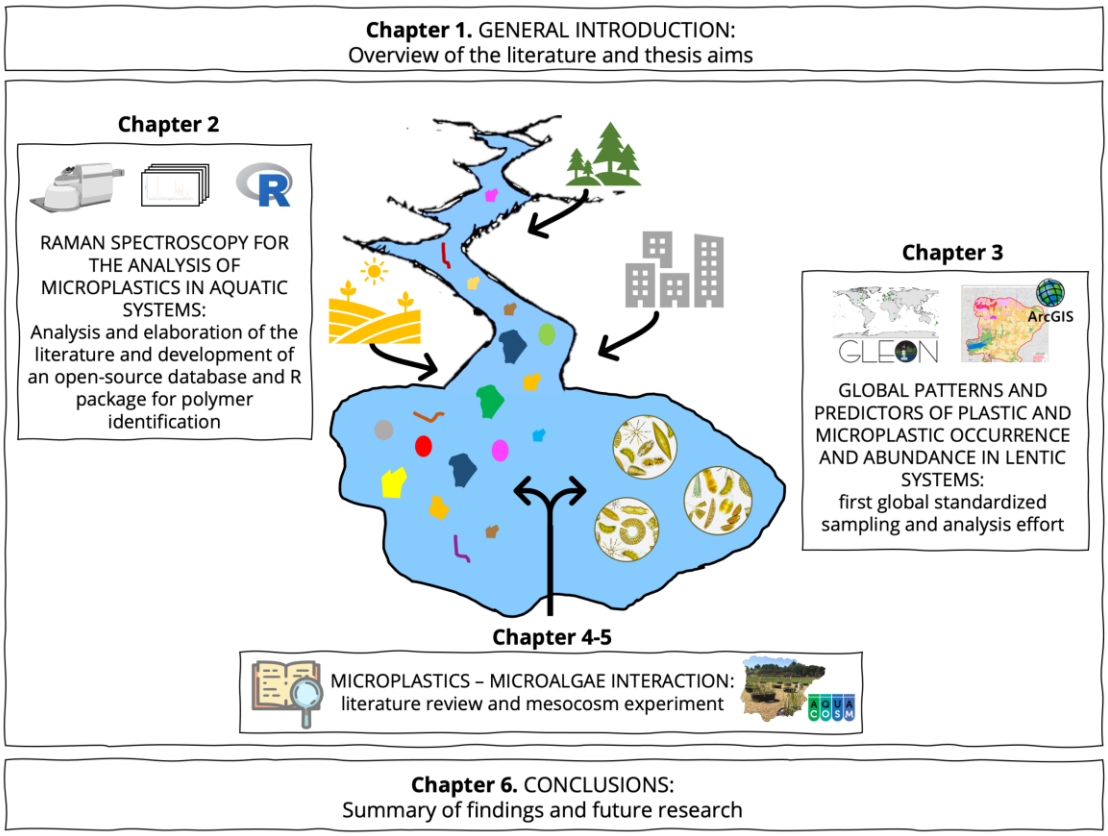


Figure 1.3. Overview of the chapters of the thesis.

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Chapter 2

Raman spectroscopy for the analysis of microplastics in aquatic systems

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Applied Spectroscopy **75** (11), 1341-1357 (2021)
doi: 10.1177/000370282110431

Abstract

Raman spectroscopy is gaining ground in the analysis of microplastics, especially due to its high spatial resolution that allows the investigation of small plastic particles, whose numeric abundance is argued to be particularly relevant in aquatic systems. Here, we aimed at outlining the status of Raman analysis of microplastics from aquatic systems, highlighting the advantages and the drawbacks of this technique and critically presenting tools and ways to effectively employ this instrument and to improve the spectra obtained and their interpretation. In particular, we summarized procedural information for the use of Raman spectroscopy, and we discussed issues linked to fluorescence interference and the analysis of weathered polymers, which may complicate the interpretation of Raman signatures. In this context, a deep understanding of the different plastic polymers and their Raman peaks and chemical fingerprints is fundamental to avoid misidentification. Therefore, we provided a catalog with detailed information about peaks of most common plastic polymers, and this represents, to the best of our knowledge, the first comprehensive resource that systematically synthesized plastic Raman peaks. Additionally, we focused on plastic additives, which are contained in the majority of plastics. These compounds are often intense in Raman scattering and may partly or completely overlie the actual material types, resulting in the identification of additives alone or misidentification issue. For these reasons, we also presented a new R package '`RamanMP`' that includes a database of 356 spectra (325 of which additives). This will help to foster the use of this technique, which is becoming especially relevant in microplastic analysis.

2.1. Introduction

The term ‘plastics’ includes a plethora of different polymers made of a wide range of semisynthetic or synthetic organic compounds. The large family of synthetic polymers has traditionally been classified into four groups: (i) thermoplastic polymers or “plastics”, *i.e.*, polymers that can be melted when heated above a specific temperature and harden upon cooling, recasting almost indefinitely; (ii) thermosetting polymers or “thermosets”, which undergo a chemical change when heated, creating a three-dimensional network, and thus cannot be re-melted and reformed; (iii) elastomers; and (iv) synthetic fibers. Strictly speaking, only thermoplastics and subsets of the other groups display thermoplastic character and should thus be referred to as “plastics”. However, the environmental science community has adopted the practice of referring to all polymers as “plastics” (Lehner *et al.* 2019).

While the benefits of plastics are undeniable, the widespread use of these polymers, namely in discardable forms like packaging materials, ultimately leads to their accumulation in the environment (da Costa *et al.* 2017). Although a fraction of this plastic waste is recycled, most of it ends up in landfills, where they may take a few hundred years to decompose (Cole *et al.* 2011). Of particular concern are plastics that enter the aquatic environment. According to a recent estimate, currently more than 5 trillion plastic particles are floating at sea, and a comparable abundance seems to be present in freshwater systems (Eriksen *et al.* 2014; Li *et al.* 2020). As plastic degradation proceeds and each particle fragments into ever smaller pieces, forming the so-called ‘microplastics’ (MPs, <5 mm), the total number of particles increases alongside the risks they pose to aquatic ecosystem functions (da Costa *et al.* 2017). Like many anthropogenic impacts on natural systems, plastic pollution is one that, despite widespread awareness of the problem, continues to escalate, and even if stopped immediately, will persist for centuries (Barnes *et al.* 2009).

Because of their small size, there are inherent difficulties in collecting, handling, identifying, and characterizing microplastics from environmental samples (da Costa *et al.* 2017). Moreover, since plastic materials are highly diverse in size, shape, color, density, and other physical and chemical properties, their identification is particularly challenging (Blair *et al.* 2019). Many analytical methods have been developed to measure microplastics in aquatic systems. Still, it is generally agreed that potential microplastics' chemical characterization represents an essential step in the microplastic analysis, especially for smaller fragments (Prata *et al.* 2019a). Among the current techniques applied for polymer identification, the most commonly used are Raman spectroscopy and Fourier-transform infrared (FT-IR) spectroscopy, which are also recommended by the Marine Strategy Framework Directive Technical Subgroup on Marine Litter in European Seas (Hanke *et al.* 2013). These spectroscopic techniques allow the discrimination of plastic and natural particles, minimizing the occurrence of false positives, are non-destructive, and require low sample amounts with minimal sample preparation (Silva *et al.* 2018). In particular, the Raman technique is rapidly gaining ground in the analysis of microplastics, especially given the higher spatial resolution (down to 1 μm while that of FT-IR is 10–20 μm), wider spectral coverage, higher sensitivity to non-polar functional groups, lower water interference and narrower spectral bands (Araujo *et al.* 2018).

Despite the relevance of Raman spectroscopy in microplastic identification from aquatic environments, comprehensive information about the use and the advantages and drawbacks of this technique for plastic polymer analysis is still missing. In particular, few information is available about additives, like colorants and plasticizers, which are contained in the majority of plastic products and can make difficult the interpretation of Raman signatures. The present paper aims to fill these gaps, giving indications and providing critical and novel perspectives for the researchers approaching Raman spectroscopy for microplastic analysis. In particular, the specific objectives

are: i) critically review and synthesize the operational settings and procedures as reported in the published literature; ii) highlight the limitations in analyzing microplastics through Raman spectroscopy and guide possible ways to overcome the difficulties; iii) discuss about additives whose presence may be identified through Raman spectroscopy and may interfere with polymer identification; iv) provide a catalog of reference spectra for the most common plastic polymers, selecting information from the literature and our laboratory experience; v) present a new R package for the processing and identification of plastic Raman spectra, implemented with a database of plastics and a variety of additives.

2.2. Raman for plastic analysis: overview from the literature

Raman spectroscopy has been successfully used to identify microplastic particles in different compartments of aquatic systems (i.e., water, sediment, biota), and a growing number of studies has been published about this topic in the most recent years (Löder and Gerdts 2015). The smallest fraction of microplastics (<300 µm) is still highly neglected in the current microplastic research (Conkle *et al.* 2018), even if it has been generally argued that mini-microplastics may be more numerically abundant than large microplastics (Erni-Cassola *et al.* 2017; Brandon *et al.* 2020). The break-down processes of plastics into smaller pieces are not likely to stop at micro-size but will continue to produce nano-sized plastics. Nanoplastics is probably the least known area of aquatic litter but potentially also the most hazardous because of their nano-specific properties, which fundamentally differ from those of the same polymer type in bulk form (Koelmans *et al.* 2015). Raman spectroscopy, given its high spatial resolution, becomes especially relevant for identifying microplastics and potentially NPs. Indeed recent studies have

proposed Raman imaging (Fang *et al.* 2020) or Raman tweezers (Gillibert *et al.* 2019) to identify nanoplastics down to 100 and 50 nm, respectively. However, identification methods for NPs are still in an early stage of development and further research is needed. Besides the high spatial resolution, Raman spectroscopy has further advantages, such as the wide spectral coverage, high sensitivity to non-polar functional groups, and low water interference (Araujo *et al.* 2018; Anger *et al.* 2018).

Different instrumental settings can influence the quality of analyses. For instance, the choice of the excitation laser wavelength can be pivotal in determining the performance of a Raman system for a particular sample. Different laser sources show specific strengths and weaknesses, but the two most widely used excitation wavelengths are 532-nm and 785-nm (Table 2.1). In plastic analysis, the most popular excitation source is the near-infrared (785-nm) laser, which is used in 57% of the studies reviewed. This laser wavelength offers an excellent balance of signal strength, fluorescence suppression, cost, and overall performance.

Raman scattering intensity is inversely proportional to the fourth power of the excitation wavelength (see Supplementary Materials for further information). Therefore, the obvious way of improving Raman sensitivity would be to use shorter wavelengths. For instance, for a given acquisition time, the Raman lines collected from a 532-nm source laser will be approximately 5 times as intense as those collected from a 785-nm source. Thus, the use of the green 532-nm laser is also considered a good compromise, and possibly other excitation systems could also be combined to better adapt to different microplastic features and improve the quality of spectra. The main problem with the 532-nm laser is that many compounds fluoresce in this region. Since Raman scattering is relatively weak compared to fluorescence, where fluorescence is present, the analyte or impurities may fluoresce sufficiently to swamp the detector (Smith and Dent 2019). Besides, it should be generally considered that the spectral resolution decreases as

the excitation wavelength decreases since the size of the focused laser beam is diffraction limited and dependent upon the laser wavelength (Smith and Dent 2019).

High Raman scattering, lower wavelength laser sources in the UV region (< 300 nm) are seldom used. Many compounds absorb UV radiation, and the high energy in this region implies an increased risk of sample degradation through photodecomposition and burning (Zhao *et al.* 2017). Also, UV lasers require specific detectors and gratings and are considerably more expensive. However, for many researchers the type of laser source and other options are determined by equipment availability already (Smith and Dent 2019). Other instrument parameters can be easily modified to improve the quality of analyses (Lopez-Reyes and Rull Pérez 2017). For instance, spectra can be recorded with different acquisition times and laser power: longer integration times increase the signal's intensity and reduce the signal-to-noise ratio. However, the detector can be saturated when using longer integration times, resulting in cut off signals. Besides, it is also possible to increase the number of acquisitions, which allows a considerable increase of the spectra's quality. The increase of both integration time and the number of accumulations, while improving the spectra's quality, leads to longer analysis times. Thus, there is the need to balance among different parameters to analyze microplastics, whose whole processing is already time-consuming (Huppertsberg and Knepper 2018).

It is not simple to provide information on the optimal parameters for acquiring spectra, since plastic particles include various polymers with different colors, additives, chemical composition, and properties (Nava and Leoni 2021), requiring to adjust specifically the instrumental set-up for each analysed particle. For instance, black samples strongly absorb laser light, heat up, and produce an intense background emission and thus require different acquisition conditions than other colored particles (Ribeiro-Claro *et al.* 2016; Asensio-Montesinos *et al.* 2020); fibers are more prone to damage

(via “burning”) if analyzed at high laser power. Therefore, lower laser power and longer acquisition times are needed (Rodríguez-Romeu *et al.* 2020).
The need of adapting the instrument parameters throughout the analysis of microplastics in samples clearly emerges from several studies, in which it is reported that the laser power, the acquisition times and the accumulations were adjusted for each sample depending on the signal-to-noise ratio and the quality of spectra (e.g., Edo *et al.* 2019; Edo *et al.* 2020; González-Pleiter *et al.* 2020; Shruti *et al.* 2020). Accordingly, it is also difficult to automate the spectra acquisition process, even if different automation techniques have been proposed. These include, for instance, full point mapping, where a whole filter section is analyzed by collecting spectra at various points along a grid, or the use of image analysis software, which creates a map of all particles in a given area and Raman spectra are collected only at those points (Araujo *et al.* 2018; Thaysen *et al.* 2020).

Since microplastic pollution is widespread and interests a diverse range of environmental matrices in aquatic systems (*e.g.,* surface water, water column, deep sediment, shoreline, biota), the procedures that are adopted to clean and prepare the samples before the spectroscopic analysis can wary widely, and can also influence the analysis results (Schwaferts *et al.* 2019).
Providing detailed information about the proper preparation of microplastics samples of different origin is beyond the scope of this study. However, some information about the substrate used to hold particles for the Raman analysis can be useful for future research. Using Raman spectroscopy, the scattering of monochromatic light is focused mainly on the surface region and, therefore, the measurement substrate is not as relevant as for other techniques, such as transmission FT-IR spectroscopy. However, vibrations of the underlying substrate can be observed in Raman spectra of thin and transparent samples. Therefore, the filter substrate for Raman analysis of microplastic samples should not exhibit any bands in the spectral range of the polymer and should not be fluorescent (Käppler *et al.* 2015). Besides,

membrane filters should also display particular optical properties to allow the use of automated particle detection (Oßmann *et al.* 2017). For manual Raman measurements and automatic particle detection, filters should be selected carefully, to fulfill some requirements. Firstly, the pore size of the membrane filter must be small enough to retain all relevant particles. Then, the surface of the membrane filter should be smooth and unstructured to ease the detection of the microplastics, especially the smaller ones. Indeed, small particles might be concealed within the membrane structure, or the structure itself might be recognized as a particle (Oßmann *et al.* 2017).

Filters used in microplastic studies include gold-coated polycarbonate (Schymanski *et al.* 2018), cellulose nitrate (Kankanige and Babel 2020; Martinelli *et al.* 2020), aluminum oxide (Allen *et al.* 2019; Tong *et al.* 2020), silicon (Alam *et al.* 2019; Jiang *et al.* 2019), and glass microfiber filters (Kaliszewicz *et al.* 2020; Kazour and Amara 2020). Advantages and drawbacks for some of these filters have been previously reported. For instance, glass microfiber filters are a cheap option, but the surface is not smooth enough to make the particles easily visible (Oßmann *et al.* 2017); aluminum oxide filters are less expensive than other options but show a weak fluorescence profile (Käppler *et al.* 2015); gold-coated polycarbonate are readily available but are expensive and, since gold is a weak Raman emitter, may have a problem of fluorescence (Oßmann *et al.* 2017); silicon filters are rigid and provide good visible images, but silicon peaks are present in spectra. When the particles are not directly analyzed on the filters, a common choice is to place and analyze plastic particles on glass slides, which has a clean Raman background (Fang *et al.* 2020) and the possibility to cover the samples easily and thus avoiding contamination and/or loss of particles (e.g., Bottari *et al.* 2019; Capillo *et al.* 2020; Dodson *et al.* 2020). However, this substrate's use is limited to the analysis of larger microplastics (i.e., >250-300 µm) that can be easily picked up and transferred to the glass slide.

The Raman analysis of microplastics, both manual and automatic, is a laborious process; thus, the identification of the polymeric composition of plastic particles is usually performed on a subset, especially for plastic-rich samples. Different approaches are typically followed to select a subset of the sample. The most commonly used approaches include, among other, the random selection of a specific area of the filter or a percentage/number of particles on the filter (Imhof *et al.* 2016; Oßmann *et al.* 2018; Deng *et al.* 2020; Thaysen *et al.* 2020). A robust procedure has recently been suggested to determine how many microplastics must be analyzed to give a representative view of the particle size distribution and chemical nature and calculate the associated margin error (Kedzierski *et al.* 2019). In particular, the authors proposed a method in which each particle collected receives a unique identifier; then, the user determines the accuracy of the results desired and, from this information, it determines the number of particles to be analyzed selecting between two different equations proposed (for details see Kedzierski *et al.* 2019). This kind of approach must be preferred since it allows a more precise estimation of the analysis's representativeness while reducing the analysis time. Best-practice should clearly define how the subset has been defined and how the results have been then scaled up to the whole sample, which is largely missing in the current microplastic research.

Table 2.1. Operational settings of Raman spectroscopy used in microplastic studies performed in aquatic systems. We report only studies for which sufficient data were available.

Laser (nm)	Objective	Acquisition time (s)	Accumulations	Spectral range (cm^{-1})	Matrix	Reference
785 (25 mW)	NA	20-45	NA	300-2500	Water and sediment (river)	(Alam *et al.* 2019)
532	20X	5	2	1135–1844; 2604–3177	Sediment (beach)	(Asensio-Montesinos *et al.* 2020)
532 (0.5 mW)	50X	1-10*	10	NA	Biota (*Zeus faber*; *Lepidopus caudatus*)	(Bottari *et al.* 2019)
532 (10-100 mW)	50X	10-50*	NA	100-3000	Biota (*Mullus barbatus barbatus*; *Trigla lyra*; *Galeus melastomus*; *Scyliorhinus canicula*; *Raja miraletus*)	(Capillo *et al.* 2020)
488 (0.75-1.5 mW)	5-20-50-100X	10	NA	NA	Water (marine); biota (*Pleuroncodes planipes*; *Bathochordaeus stygius*)	(Choy *et al.* 2019)
785 (10-100 mW)	10-20X	NA	NA	90-3200	Sediment (marine coastline)	(Clunies-Ross *et al.* 2016)
514.5 (<5 mW); 784.7 (<30 mW)	50-100X	5-50	NA	NA	Biota (*Squalius cephalus*)	(Collard *et al.* 2018)
514.5 (≤5 mW)	50-100X	10-40	NA	NA	Biota (*Engraulis encrasicolus*)	(Collard *et al.* 2018)
785	NA	90	2	100–3200	Sediment (mangrove wetlands)	(Deng *et al.* 2020)
633 (3.15 mW); 532 (4.1 mW)	10-50X	10-500	NA	50-3700	Biota (*Unio pictorum*)	(Domogalla-Urbansky *et al.* 2019)
532; 633	NA	10	2	300-3300	Sediment (lake)	(Dong *et al.* 2020a)
532	10-100X	1-10	1-10	200-3500	Sediment (river)	(Dong *et al.* 2020b)
780 (7-8 mW)	10-20-50X	*	*	200-3100	Sediment (beach)	(Edo *et al.* 2019)
455	10-50X	20 - mins	NA	100-3500	Water (marine)	(Enders *et al.* 2015)

785 (1-100 mW)	20-100X	10-60	NA	NA	Water (marine); biota (fish)	(Ghosal *et al.* 2018)
780	10-20-50X	*	NA	200-3100	Water (lake)	(González-Pleiter *et al.* 2020)
532; 785	50X	2	10	300-3200	Biota (*Chelon saliens*; *Mullus barbatus barbatus*; *Mullus surmuletus*; *Trachurus mediterraneus*; *Lithognathus mormyrus*)	(Gündoğdu *et al.* 2020)
785	50X	10-30	1-3	NA	Biota (*Acanthopagrus australis*, *Mugil cephalus*, *Gerres subfasciatus*)	(Halstead *et al.* 2018)
785	50X	30	2	600-3200	Sediment (river)	(Horton *et al.* 2017)
632.8 (~14 mW)	10-50X	NA	NA	50-400	Sediment (lake shore)	(Imhof *et al.* 2016)
532 (<2 mW)	20X	0.5-1	30-120	NA	Water (river, lakes)	(Kaliszewicz *et al.* 2020)
532; 785	10-100X	0.2; 1; 2; 5	2; 5; 10; 15	200-3400	Biota (*Mytilus edulis*)	(Kazour and Amara 2020)
785	10-100X	NA	NA	200-3500	Biota (*Platichthys flesus*)	(Kazour *et al.* 2020)
532; 785	10-100X	NA	NA	200-3400	Water, sediment (marine), biota (*Engraulis encrasicolus*; *Spondylus spinosus*)	(Kazour *et al.* 2019a)
532; 785	10-100X	NA	NA	200-3400	Wastewater, water, sediment, biota (marine)	(Kazour *et al.* 2019b)
785 (0.75-37.25mW)	20X	10-30	NA	100-3400	Biota (benthic fauna)	(La Beur *et al.* 2019)
785	NA	40	2	200-1800	Sediment (beach)	(Lots *et al.* 2017)
632.8 (20 mW)	50X	1	10-100	NA	Biota (*Scyliorhinus canicula*)	(Mancia *et al.* 2020)
785 (90 mW)	50-100X	12	10-30	150-3200	Water (river)	(Mani *et al.* 2019)
785	10-50X	30	10	NA	Sediment (marine), biota (*Hediste diversicolor*)	(Missawi *et al.* 2020)

532 (<5 mW)	50X	10	NA	NA	Biota (*Perna viridis*)	(Naidu 2019)
532 (<5 mW)	50X	10	NA	NA	Biota (*Sternaspis scutata*, *Magelona cinta*, *Tellina* sp.)	(Naidu *et al.* 2018)
532; 785 (1-60 mW)	20-50X	10-60	NA	400-4000	Water (marine)	(Pan *et al.* 2019a)
532; 785 (1-60 mW)	20-50X	10-60	NA	400-4000	Water (marine)	(Pan *et al.* 2019b)
532; 785 (1-60 mW)	20-50X	10-60	NA	400-4000	Water (marine)	(Pan *et al.* 2019c)
488 (1.5 mW)	40X	0.1	NA	NA	Biota (*Mullus barbatus*)	(Rodríguez-Romeu *et al.* 2020)
532 (0.5 mW)	50X	50	10	NA	Biota (*Engraulis encrasicolus*; *Sardina pilchardus*)	(Savoca *et al.* 2020)
532 700-1100 (<100 mW)	50X	NA	NA	100-3000 300-3200	Water (marine), biota (*Pagellus erythrinus; P. bogaraveo*)	(Savoca *et al.* 2019)
785	NA	40	2	200-3200	Sediment (lake)	(Turner *et al.* 2019)
785	20-50X	10- min	NA	50-4000	Water (marine)	(Wang *et al.* 2020)
785	50-200-500X	3	10	NA	Biota (*Lasmigona costata*)	(Wardlaw and Prosser 2020)
785	50X	NA	NA	150-1850	Biota (sea turtles)	(White *et al.* 2018)
473, 532 (5-45 mW); 632.8 (5-37 mW)	100X	5-500	*	600-1750	Water (marine)	(Zobkov *et al.* 2019)

*Parameters adjusted depending on signal-to-noise ratio.

2.3. Limitations in Raman analysis of plastics

One of the leading causes of poor Raman signal quality for the analysis of microplastics is the presence of concurrent fluorescence interference, which may be either intrinsic to the main constituent of the plastic fragment or due to impurities such as coloring agents, degradation products, and biological material (Lenz *et al.* 2015; Löder and Gerdts 2015; Primpke *et al.* 2020). The fluorescence intensity can be orders of magnitude larger than the Raman scattering, thus impeding the interpretation of Raman spectra of polymers (Zhao *et al.* 2017). Indeed, fluorescence background usually manifests as an increased baseline, which covers the Raman signatures (Renner *et al.* 2019). Since fluorescence strictly depends on the analyzed particle features, the intensity of fluorescence can vary depending on several factors. For instance, the color of the particles can influence the quality of the spectra, and it is reported that measurements of red and yellow colored items are more affected (Lenz *et al.* 2015). Moreover, organic residues of algae or plant residues attached to microplastic surfaces may be causes of a strong fluorescence. This can be reduced by adopting an adequate procedure of purification. One of the chemicals widely used for this scope is hydrogen peroxide (H_2O_2), used alone (usually at a concentration of 15-30% v/v) or in combination with other chemicals like for instance iron (Prata *et al.* 2019b; Wiggin and Holland 2019). Indeed, it is reported that H_2O_2 is particularly efficient in removing organic matter coating from the polymer matrix (Prata *et al.* 2019b). However, even if proper pre-treatments are applied, removing all the organic materials is difficult, and fluorescence can still occur for organic-rich samples. In this case, Raman microspectroscopy's high spatial resolution may be used to circumvent the problems posed by deposits on the microplastic particles. Indeed, employing a high-magnification, high-numerical-aperture objective lens, and a low confocal hole, fluorescence can be significantly suppressed (Lenz *et al.* 2015; Ribeiro-Claro *et al.* 2016). Another way to overcome the fluorescence issue is by photo-bleaching the

sample, which consists of constant sample irradiation for an extended period before acquiring a spectrum to degrade the fluorescent agent (Zięba-Palus and Michalska 2014). However, this procedure, cannot be used for samples susceptible to photo-degradation, and, even when applied, it is not always efficient (Araujo *et al.* 2018).

There are also well established computational approaches for baseline treatments, which allow removing the fluorescence background and reveal the underlying polymer spectrum, and several methods (e.g., weighted least squares, asymmetric least squares) have been proposed with this aim (Renner *et al.* 2019; Xu *et al.* 2019). Renner *et al.* (2019) proposed the use of Savitzky–Golay differentiation, which is based on Savitzky–Golay smoothing, but instead substitute a raw data point with a center point of polynomial fit of a moving smoothing window, the n^{th} derivative of this polynomial is used. Ghosal *et al.* (2018) suggested an algorithm builds upon the modified multi-polynomial fitting method (ModPoly) developed by Lieber *et al.* (2003), which iteratively fits a polynomial function to a spectrum's fluorescence background and thereby subtracts the auto-fluorescence signal. In some cases, the fluorescence is so intense that none of those mentioned above methods can overcome the problem; thus, the only remaining possibility is to adopt a different excitation source using lasers with higher wavelengths (see section 2.2).

Microplastics in the environment are subject to various weathering processes, including ultraviolet (UV) radiation, biodegradation, physical abrasion, and chemical oxidation. Differences between the Raman spectra of weathered microplastics and the standard spectra can be observed, which may lead to the inaccurate identification of certain microplastics (Dong *et al.* 2020b). Several studies reported that these dissimilarities arise mainly from different intensities in the characteristic peaks and in the occurrence of several new oxidative functional groups developed after weathering processes (Cai *et al.* 2018). Specifically, the weakened stretching vibrations of

methyl, methylene, and methine at 2800–3000 cm^{-1} and a broad and strong band at 2100–2200 cm^{-1} are commonly reported (Dong *et al.* 2020b). Therefore, considering that the Raman spectra of weathered microplastics are prone to change, when identifying unknown microplastics in environmental samples, comparison with commercial libraries of Raman spectra, which generally only includes virgin material, may lead to unsatisfactory results. Thus, including databases of weathered microplastics may be beneficial, and some studies that provide Raman database of weathered microplastics already exist (Munno *et al.* 2020; Dong *et al.* 2020b).

2.4. Raman analysis of plastics with additives

Another issue for Raman analysis of microplastics is the presence in MP particles of additive compounds (e.g., fillers, anti-aging additives, colorants, plasticizers), which, besides inducing fluorescence, impede the determination of the plastic polymer composition (Fig. 2.1). Indeed, many of these additives are intense in Raman scattering and may partly or even completely overlie the actual material types, resulting in the identification of additives alone or to misidentification issue (Lenz *et al.* 2015; Käppler *et al.* 2016; Zhao *et al.* 2017). Nevertheless, additional information about the additives contained in plastics, usually not detected by FT-IR microspectroscopy, can be obtained through Raman spectroscopy (Käppler *et al.* 2016).

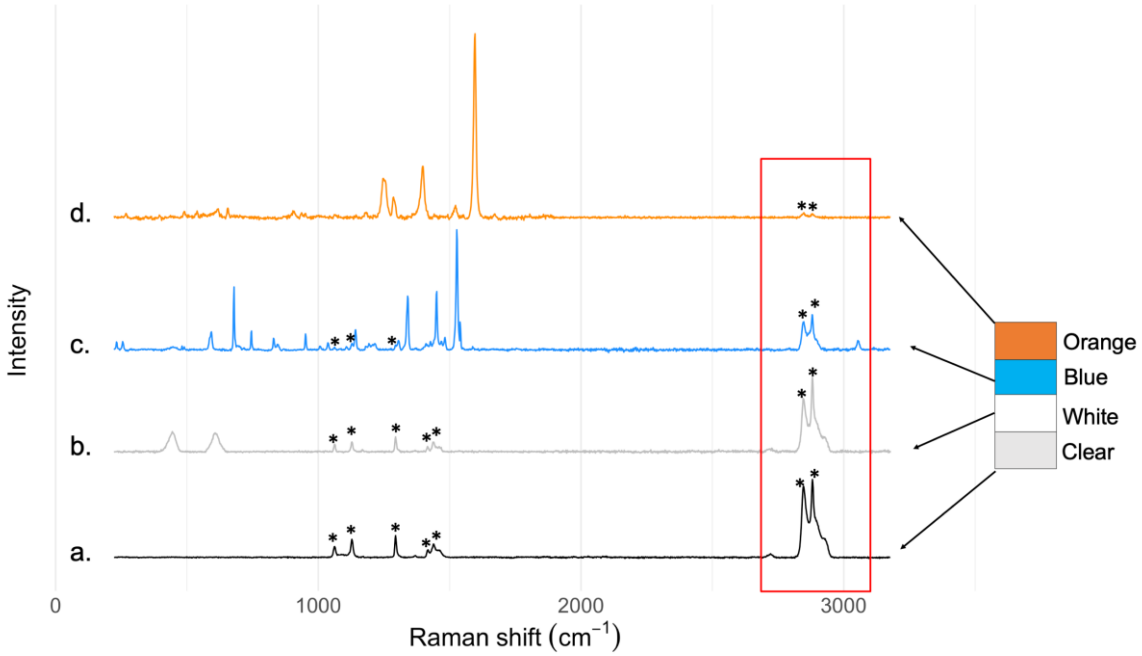

Figure 2.1. Raman spectra of Polyethylene (PE) packaging with different printed colors: a) clear; b) white; c) blue; d) orange. It is possible to notice the presence of pigments of the different colored pigments, which in some cases almost completely conceal the diagnostic Raman peaks of PE. The asterisks indicate the characteristic peaks of PE. Acquisition conditions: laser 532nm; 30 mW; 30s x 1 accumulation; 50X; grating 600; hole 200; spectral range 223-3177 cm^{-1}.

Plastics can have color applied by various methods, including surface printing or surface coating (painting), applying a decorative film, or by incorporating colorant additives into the polymer mass via compounding. Mass coloration of plastics uses colorant additives: these can be dyestuffs, colored substances that are soluble or go into solution during the application process, or pigments (organic or inorganic), which are usually insoluble in polymers and are dispersed in the plastic (Gürses *et al.* 2016; Al-Malaika *et al.* 2017). Organic pigments can be, in turn, classified into classical azo pigments (monoazo, diazo), special azo pigments (naphthols, azo condensation, benzimidazolone), and polycyclic pigments (e.g., phthalocyanine, anthraquinone, dioxazine). Among the inorganic pigments, titanium dioxide (TiO_2, Pigment White 6) is the most widely used in the plastics industry. This is a white pigment used in plastics in two crystal forms (Fig. 2.2a): the rutile grade, most commonly used, and the anatase grade. Different studies

reported the presence of this pigment in microplastic samples (Lenz *et al.* 2015; e.g., Imhof *et al.* 2016; Schymanski *et al.* 2018). Another pigment commonly reported in microplastic studies is copper phthalocyanine (Pigment Blue 15, Fig. 2.2b), a common blue pigment used for dyeing plastics (Weber *et al.* 2020). In microplastic particles, colorants vary widely among studies since pigments and dyes used for plastic materials are abundant and different. For instance, Imhof *et al.* (2016), studying paint particles abundance from beach sediment of a lake, highlighted that the most common colorants found were, beside TiO_2 and phthalocyanine blue, Pigment Green 7 (phthalocyanine green) and Pigment Yellow 83 (Fig. 2.2c); Oßmann *et al*. (2018) investigating the presence of microplastics in samples of mineral water found colored particles attributed to Pigment Yellow 83, Pigment Violet 23, and Pigment Blue 15; Van Cauwenberghe *et al.* (2013) identified three different colorants in particles collected from deep-sea sediments, i.e., copper phthalocyanine, polychloro copper phthalocyanine, and permanent red; Weber *et al*. (2020) studying tap water found, besides copper phthalocyanine, the presence of reactive black 5 and indigo dye, which are frequently used for dyeing clothing (Fig. 2.2d).

Colorfulness is not exclusively a feature of plastic products; consequently, when a Raman spectrum revealed the colorant only, defining the polymer composition is not possible. Therefore, these particles are usually classified as “anthropogenic” (Imhof *et al.* 2016). However, even if the Raman spectra of the polymer cannot be determined, some information may still be obtained from the colorant used. Indeed, while colorants can be used to stain several polymers, some polymers stain better with one colorant over another, and hence the plastic industries tend to use specific colorants for specific polymers (Al-Malaika *et al.* 2017; Zhu *et al.* 2019). This is especially true for fabrics. For instance, the vat dyes indigo is used exclusively to dye denim, while disperse dyes are used for synthetic plastic polymers, namely, polyester and polyolefin fibers (Zhu *et al.* 2019). From this knowledge, Zhu *et al.* (2019) developed a multistep method for analyzing microfibers to material

type based on the dye used, which may help in the identification of dyed textile fibers.

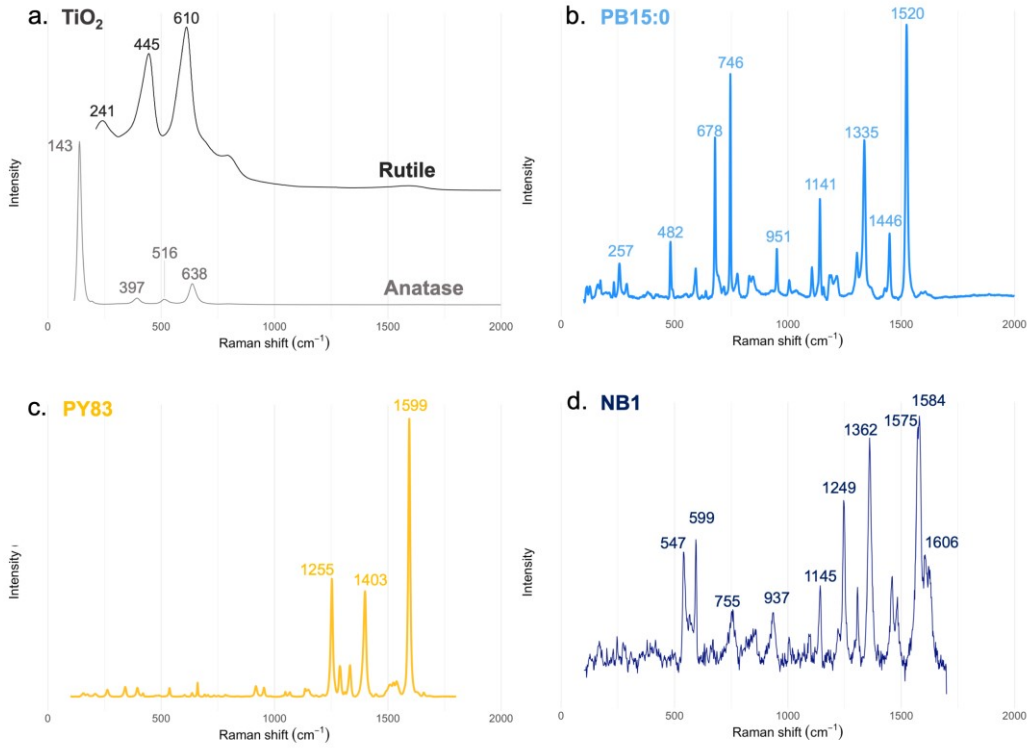

Figure 2.2. Raman spectra of some common additives and main peaks highlighted. a) TiO_2, white pigment (PW6) in the two crystal forms (i.e., rutile, most widely used, and anatase); b) Copper Phthalocyanine (Pigment Blue 15:0, PB15:0); c) Diarylide Yellow (Pigment Yellow 83, PY83); d) Indigo dye (Natural Blue 1, NB1). Spectra of PW6 were obtained from Lafuente *et al*. (2016); of PB15:0 and PY83 from Fremout and Saverwyns (2012), and NB1 from Caggiani *et al.* (2016).

Besides colorants, plasticizers are used in a variety of polymers for different purposes, such as increase moldability and flexibility or reduce melt viscosity. Their primary use is in the production of flexible PVC, and polymers other than PVC account for less than 10% of plasticizers used (Al-Malaika *et al.* 2017). For this reason, PVC polymer identification through Raman spectroscopy should take into account the presence of different plasticizers. Currently, 500 types of plasticizers have been industrialized, and diesters of phthalic acids, also known as phthalates, are the most extensively employed plasticizers (e.g., dioctyl phthalate, DOP, also known as Di(2-ethylhexyl)

phthalate, DEHP, Fig. 2.3) for PVC and covers more than 80% of the overall plasticizer utilization (Kumar 2019).

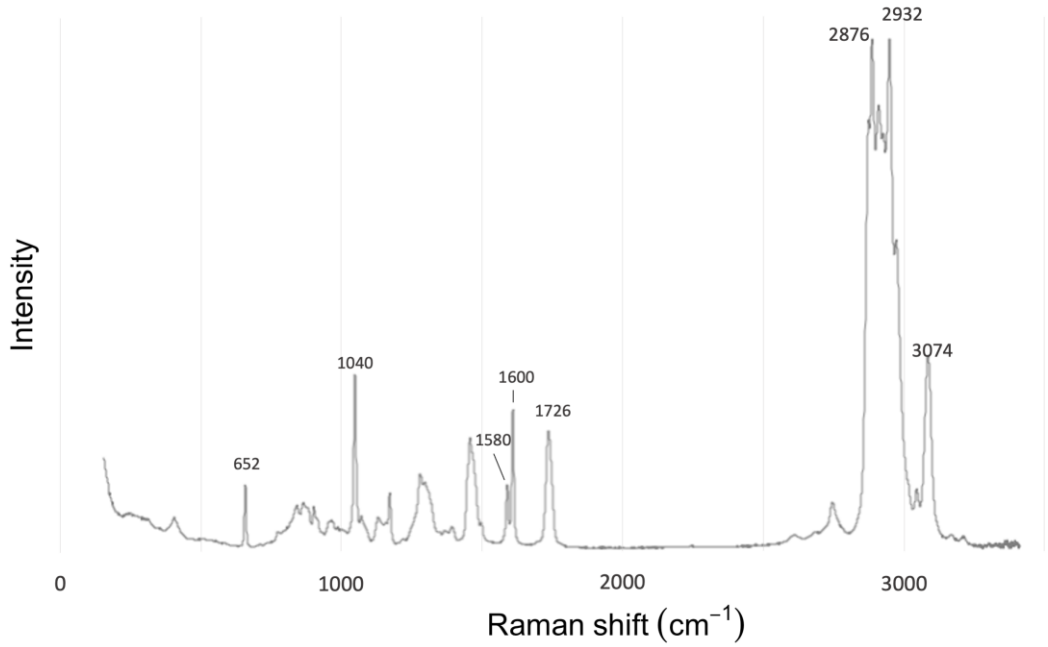

Figure 2.3. Raman spectra of dioctyl phthalate (DOP), also known as Di(2-ethylhexyl) phthalate, DEHP, extensively employed as plasticizer in PVC polymers.

2.5. Band assignment in the Raman spectra of most common plastic polymers

As mentioned, plastic molecules may be widely altered, and the presence of additives, the occurrence of degradation, or the coating of plastic surface with organic residues may differently alter the Raman spectra of these polymers, making it difficult to obtain a good fitting when employing automatic procedure of comparison with spectral libraries. Therefore, the knowledge of the main peaks of the most common plastics becomes pivotal to avoid misidentification of polymer composition. To assist in this, we reported the Raman peaks for many common plastic polymers in Table 2.2.

Table 2.2. Main Raman vibrations (cm^{-1}) of most common plastic polymers.

Polymer	Vibration frequency (cm^{-1})	References
Acrylonitrile butadiene styrene (ABS)	621-745-780-902-994-**1002**-1032-1077-1099-1156-1183-1200-1330-1441- 1453-**1583-**1603-1640-1653-1668-2240-**2854**-2906-2939-2981-3004-**3060**	(Bikulčius *et al.* 2014)
Isotactic polypropylenee (iPP)	252-321-**398**-458-530-**809**-**841**-900-941-973-998-**1040**-1102-**1152-**1167sh-1219-1257-1296-1306-**1330**-1360-1371sh-1435-**1458**-2840-2871-**2883**-2905-2920-2952	(Andreassen 1999)
Nylon 6 – α polymorph (αNY6)	932-1065-**1130**-1203-1283-1310-**1444**-1470-1480-1203-**1636**-**2869**-**2900**-**2929**-3302	(Milani 2015)
Nylon 6 – γ polymorph (γNY6)	925-962/977-1060-**1080**-1234-1276-1298-**1440**-**1636**-**2869**-**2900**-**2929**-3302	(Milani 2015)
Nylon 66 (NY66)	601-952-1063-1128-1235-**1298**-1382-**1441**-1475-1550-**1637**-2732-**2867**-**2919**-3304	(Larkin 2018)
Polyacrylonitrile (PAN)	264-515sh-531-822-1082-1104-1223-1317-1354-1453-**2237**-2872sh-2909-2940	(Huang and Koenig 1971)
Polybutylene-1 (PB-1)	372-523-535-973-1001-1094-1150-1299-1341-1363-1376-**1447**-1461-2730-2854-**2879**-2908-2932-2960	(Cornell and Koenig 1969)
Polycarbonate (PC)[2]	400-480-575-635-704-733-760sh-815-828-**887**-919-936-1007-1080-1110-1145-1178-1235-1290-1308-1444-1464-**1602**-1772-2720-2760-2872-2912-2940-2974-**3074**	(Gedler *et al.* 2013)
Polyethylene (PE)	1062-1130-1170-1295-1417-1440-1460-**2850**-**2883**	(Sagitova *et al.* 2016; Zanocco *et al.* 2020)

Polyethylene Terephthalate (PET)	278-626-701-800-857-950-1000-1096-1119-1192-1295-1310sh?-1418-1462-**1615**-**1730**-2912-2968-3085	(Boerio *et al.* 1976)
(Poly)Ethylene vinyl acetate ((P)EVOH/EVA)[1]	380-421-477-521sh-600-738-825sh-851-902-1024-1069-1088-1114-1144-1301-1365-**1438**-2186-2721-2851sh-**2901**-2935sh-3006-3318	(Cooney *et al.* 1994)
Polymethyl methacrylate (PMMA)	304-370-400-484-504-537-**601**-736-796-813-833-857-972-986-1125-1161-1188-1234-1276-1400-1453-1494-1728-2846-2849-2920-2957	(Willis *et al.* 1969)
Polystyrene (PS)	621-795-**1001**-1031-1155-1450-1583-**1602**-2854-2904-**3054**	(Mazilu *et al.* 2010; Yan *et al.* 2012)
Polytetrafluoroethylene or Teflon (PTFE)	198-291-383-575-595-**729**-1215-1295-1379	(Koenig and Boerio 1969)
Polyvinyl alcohol (PVOH or PVA)	369-413-480-521-592sh-628-**853**-891sh-915-1023-1070sh-1093-1125-1146-1237-1356-1371-**1441**-1711-1727-2245-2712-2835sh-**2910**-2934sh-3025-3380	(Cooney *et al.* 1994)
Polyvinyl chloride (PVC)	310-345sh?-**363**-420-496-544-571-599-615-**638**-682-**694**-752-838-930-964-972-1066-1101-1119-1172-1187-1216-1257-1316-1335-1357-1379-**1430**-1437-1498-**2914**-**2935**-2969-2994	(Koenig and Druesedow 1969; Prokhorov *et al.* 2016)
Thermoplastic Polyurethane – (TPU)[3]	715-782-**866**-902-974-1020-1048-1069-1119-**1185**-1197-1252-1313-1439-1539-**1617**-1701-1730-2875-**2926**-3065-3337	(Bruckmoser and Resch 2014)

'sh' = shoulder. Most intense vibrations are listed in bold characters.

[1]Ethylene copolymer ratios of 32.

[2]Polycarbonate peaks refer to bis-phenol A type polycarbonate, also known as Lexan (Macrolon®), which is the most common polycarbonate.

[3]Polyester type polyurethane.

What follows is an account of bands' assignment for the polymers most commonly found in aquatic systems, i.e., polyethylene, polypropylene, polystyrene, polyamide, polyesters, and polyvinyl chloride (Schwarz *et al.* 2019; Erni-Cassola *et al.* 2019), to get a better understanding of the chemical fingerprint of these polymers.

Raman spectra of polyethylene (PE) shows main vibrations at 1062, 1130, 1170, 1295, 1417, 1440, 1460, 2850, and 2883 cm^{-1} (Fig. 2.4a). The bands at 1062 and 1130 cm^{-1} have been assigned to the asymmetric (ν_{as}(C–C)) and symmetric (ν_s(C–C)) stretching vibrations of the C–C bonds, respectively. The bands at 1170 and 1460 cm^{-1} represent bending modes (rocking ($\rho(CH_2)$) vibrations) of the CH_2 bonds, and the bands at 1295 and 1417 cm^{-1} are twisting ($\tau(CH_2)$) and wagging ($\omega(CH_2)$) vibrations of the CH_2 groups, respectively. The bands at 2850 and 2883 cm^{-1} belong to the symmetric ($\nu_s(CH_2)$) and asymmetric ($\nu_{as}(CH_2)$) stretching vibrations of the CH_2 groups, respectively (Sagitova *et al.* 2016; Zanocco *et al.* 2020). It is also possible to observe a weak peak at 2725 cm^{-1} and a smooth shoulder at 2935 cm^{-1}: the first is an overtone of wavenumbers in the range of 1400-1495 cm^{-1} (–CH_2– bonds); the latter is reported to be the Fermi resonance between the CH_2 symmetric stretching and the overtone from the CH_2 bond (da Silva and Wiebeck 2019).

Raman spectroscopy is sensitive to changes in the molecular structure level of PE, such as the degree of crystallinity, which is the key determining factor of PE density (i.e., higher the degree of crystallinity, higher the density). Thus, it is argued that Raman spectroscopy may distinguish low-density (LDPE) and high-density (HDPE) polyethylene. The Raman spectra of LDPE and HDPE are very similar, but differences are reported in the C–H stretching region. Moreover, the intensity of the symmetric CH_2 stretching mode at 2850 cm^{-1} relative to the asymmetric CH_2 stretching mode at 2883 cm^{-1} appears to be higher for LDPE compared to HDPE. Therefore these two regions, i.e. C-H stretching (2825-2970 cm^{-1}) and the CH_2 bending regions (1398-1470 cm^{-1}),

have been used to discriminate between HDPE and LDPE (Ibrahim and He 2017). However, most studies about microplastics do not differentiate among the two density types of PE (Di and Wang 2018; e.g., Ghosal *et al.* 2018).

Commercial polypropylene is usually isotactic. The most intense vibrations in isotactic polypropylene (iPP) are at 398, 809, 841, 973, 1040, 1152, 1219, 1330, 1360, 1458, 2883 cm^{-1} (Fig. 2.4b) (Andreassen 1999). The band at 398 cm^{-1} has been assigned to CH_2 wagging and CH bending, vibration at 809 cm^{-1} to CH_2 rocking and stretching of C–C and C–CH_3, at 841 cm^{-1} to rocking of CH_2 and CH_3 and stretching of C–C and C–CH_3, and at 973 cm^{-1} to CH_3 rocking and C–C stretching (Andreassen 1999). The remaining bands have been assigned as follows: 1040 cm^{-1} band to stretching of C–CH_3 and C–C stretching and CH bending; 1152 cm^{-1} to C–C and C–CH_3 stretching, CH bending and CH_3 rocking; 1219 cm^{-1} to CH_2 twisting, CH bending, and C–C stretching; 1330 cm^{-1} to CH bending and CH_2 twisting; 1360 cm^{-1} to symmetric bending of CH_3 and CH bending; 1458 cm^{-1} to asymmetric bending of CH_3 and CH_2 bending and; 2883 cm^{-1} to symmetric stretching of CH_3 group (Andreassen 1999).

Another commonly used plastic polymer is polystyrene (PS), whose Raman spectrum shows an intense band at 1001 cm^{-1}, linked to the breathing mode of the aromatic carbon ring (Fig. 2.4c). Other PS vibrations can be observed at 621, 795, 1031, 1155, 1450, 1583, 1602, 2850, 2904, 3054 cm^{-1} (Mazilu *et al.* 2010; Yan *et al.* 2012). These bands correspond to the ring deformation mode (621 cm^{-1}), CH deformation out-of-plane (795 cm^{-1}) and in-plane (1031 cm^{-1}), C−C stretch (1155 cm^{-1}), CH_2 scissoring (1450 cm^{-1}), ring-skeletal stretch (1602 cm^{-1}), CH_2-aliphatic stretching modes symmetric (2852 cm^{-1}) and antisymmetric (2904 cm^{-1}). PS also contains overlapped bands at 3054 cm^{-1} due to the C–H bonds stretching on the benzene ring (Mazilu *et al.* 2010; Yan *et al.* 2012).

Among the polycondensation polymers, the most important and widely used today are polyesters, followed by polyamides. Polyesters are widely used as packaging materials and also as a fiber, filament, fabrics in textiles. The most

important commercial polyester is polyethylene terephthalate (PET) (Krishnan and Kulkarni 2008). However, both IR and Raman spectra are considered difficult to subgroup polyesters, and thus the majority of the study used to group all the polyester polymers (Cai *et al.* 2012; Schymanski *et al.* 2018). Here, we reported the Raman spectrum of PET since it is the most widely used. The main Raman frequency shifts of PET are 857, 1096, 1295, 1615, 1730 cm^{-1} (Fig. 2.4d; Boerio *et al.* 1976). The Raman line observed near 857 cm^{-l} could be assigned to an A_g mode consisting of ring CC and C(O)–O stretching. The band at 1615 cm^{-1} is a ring mode 8a (in Wilson's notation), while the band at 1096 cm^{-1} has been assigned to the anti-symmetric stretching vibration of C–O–C. The strong bands near 1730 and 1295 cm^{-l} are principally associated with stretching of the C=O and C(O)–O bonds, respectively. The vibration observed near 1096 cm^{-1} for PET consists mainly of the stretching of ring CC, ester C(O)–O, and ethylene glycol CC bonds (Boerio *et al.* 1976).

A variety of polyamides are presently manufactured and marketed under several different trade names. Among them, Nylon-6 and Nylon-66 are the two most manufactured. Nylon-6 (NY6) has characteristic band at 3302 cm^{-1}, correlated to NH stretching vibrations, and 1636 cm^{-1}, which is the amide I band containing mainly the C=O stretching mode. Other strong vibrations can be identified at 2869, 2900, and 2929 cm^{-1}, assigned to the stretching of the CH_2 group. Two main polymorphs of NY6, the α and γ phases, exist. The most common structure found in most commercially available Nylon-6 is the α -form. α-NY6 has principal bands at 932, 1065, 1130, 1203, 1283, 1310, 1444, 1470, 1480 cm^{-1}. The bands that constitute the main difference from the other polymorph, as reported by Milani (2015) are at 1130, 1203, 1470, and 1480 cm^{-1}. Instead, γ-NY6 has a dominant vibration at 1080 and additional bands at 962 and 1234 cm^{-1} (Milani 2015). Nylon-66 (NY66) has major bands at 952, 1063, 1128, 1235, 1298, 1441, 1637, 2867, 2919, 3304 cm^{-1} (Fig. 2.4e; Larkin, 2018). The specific vibration at 1283 cm^{-1} for nylon 6, linked

to an amide III band (C–N stretch and N–H bend), is the major difference with respect to NY66 (Miller and Bartick 2001).

Polyvinyl chloride (PVC) shows high-intensity Raman bands at 638 and 694 cm^{-1}, assigned to stretching vibrations of C–Cl bonds. An intense Raman band at 2914 cm^{-1} can also be observed, which corresponds to asymmetric CH_2 stretching vibration (Fig. 2.4f). Other important bands can be observed at 363, 615, 1430, and 2935 cm^{-1} (Koenig and Druesedow 1969; Prokhorov *et al.* 2016). As mentioned before (see section 2.4), PVC is one of the polymers most commonly produced with additives; thus, this should be considered when unknown peaks are identified in spectra of this polymer.

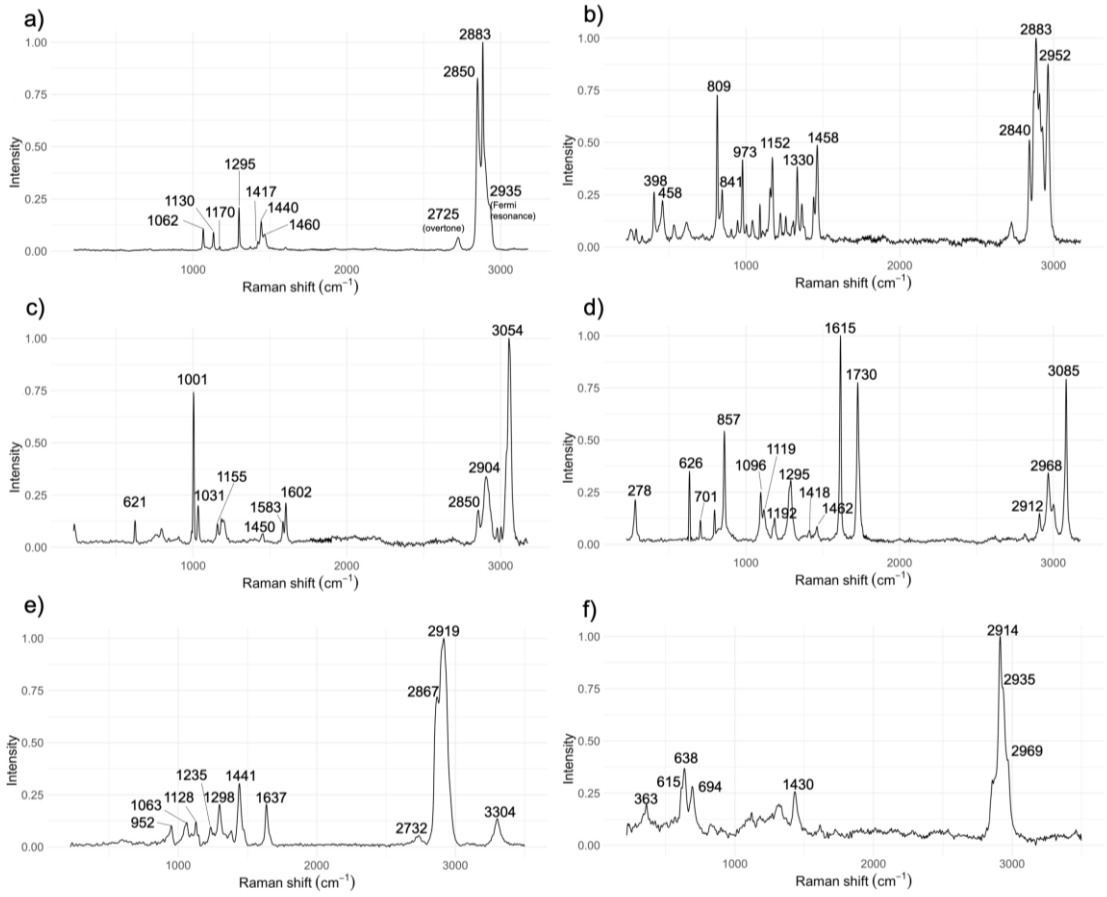

Figure 2.4. Illustrated Raman spectra of common plastic polymers. a) PE, polyethylene; b) iPP, isotactic polypropylene; c) PS, polystyrene; d) PET, polyethylene terephthalate; e) PA – NY66, polyamide; f) PVC, polyvinyl chloride.

2.6. R package for analysis and identification of virgin and with additives plastics

The need to quantify small plastic particles is increasingly requiring the adoption of Raman spectroscopy. However, the analysis of plastic composition through this technique is still far from widespread, and, accordingly, comprehensive, and complete Raman spectral libraries of plastic polymers are not very common. Commercial spectral libraries exist, but these are not freely available (Araujo *et al.* 2018). Moreover, these spectral libraries focus on virgin plastics, while particles collected from the environment may be differently altered and present several additives, especially colorants, as widely discussed in the previous sections (Dong *et al.* 2020b).

For these reasons, we created a new R package, called '`RamanMP`', which includes some easy-to-use and simple tools for comparing spectra and a Raman spectral library of additives and plastics collected from aquatic systems. We selected the R software because it is one of the most popular and widely used free software. This application is freely downloadable from CRAN (https://cran.r-project.org/web/packages/RamanMP) and the database, which currently includes 356 spectra, with several spectra of colorants, provided by Fremout and Saverwyns (2012), is freely accessible and downloadable. This spectral library is an open platform that allows searching and submitting spectra (https://github.com/VeronicaNava/RamanMP). Contributed spectra will be checked prior to uploading to ensure the quality of the database's contents. Eventually, this will help achieve a high degree of quality and completeness of the database, which may benefit many future studies in plastic analyses.

A workflow displaying the functioning of the 'RamanMP' package, which includes 10 different functions, is reported in Fig. 2.5.

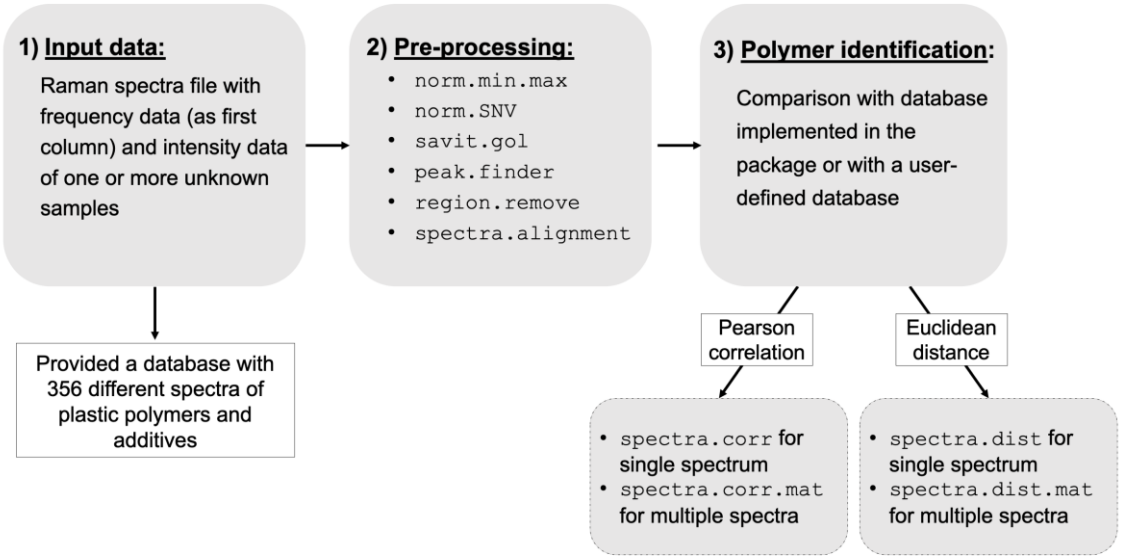

Figure 2.5. Workflow for the '`RamanMP`' package.

The package provides some tools to perform preliminary analyses of the spectra: `norm.min.max` function allows normalization by minimum and maximum peaks, while `norm.SNV` performs Standard Normal Variate (SNV) transformation; `savit.gol` function applies `Savitzky-Golay` smoothing filter; `peak.finder` allows peak identification based on local maxima; `region.remove` allows removal of spectral regions with no relevant information and `spectra.alignment` aligns spectra with different spectral resolutions with a user-defined tolerance span. These operations may be also performed in other free software, which in some cases implement more complex elaborations. However, some basic functions have been implemented in this package to let the user performing all the operations in a unique software, reducing the analysis time. The core of the package are the `spectra.corr` and `spectra.dist` functions, which allow to match the spectra on an unknown polymer with the implemented database or even with a user-developed database. Two different ways to measure the matching of the spectra can be used: the Pearson correlation coefficient (`spectra.corr` function) or the Euclidean distance (`spectra.dist` function). The function by default also returns a plot, displaying the unknown

polymers and the spectra of the database for which the highest matching score was found, thus verifying the results obtained.
Moreover, the package implements the functions `spectra.corr.mat` and `spectra.dist.mat`, which compare a whole matrix of unknown polymers with the database, thus reducing the analysis time. In this case, no plot is shown, and, therefore, the results should be considered with caution. A manual of the package with an extensive explanation of the different functions is available at this link (https://cran.r-project.org/web/packages/RamanMP).

2.7. Conclusion and future perspectives

Scientific focus on microplastic pollution has increased markedly over the last decades, and this issue has been recognized as a critical environmental problem also by the public and policy makers, becoming an emerging issue of global concern. In this context, being able to provide proper and quality data about microplastics is pivotal. Accordingly, reliable tools for MP identification and characterization, like spectroscopic analyses, become fundamental since visual inspection alone has been widely claimed to be insufficient. The need to identify smaller and smaller plastic particles, up to 1 μm, whose presence is reported to be widespread and even more numerically significant than larger microplastics, requires the use of micro-Raman spectroscopy. However, studies investigating plastics through Raman spectroscopy are still limited. This review outlined the status of Raman analysis of microplastics, highlighting the advantages and the drawbacks of this technique and critically presenting tools and ways to effectively use this instrument and improve the results that can be obtained.

Raman spectroscopy is a straightforward technique, with many advantages. Still, problems can arise when analyzing plastics collected from environmental matrices, and the identification can be, in some instances,

more complicated than expected. Therefore, we claimed that a deep understanding of the different plastic polymers and their Raman bands and chemical fingerprints is fundamental to avoid misidentification. Therefore, we provided a catalog of reference spectra, which is, to the best of our knowledge, the first comprehensive resource that systematically synthesized Raman bands of most common plastic polymers. Alongside we implemented a tool for the analysis and identification of plastic polymers and additives, including a freely available database. We encourage the whole community to contribute (https://github.com/VeronicaNava/RamanMP); this will help create a free and complete Raman database of plastic polymers, fostering the use of this technique, which may become especially relevant in the field of microplastic analysis. In general, we suggest, as a best practice, that future MP studies should include and share the Raman spectra acquired. Indeed, this will increase access to spectroscopic data about plastics collected from the environment.

Raman spectroscopy, as analogous spectroscopic techniques, is time-consuming, and therefore, the analysis is usually performed on sub-samples. Automatic procedures may help reduce the analysis time. However, these approaches are generally more challenging to apply when analyzing more complex matrices (like sediments or biota), for which steps of purification can reduce the not-plastic materials but not completely eliminate them. Moreover, there is a lack of clarity about how the results are scaled up from sub-sampling procedures to the whole sample, and, thus, future studies should be more explicit in these regards. Regardless of the technique adopted, it is crucial to verify the results obtained, and this can only be done when the features of Raman spectra of plastics and issues that may arise from their analysis are well known.

Supplementary Materials

Principles of Raman spectroscopy

Raman spectroscopy is based on the inelastic scattering of light by matter in its solid, liquid, or gas state. Monochromatic light scattered by matter contains radiations with frequencies different from the exciting light. (Frezzotti *et al.* 2012) The property involved is the change in the polarizability of the molecule with respect to its vibrational motion (Nafie 2001). This effect, predicted by Smekal (1923), was experimentally observed by Raman (1928) and named after him.

The Raman effect arises when light hits a molecule and interacts with the electron density of the chemical bond. When intense monochromatic radiation (usually a laser) hits a sample, part of the light is scattered over all directions after its interaction with sample molecules. Much of this scattered radiation has a frequency equal to that of the incident radiation: the transition starts and finishes at the same vibrational energy level without gain or loss of energy (Rayleigh scattering) (cf. Vandenabeele 2013). Only a small fraction of the scattered radiation *(i.e.*, 10^{-6}–10^{-8} of incident photons) has a frequency different from that of the incident radiation after interacting with the sample molecules (Raman scattering). The Raman effect induces a shift to lower and higher frequencies in scattered light by an amount depending upon the vibrational state of the molecule (Stokes and anti-Stokes scattering). Frequency changes depend on the energy levels of different molecular vibrations and are independent of the wavelength of the light source (cf. Frezzotti *et al.* 2012 and references therein).

As molecular vibrations are different for every molecule, Raman spectra represent the "molecular fingerprint" of the analyzed matter. In a Raman spectrum, the intensity of Raman scattering, expressed as arbitrary units, or counts, is plotted as a function of the shift of frequencies relative to that of the incident electromagnetic radiation, ω, in wavenumbers expressed in cm^{-1}:

$$\tilde{\omega} = v_m - v_0 = \frac{v_m}{c} - \frac{v_0}{c} \qquad (1)$$

where v_m and v_0 stand for the frequency of the scattered and incident radiation, respectively, and c is the light speed. Usually, only Raman Stokes shifts are presented, since these are about ten times more frequent than their anti-Stokes equivalents (Frezzotti *et al.* 2012).

The spectrum bands reflect the vibrational energies of the molecules, and their functional groups, within the analyzed sample, which are linked to the nature of the bonding. Main molecular vibrations include stretching (symmetric and anti-symmetric) and bending (or deformation) modes, stretching frequencies being usually higher than bending frequencies. The basic selection rule is that a vibration is Raman-active if the polarizability is changed during the vibration. Usually, symmetric vibrations cause the largest changes (Smith and Dent 2019).

Methodology for bibliographic research

Most papers were retrieved from the Web of Science (https://webofknowledge.com) database. Search string used was *microplastic* AND *Raman spectroscopy*. Searches were performed until January 2021. We retrieved two kind of studies: studies that provide useful information about the use of Raman spectroscopy for microplastic analysis (46 studies), and studies reporting microplastics analysed through Raman spectroscopy collected from aquatic matrices (62 studies). In total, 108 papers and book chapters reporting study about microplastics investigated through Raman spectroscopy were reviewed.

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Chapter 3

Global patterns and predictors of microplastic and plastic occurrence and abundance in lentic systems

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In preparation

Abstract

The majority of microplastic research has focused on seawater, with fewer than 4% of microplastic-related studies occurring on freshwaters. The limited available information suggests that the abundance of microplastics in freshwaters is often as high or even higher than in marine environments. However, comprehensive investigations on the occurrence and fate of microplastics in freshwaters are scarce and highly fragmented, partly because detection and identification of microplastic particles are rather complex. In addition, up to now, there is a lack of consensus on sampling and analytical procedures for microplastic identification and quantification, and studies with different research aims and hypotheses often report unstandardized results, making comparison among studies difficult. Here, we performed the first global standardized sampling and analysis effort to investigate (micro)plastics (>250 µm) in surface waters of 38 lakes located in 28 different countries covering an assortment of limnologically diverse freshwater ecosystems under varying levels of anthropogenic stress. We aimed at characterizing and quantifying plastic contamination in different ecosystems and at relating the occurrence of these pollutants to lake hydro-morphological and urban-associated attributes. Samples have been collected and analyzed following a common protocol, which established the collection of samples by horizontal trawling of a plankton net and, after treatment with hydrogen peroxide (15%, 24h, 55°C), the polymer identification through micro-Raman spectroscopy. Our results showed that concentration of plastics spanned among different orders of magnitude (from 10^{-3} to 10^{1} particles/m^3), with a mean abundance of 1.82 ± 0.37 (standard error, SE), and values were comparable to those found in previous research with similar sampling methodologies. Fibers and fragments were the most frequently detected particles, suggesting a secondary origin of plastic contamination. Polyester, polypropylene, and polyethylene, which are polymers commonly used in short life-cycle products and accounted for the large majority of

global plastic production, constituted the polymer most commonly identified in surface water with an overall recurrence of 30.4, 20.3, and 15.7%, respectively. The results of this study suggested a relationship between urban-related attributes of lakes/watersheds (i.e., urban land cover and population density) and the plastic contamination but also highlighted that larger and deeper lakes with higher retention times are accumulating plastic debris at higher concentrations. Results from this study add greatly to the current body of information on plastic contamination in surface water of lakes, especially given the largely claimed urgency of comparable data in the field, providing information that will help contextualize future research and define the role of lakes in the plastic cycle.

3.1. Introduction

A vast quantity of discarded plastic waste is accumulating in aquatic ecosystems, where it breaks down to form microscopic fragments, called "microplastics" (Cole *et al.* 2011; Eerkes-Medrano *et al.* 2015). Defined as particles <5 mm, microplastics can be ingested more readily than larger particles by a wide range of aquatic organisms, such as zooplankton, bivalves, fishes, which represent keystone organisms, thus constituting a threat to aquatic biota across all trophic levels (Oberbeckmann *et al.* 2014; Eerkes-Medrano *et al.* 2015; Hermabessiere *et al.* 2017). This ingestion can result in physical damage such as obstruction or internal abrasions. Besides, microplastics can potentially concentrate and transfer chemicals and persistent, bioaccumulative, and toxic substances adsorbed on their surface to organisms or plastic additives, mainly used as plasticizers, flame retardants, stabilizers, antioxidants, and colorants (Eerkes-Medrano *et al.* 2015; Horton *et al.* 2017). Microplastics in aquatic systems can be transported both horizontally, over long distances, and vertically, through the water column, after changes in biofouling that affect particle density, thus acting as vectors for the selection and spread of attached pathogenic bacteria, harmful algae, and invasive species (Arias-Andres *et al.* 2018; Nava and Leoni 2021). Furthermore, ecotoxicological risks of microplastics to organisms have been suggested, even if these remain highly uncertain (Kong and Koelmans 2019). Therefore, microplastics are amongst the contaminants of emerging concern for aquatic systems. As these polymers are highly resistant to degradation, quantities of microplastics in aquatic environments will most likely continue to increase over time and, consequently, microplastics will represent a long-lasting problem that future generations will have to face (Galloway and Lewis 2016).

Although marine microplastic research remains at the forefront, in recent years researchers, recognizing the comparative lack of studies on microplastics in freshwater environments (less than 4% of microplastics-

related studies are associated with freshwaters), have begun to address this field as a matter of priority (Horton *et al.* 2017; Li *et al.* 2018). These studies reveal that microplastics are present in freshwater lakes sometimes in densities comparable to the oceans, and a high-level of contamination can be also detected in lakes in remote areas (Free *et al.* 2014). However, investigations about microplastics in freshwaters are still scarce, highly fragmented, and little quantitative data are available. Besides, the detection and identification of microplastic particles are rather complex, and different methods have been proposed for their investigation in water samples. However, up to now, there is a lack of consensus on sampling and analytical procedures for microplastic identification and quantification and different ways of reporting the results make the comparison among studies hardly interpretable (Horton *et al.* 2017).

In the present study, we performed the first global standardized sampling and analysis effort to investigate the occurrence and the features of microplastics in lakes and reservoirs covering an assortment of limnologically diverse freshwater ecosystems under varying levels of anthropogenic stress. We collected and analyzed microplastic samples from 38 lakes with different features (e.g., surface area, depth, thermal regime, watershed characteristics), following a common protocol. With this global dataset, we address the following questions: i) what is the concentration of plastics and microplastics in different freshwater systems worldwide? ii) what are the features (e.g., shape, color, dimension, polymeric composition) of these particles? iii) is there a relationship between the abundance of microplastics and watershed and lake features; iv) are there some factors that are likely to greatly influence the number of microplastics in surface water of lentic systems?

3.2. Materials and methods

3.2.1. Study area and sample collection

The samples have been collected from 38 lakes and reservoirs located in 28 different countries with a worldwide distribution (Fig. 3.1). Lakes have been selected to cover an assortment of limnologically diverse freshwater ecosystems. They varied in size from 0.04 to 32,600 (median = 19.50) km^2, with a mean depth of 0.5 to 580 (median = 9.7) m and a volume of $1.8 \cdot 10^{-5}$ to 18,980 (median = 0.18) km^3. Lakes spanning over different thermal regime were included (i.e., polymictic, 11; monomictic, 12; dimictic, 8; and meromictic, 5) and covering a different trophic status (i.e., ultra-oligotrophic, 3; oligotrophic, 10; mesotrophic, 12; eutrophic, 11; hyper-eutrophic, 2). Detailed information about lake hydro-morphological features and attributes is reported in Table S1.

Sampling work was carried out in 2020-2021. Surface water has been collected by horizontal trawling of a plankton net (mesh size between 50 and 300 µm, mouth diameter equal to or larger than 30 cm). Sampling occurred in the pelagic zone, near the major outflowing stream, with a direction of the trawls perpendicular to the outflow. The samples have been collected when lake conditions were as calm as possible and weather data (i.e., wind speed, wind direction, air, and water temperature) were collected to get comprehensive information about the sampling. Three parallel trawls have been performed in each lake using a plankton net. The net was placed at the port side and the boat speed was kept at around 2-3 kts, following the indication of GESAMP (2019). At least 50 m^3 of water have been filtered for each trawl and GPS tracks have been recorded for each sample to estimate the exact volume filtered for each sample. Attention was given to avoid clogging of the net. If this happened, the trawls have been divided into different sub-trawls to allow for the cleaning of the net.

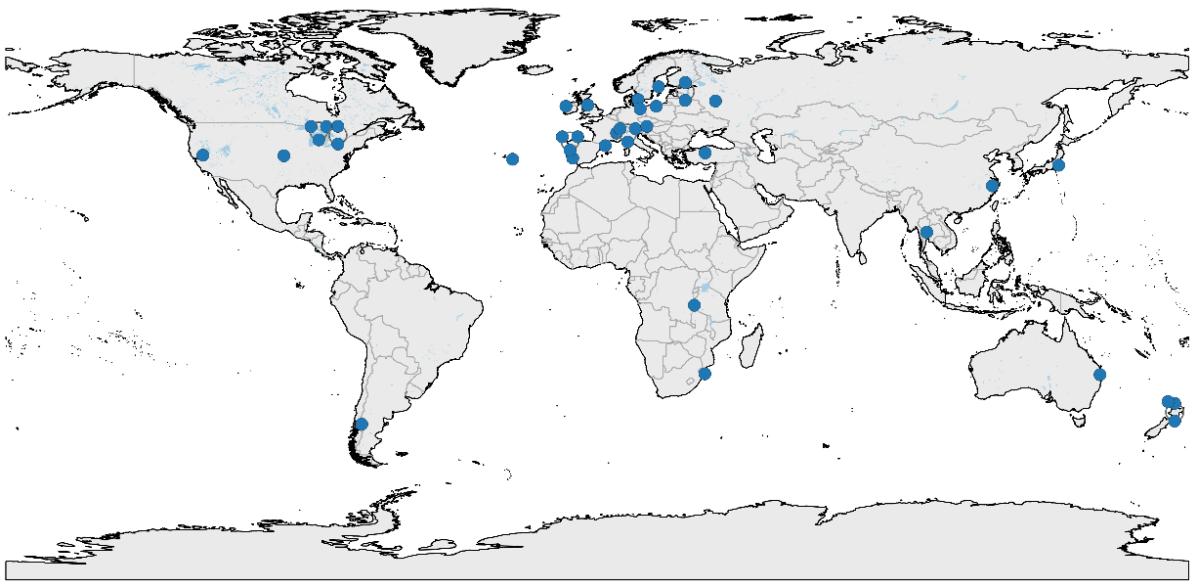

Figure 3.1. Map showing the locations of lakes and reservoirs included in the study. Detailed information about each lake is reported in Supplementary Materials (Table S1).

3.2.2. Sample analysis

Once collected, all the samples have been analyzed at the Laboratory of Freshwater Ecology of the University of Milano-Bicocca (Italy) following a common procedure. All the samples have been wet sieved on a 250 µm mesh in order to align the lower limit size across the different samples. Then, the samples have been processed with 15%H_2O_2 for 24h at 60°C, following the indication of previous studies that suggest avoiding higher concentration and/or temperature to reduce potential damages to plastic particles (Zhao *et al.* 2017; Hurley *et al.* 2018; Wiggin and Holland 2019). After the oxidation process, the samples have been filtered on 0.45 µm glass microfiber filters (GF/F, 47 mmØ, Whatman) and then the filters were placed in clean glass Petri dishes. The filters were analyzed under a dissecting microscope (40X) and particles recognized as plastics were transferred on glass slides for the subsequent spectroscopic analysis. Pictures of all the plastic particles were acquired using the high-resolution camera Leica ICC50. All the particles were counted and measured in length (the longest dimension) using the software ImageJ (1.52q). Based on their dimension, plastics have been assigned to four

different categories: small-microplastics (250 µm-1 mm), microplastics (1-5 mm), mesoplastics (5-10 mm), and macroplastics (>1 cm). This classification has been adopted to be consistent with previous literature, for which upper size limit for microplastics was set at 5 mm (e.g., Frias and Nash 2019; GESAMP 2019), and also to other size classifications that suggest consider microplastics those particles in the micrometer size (<1 mm) (e.g., Hartmann *et al.* 2019). For shape categorization, a modified version of the classification proposed by Hartmann *et al.* (2019) was adopted. In particular, plastics were classified as fiber, fragment, film, spheres/pellet, and filament. We added the filament class to identify those plastics that have a shape similar to fibers (longer in one dimension) but that have a larger diameter in order to differentiate them from fibers deriving from textiles (for an example of the shape categorization see Fig. S3.1). Moreover, plastic particles have been classified based on color (i.e., red, orange, yellow, green, blue, violet, black, white, clear, and multicolored), following a RAL standard color scale, according to Lusher *et al.* (2020).

3.2.3. Quality assurance and quality control (QA/QC)

Laboratory-based quality assurance and quality control (QA/QC) included procedural blanks. To assess potential contamination from laboratory materials or air, laboratory blanks were collected for environmental water samples analyzed. Moreover, during the lab procedure, all equipment was rinsed three times with ultrapure water and all plastic materials were avoided, preferring instead glass equipment. Cotton lab coats were used, and all the surfaces were accurately cleaned before use. In addition, the samples were always kept covered with tin foil.

Blank levels have been subtracted from environmental samples, thus results of plastic concentration reported already took into account the contamination. Furthermore, results of blank sample analysis were reported in Table S2.

3.2.4. Raman analyses

We employed Raman microscopy to define the chemical identity of a random subsample of microplastic particles. We performed polymer identification on sub-samples, as widely suggested and performed in literature (e.g., Käppler *et al.* 2016; Kazour *et al.* 2019; Alfonso *et al.* 2020). In particular, we adopted a robust procedure to first determine the minimum number of particles to be studied to reach a certain confidence level in the estimated proportion, following what was reported by Kedzierski *et al.* (2019). The number of microplastics in the sampled population was obtained as follows:

$$n = \frac{\frac{1}{4} + \frac{\varepsilon^2}{\left(u_{1-\frac{\alpha}{2}}\right)^2}}{\frac{\varepsilon^2}{\left(u_{1-\frac{\alpha}{2}}\right)^2} + \frac{1}{4N}}$$

with ε the accuracy; $u_{1-\alpha/2}$ the fractal of order α of the standardized normal law; N the global population size. We chose as degree of confidence 95% (*i.e.*, α = 0.05; $u_{1-\alpha/2}$ = 1.96) and ε =0.1.

Raman spectra have been acquired by the Horiba Jobin Yvon LabRAM HR Evolution Raman System at the Dep. Earth and Environmental Sciences, University of Milano – Bicocca (Italy), characterized by 800 mm of focal distance and coupled with an air-cooled 1024×256 px CCD detector. The record of plastic spectra has been performed by using a green Nd 532.06 nm laser source (300 mW) with a 50× magnification (Olympus BXFM). The grating was 600 gr/mm and the spectral per pixel resolution was about 1.6 cm^{-1}/px. Two spectra were acquired for each particle with a spectral interval from 222.86 to 1899.01 cm^{-1} and from 1762.24 to 3177.02 cm^{-1}. Depending on the particles analyzed, acquisition parameters were changed: accumulation ranged between 1-3; integration time between 20-60 s; and power between 0.3-300 mW. Calibration was daily performed based on the auto-calibration process performed by the Ryman system Service in respect to the zero line

and the silicon standard (520.7 cm^{-1}), according to the ASTM 1840–96 normative (Hutsebaut *et al.* 2005; Remigi *et al.* 2021).

Raman spectra were baseline corrected and processed by statistical analysis using Fityk software (Wojdyr 2010; Frezzotti 2019). Further analyses on polymer spectra were performed in R, using the package 'RamanMP' (Nava *et al.* 2021).

3.2.5. Watershed and lake attributes

Additional information was retrieved in order to characterize the level of anthropogenic disturbances across the different lakes, collecting in total 14 variables for each lake. Watersheds of different lakes (i.e., the land and water areas that drain toward the lake) have been calculated in GIS systems, with ArcMap 10.7 (Spatial Analyst tool), using the ASTER Global Digital Elevation Model (GDEM) Version 3 (ASTGTM), which provides a global digital elevation model (DEM) of land areas on Earth at a spatial resolution of 1 arc second (approximately 30 meter horizontal posting at the equator) (NASA/METI/AIST/Japan Spacesystems and U.S./Japan ASTER Science Team 2019).

Information about land cover has been then retrieved by clipping the 100 m resolution land cover map provided by the Copernicus Global Land Service (Buchhorn *et al.* 2020). Moreover, we collected information about the population in the watershed using the UN WPP-adjusted population estimates for the year 2020 (Center for International Earth Science Information Network - CIESIN - Columbia University 2020).

3.2.6. Statistical analyses

To avoid problems of multicollinearity, the correlation among the 14 variables collected has been tested through Pearson correlation test. Variables that resulted highly correlated (r>0.85) have been dropped out from subsequent analyses.

The selection of most relevant features related to plastic concentration has been performed using the Boruta algorithm based on 500 permutations, which is a wrapper built around the random forest (RF) classification algorithm (Kursa and Rudnicki 2010). The main idea of this approach is to compare the importance of the real predictor variables with those of random so-called 'shadow' variables, whose values are obtained by shuffling values of the original attribute across objects, using statistical testing and several runs of RFs. In each run, the set of predictor variables is doubled by adding a copy of all variables. A RF is trained on the extended data set and the variable importance values are collected (Z scores). For each real variable, a statistical test is performed comparing its importance with the maximum value of all the shadow variables. Variables with significantly larger or smaller importance values are declared as important or unimportant, respectively. All unimportant variables and shadow variables are removed, and the previous steps are repeated until all variables are classified or a pre-specified number of runs has been performed.

Principal component analysis (PCA) was then performed using the variables selected through the Boruta algorithm. All data were centered and scaled to allow comparison among parameters. Only those components with eigenvalues higher than or equal to one were considered as significant components (Kaiser 1958).

Statistical analyses and figures were produced using different packages (base packages and "ggplot2", "RamanMP", "Boruta", "randomForest", "factoextra") in R (4.1.1.) (Liaw and Wiener 2001; Kursa and Rudnicki 2010; Wickham 2016; Kassambara and Mundt 2016; Nava *et al.* 2021).

3.3. Results

3.3.1. Plastic abundance

Overall, 9425 plastic particles have been collected and analyzed. Concentrations of plastic particles identified in surface water of the 38 lakes spanned from 0.09 particles/m^3 (Lake Avery, USA) to 11.5 particles/m^3 (Lake Lugano, Switzerland-Italy), with a mean value of 1.82 ± 0.37 (standard error, SE) and a median of 0.85 particles/m^3 (Fig. 3.2a). Besides Lake Lugano, whose concentration represents an outlier of the considered population, high values of plastic abundance were also recorded in Lake Maggiore (Italy), where the concentration was equal to 8.24 particles/m^3, and Lake Tahoe (Nevada, USA), with a concentration of 5.44 particles/m^3. The majority of locations (>55%) had abundances below 1 particle/m^3. When considering only fragments with dimensions below 5 mm, mean concentration and median were respectively 1.70 ± 0.35 and 0.77 particles/m^3; while, considering particles lower than 1 mm, mean was equal to 0.82 ± 0.19 and median to 0.42 particles/m^3.

We reported plastic concentration also on surface area other than on volume unit in order to facilitate comparisons with previous studies, and we recorded values ranging from 0.009 to 2.59 particles/m^2 (mean ± SE = 0.35 ± 0.08, median = 0.17 particles/m^2; Fig. 3.2b).

The aforementioned results refereed to the sum of plastics identified in the volume (or surface area) sampled among the three trawls performed for each site. When analyzing the three trawls distinctly (Fig. 3.2c), we observed that replicates showed a low variance for many lakes, especially when the mean concentration detected was low. However, some lakes showed consistent differences among the trawls, like for instance Lake Lugano, Maggiore, Azul (Portugal), and Sau (Spain).

Even if all the samples have been sieved through a 250 µm mesh, we tested whether an effect of different mesh sizes used during the sampling activities

was present. However, we did not find any correlation between the concentration of plastics and the mesh used.

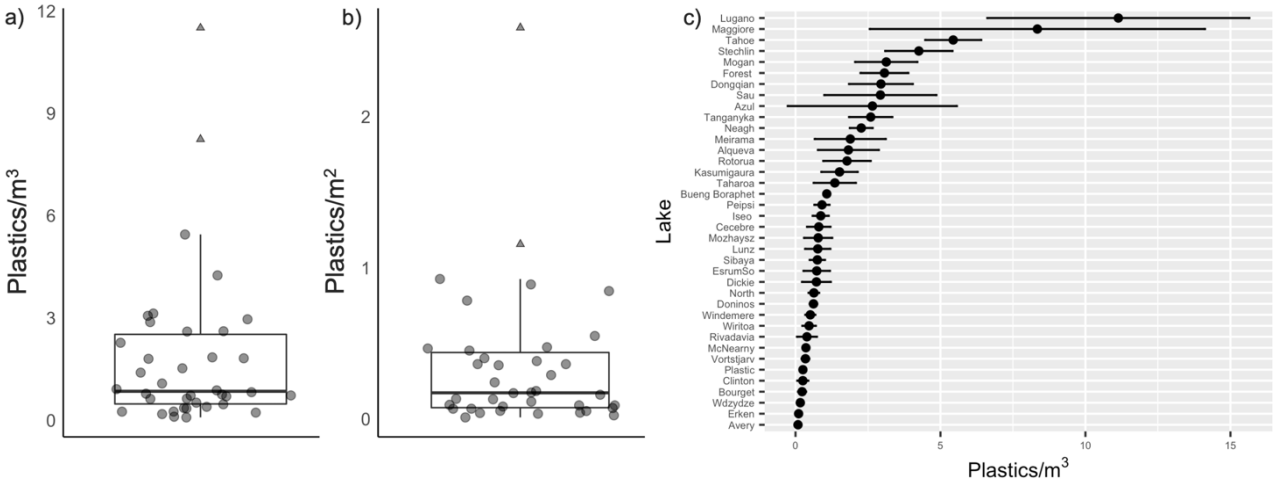

Figure 3.2. Concentrations of plastic particles across the 38 lakes analyzed: a) Tukey boxplot with concentration over the volume (Plastics/m^3); b) Tukey boxplot with concentration over the surface (Plastics/m^2). Triangular-shaped points indicate outlier values. c) Mean and standard deviation as a result of the three trawls in each lake.

3.3.2. Plastic characterization

3.3.2.1. Physical characteristics

More than 90% of the plastic particles identified in this study belong to two shapes, namely fiber (49.4%) and fragment (41.0%). The remaining 9.6% comprised films (4.8%), filaments (4.0%) and spheres/pellets (0.8%) (Fig. 3.3). The color with the highest recurrence among all samples was black (29.8%), followed by clear (23.7%), blue (17.9%), and white (13.5%). The remaining colors were present in low abundances: red (4.8%), green (2.6%), violet (2.5%), orange (2.1%), yellow (1.7%), and multicolored (1.4%). Observing the distribution of colors across the different shapes, we observed that the majority of fibers were black (43.5%) and blue (28.6%), while the majority of fragments were clear (37.5%) and white (23.8%).

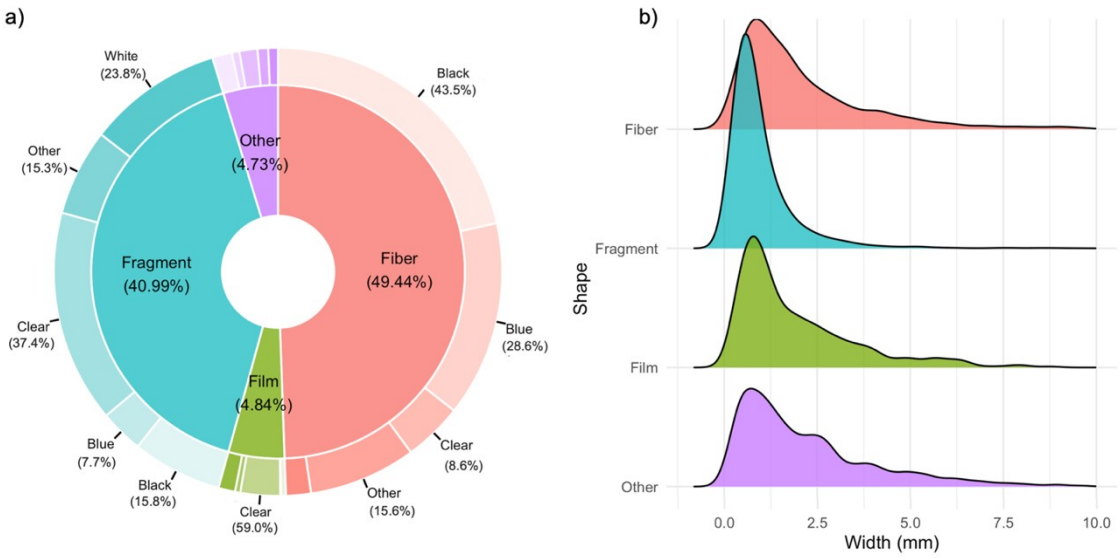

Figure 3.3. a) Donut pie chart with percentage abundances of the different shapes and relative colors for all the plastic particles analyzed across the different lakes; b) Width distribution (from 0 to 10 mm) divided by the different shapes.

The majority of plastics were classified as small-microplastics (<1 mm, 46.9%) and as microplastics (1-5mm, 46.9%). Only 4.7% of plastics were mesoplastics (5-10 mm), while we only observed a few macroplastics (>10 mm, 1.5%). Fragments had generally smaller dimensions, with a mean width across all plastics identified of 1.02 (median = 0.71) mm, while that of fibers was 2.31 (median = 1.56) mm. Indeed, dimension was statistically different when tested among the different shapes (P<0.001).

3.3.2.2. Chemical composition

Raman spectra of 2294 plastics have been acquired, representing more than the 24% of the plastics identified. The polymers with the highest abundances were polyester (30.4%), followed by polypropylene (20.3%), and polyethylene (15.7%). Additional polymers with a high recurrence were polyamide (6.7%), polyvinyl chloride (4.7%), and polystyrene (0.8%; Fig. 3.4a). Other polymers that were identified, but with a low frequency, were for instance polyurethane, polybutene, polyvinyl alcohol.

Analyzing the distribution of the polymeric composition based on the particle shape, we observed that fibers had a distinct composition from that of the other shapes. Indeed, the large majority of plastic fibers were made of polyester, while polypropylene and polyethylene prevailed for fragments and films (Fig. 3.4b).

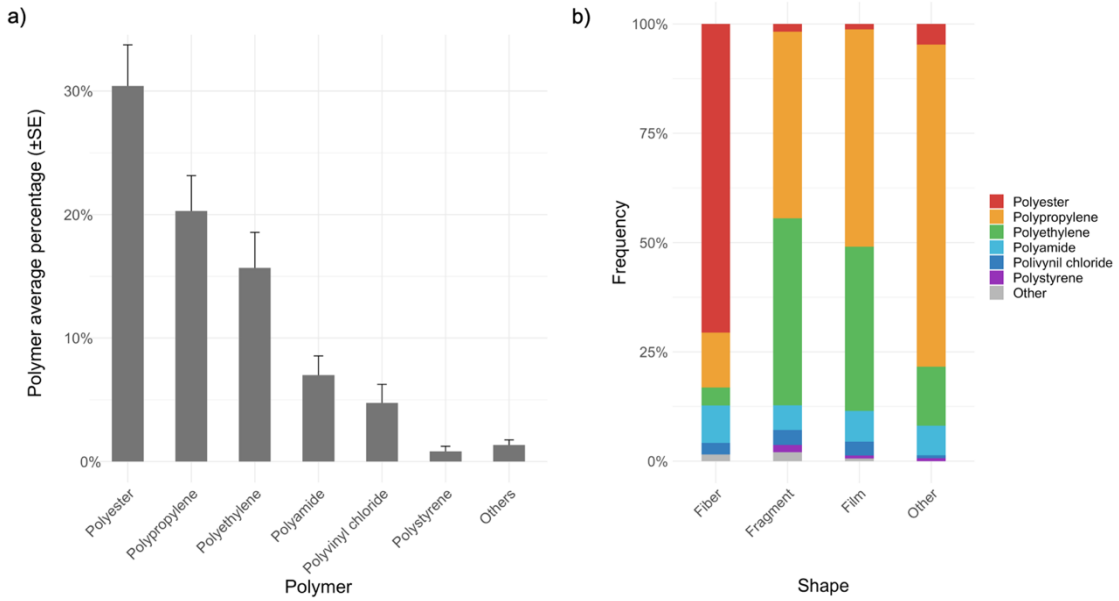

Figure 3.4. a) Average percentage ± standard error (SE) of polymer composition for all the 38 lakes analyzed. b) Frequency of polymeric composition for the different shapes.

3.3.3. Predictors of plastic concentration

The preliminary analysis of the relationship among the possible predictors of plastic concentration showed a strong correlation among lake area, lake volume, watershed area, and shoreline length (P<0.001). Therefore, to avoid problems of multicollinearity, we removed from the subsequent analysis the following variables: lake volume, watershed area, and shoreline length.

Boruta selection algorithm showed that the most important predictors for plastic concentration data were, in descending order, average lake depth, population density in the watershed, lake area, residence time, and percentage of urban-land use in the watershed. The algorithm was indecisive about one additional variable, namely the percentage of cropland in the

watershed (boxplots with the results of the Boruta algorithm are reported in Fig. S3.2). The random forest model was performed both including all the six variables identified through the Boruta algorithm and dropping out the variable in the irresolution area. The best results were obtained when adding only the five important variables.

Principal component analysis, based on the variables identified through the Boruta-algorithm, showed that the lakes were mainly distributed along the two first components, which explained 75.4% of the variance (PC1: 40.6%; PC2: 34.8%; Fig. 3.5). In particular, the first component was primarily explained by lake morphological and hydrological features (i.e., lake area, depth, and residence time); while population density and percentage of urban land-use in the watershed were correlated and mainly explained by the second component. We observed that the higher anthropogenic impact (expressed by the population density and/or urban coverage) was reflected in the concentration of plastic debris, with a positive relationship. Moreover, we observed that concentrations of plastics were high also in a portion of lakes characterized by high surface area and high average depth, like for instance Lake Tanganyika (Burundi), Lake Tahoe (USA), Lake Maggiore (Italy), and Lake Lugano (Switzerland/Italy). In Lake Bourget (France), we observed a low concentration of plastic particles (0.22 particles/m^3) even if morphological features of this lake and the level of anthropogenic impact make it more similar to lakes in which we detected a higher concentration of plastics. Moreover, we observed some lakes with a high concentration of plastics (>1.5 particles/m^3) where the anthropogenic impact captured by our predictors was identified as being low (i.e., Lake Azul, Portugal).

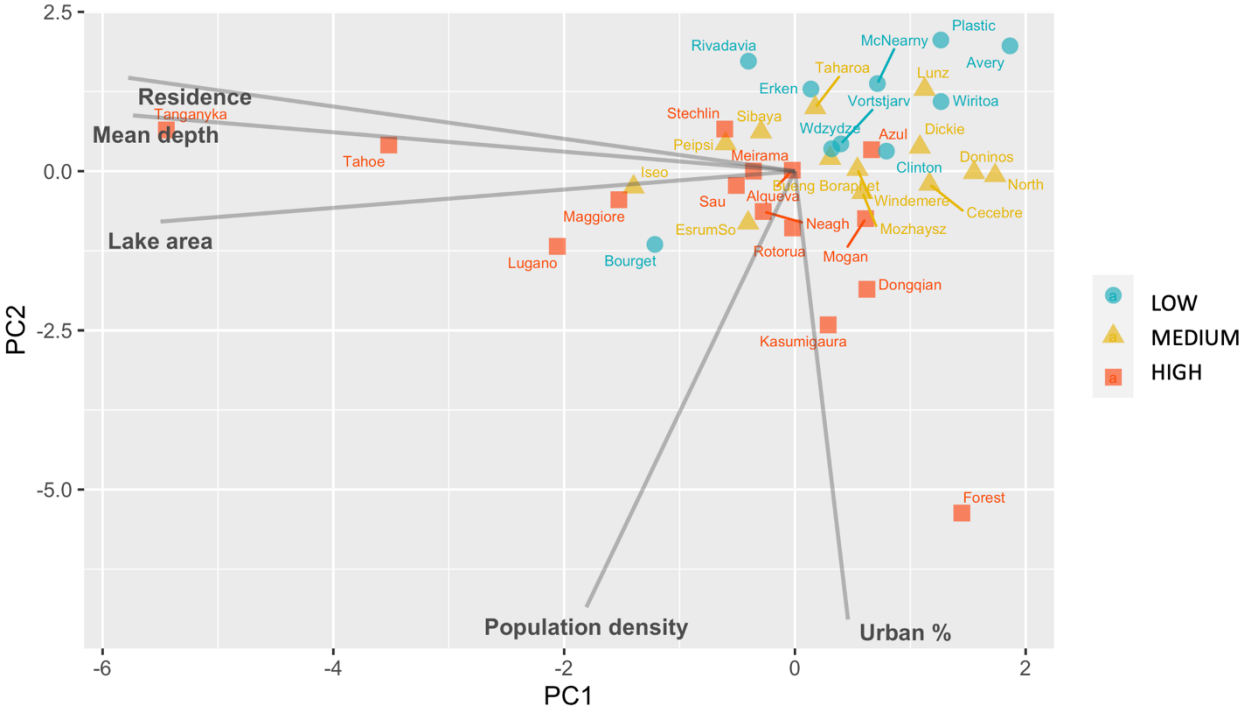

Figure 3.5. Principal component analysis of the lakes studied based on the variables selected through Boruta algorithm: 'Lake area', lake area; 'Mean depth', lake average depth; 'Residence', lake residence time; 'Population density', population density in the watershed and 'Urban %', percentage of urban land-cover in the catchment. Scores of lakes are colored based on concentration of plastic detected: 'low', low concentration of microplastics (<0.5 particles/m^3); 'medium', medium concentration of plastics (0.5-1.5 particles/m^3); 'high', high concentration of microplastics (>1.5 particles/m^3).

3.4. Discussion

The present study represents, to the best of our knowledge, the first survey to analyze (micro)plastics (>250 μm) in surface water of lakes with a worldwide distribution following a standardized methodology. This was achieved by filtering large volumes of water samples to get representative data for 38 lakes (3 samples for each lake, totally 114) located in 28 different countries and allowed obtaining data about the concentrations and characteristics of plastic debris in lakes and reservoirs covering an assortment of limnologically diverse freshwater ecosystems under varying levels of anthropogenic stress. The role of freshwater systems in global

plastic pollution is increasingly recognized. Indeed, these systems on one side represent a source of plastic debris, as land-based sources rather than marine-based sources are considered the primary input of plastics into the ocean, and on the other side, they can also represent a sink where plastic debris can accumulate and impair freshwater organisms and ecosystem services (Ballent *et al.* 2016; Lebreton *et al.* 2017; Sridharan *et al.* 2021). Moreover, lakes within fluvial networks can also receive more plastic particles overall than coastal areas because they are closer to sources of pollution (Tanentzap *et al.* 2021). This makes the study of plastic pollution in freshwater systems even more relevant for management purposes, since the vicinity to sources of pollution can allow better identification and thus intervention to prevent plastics from entering waterways (Schmaltz *et al.* 2020). However, data about the occurrence of plastics in freshwater systems are still quite limited and scattered, and it is generally claimed that the comparison of results from already existing studies is difficult due to a lack of consensus in procedures of classification, analysis, and quantification (e.g., Wong *et al.* 2020; Li *et al.* 2020). Therefore, our study is a valuable contribution to the field as sampling methodologies have been standardized as much as possible and sample pre-treatment and analyses have been performed following the same methodologies, allowing obtaining truly comparable data.

Our results showed that the concentration of plastics spanned among different orders of magnitude (from 10^{-3} to 10^{1} particles/m^3). For the reasons stated above, the comparison of the results obtained with those of previous studies should be done with caution. Restricting the comparison to studies that have investigated plastic abundance in surface water of lakes addressing the same dimensional range of our study, we observed that our data falls within ranges previously reported (Dusaucy *et al.* 2021; Tanentzap *et al.* 2021). For instance, the study of Fischer *et al.* (2016), investigating the occurrence of plastics with a dimension between 0.355 and 5 mm in Lake Bolsena and Chiusi (Italy), reported a concentration of 0.82-4.41 particles/m^3

and of 2.68-3.36 particles/m^3, respectively; while samples collected through a manta net (333 µm) in Lake Kallavesi (Finland) showed a concentration of 0.27 particles/m^3 (Uurasjärvi *et al.* 2020). Concentrations in the marine environment also varied widely (see Lusher 2015 and references therein). Still considering studies that have collected samples through filtering procedures, concentrations spanned from $1.6 \cdot 10^{-5}$ (Bering Sea; Day and Shaw 1987) to 47 (Geoje Island, South Korea; Song *et al.* 2014) particles/m^3. Mean and maximum concentration detected in the South Pacific subtropical gyre was equal to 0.17 and 2.48 particles/m^3 (Eriksen *et al.* 2013), while in the North Pacific Subtropical Gyre were equal to 2.23 and 6.46 particles/m^3 (Moore *et al.* 2001), showing that the concentration that we have found in some lakes can exceed values detected in considerable accumulation zones in the ocean. Different sampling methods, and in particular the adoption of different lower size limits, may lead to concentrations of plastics that largely outpaced the maximum value recorded in our study (i.e., 11.5 particles/m^3). For instance, data for Lake Poyang (China) showed a concentration ranging from 5,000-34,000 particles/m^3 (Yuan *et al.* 2019); however, the samples have been collected using a grab method, which has in general the advantage of allowing capturing plastic at the micro- and nano-scale, but the small volume of water sampled may result in high variability among samples and less representativeness compared to studies where neuston nets are used (Barrows *et al.* 2017).

The majority of plastics that we have identified were fibers, mainly constituted by polyester, with an overall occurrence of 49.4%. Indeed, plastic fibers from textile materials have been indicated as a major source of plastic contamination (Carney Almroth *et al.* 2018). The release of fibers as a result of washing of textiles has been widely reported as one of the main sources. A study, investigating the number of fibers released from washing 6 kg of laundry, indicated that more than 700,000 fibers could be released (Napper and Thompson 2016). We identified the presence of textile fibers even in

lakes with limited human impact, like for instance in Lake Erken (Sweden) or Lake Dickie (Canada), where the urban land-cover was less than 0.2%. This may suggest that atmospheric deposition can be a relevant source of fibers since previous studies already reported that atmospheric fallout might be an important contributor (Dris *et al.* 2018). However, we have no data for this contribution and thus it can only be hypothesized. Besides fibers, the most abundant shape was fragments (40.9%), which likely derive from the fragmentation of larger plastic items (i.e., secondary microplastics). Indeed, field studies have shown that predominantly secondary microplastics are found in the environment (Shim *et al.* 2018). In our study, pellets and spheres, whose shape suggests a primary origin, accounted for less than 1%. The shape is a relevant feature to understand the effect and bioavailability of these pollutants to aquatic organisms as microplastics could potentially have different shape-dependent effects (Frydkjær *et al.* 2017; Botterell *et al.* 2020). For instance, Ziajahromi *et al.* (2017), analyzing the acute and chronic effects of microplastic fibers and beads on freshwater zooplankton *Ceriodaphnia dubia*, showed greater adverse effects of fibers, with reduced reproductive output observed at concentrations within an order of magnitude of reported environmental levels.

Another feature that can also be relevant in influencing the bioavailability of plastic particles is color. Indeed, selective feeding for different colors of microplastics has been observed previously in fish and other organisms since microplastics can be mistaken for foods with similar colors (Santos *et al.* 2016). Color assignation during sample analysis can be difficult due to weathering of particles, color blindness, and different color perception amongst researchers (Lusher *et al.* 2020). Despite this, it is still recommended to record particle color during the visual assessment. Indeed, whilst source derivation is not likely possible based on color alone, recording color may help to identify broad trends, such as ingestion preference. The majority of plastics that we have identified were black (43.5%) followed by clear (23.7%),

and blue (17.9%). In particular, we found a considerable number of bluish items, contrasting with the very low number of reddish items (i.e., red, orange). Interestingly, Martí *et al.* (2020) found similar results reporting that white, transparent/translucent, black/gray and blue particles were particularly common (31%, 16%, 12%, and 11% of the total, respectively), with a very low number of red particles. As an explanation, it has been hypothesized that a lower removal of bluish plastics from the surface water by plastic-ingesting predators can occur since blue has been suggested as a common camouflage color. Thus, a higher probability of detection and ingestion of items with non-blue colors, like red-colored, by visual surface predators would lead to a progressive enrichment in blue microplastics on the ocean surface (Shaw and Day 1994; Martí *et al.* 2020). Moreover, plastics with different colors imply the presence of different additives (i.e., colorants) which may have potentially different effects for the biota (Nava *et al.* 2021).

Regarding the polymeric composition, polyester, polypropylene and polyethylene constituted the large majority of polymer identified (overall 66.4%). This is not surprising as these materials, accordingly to the analysis of Geyer *et al.* (2017), accounted for the large majority of global plastic production, with PE accounting for 36%, followed by PP (21%), while polyester, most of which is PET, accounts for 70% of all polyester, polyamide, and acrylic fiber production; moreover, these polymers are commonly used in short life-cycle products. Our results are also in agreement with previous findings both in marine (Erni-Cassola *et al.* 2019) and freshwater ecosystems (Li *et al.* 2020; Dusaucy *et al.* 2021).

Sources of microplastics, especially secondary microplastics, are both diverse and numerous. Identify these sources for different aquatic systems is increasingly important, especially for management purposes. The investigation of microplastics in freshwater environments allows to more likely relate the occurrence of microplastics with the surrounding catchment given the closer proximity to the possible sources than in marine

environments. Moreover, it is still not clear if lake features (morphological and hydrodynamics) can influence the distribution of plastics. Here, we identified different predictors that should capture different possible sources of plastics. In particular, we showed that lakes with higher population density and higher urban land coverage in the watershed are likely to have higher concentrations of microplastics in the surface water. This was observed also in previous research, which reported a correlation between microplastic concentrations and urban-related attributes (e.g., Baldwin *et al.* 2016; Tanentzap *et al.* 2021). We also showed that lakes with larger surface area and deeper and with higher residence time accumulate more plastics in the surface water compared to smaller lakes. This may be linked to the greater area that is drained by larger lakes and thus to a higher number of possible contamination sources. Moreover, the higher residence time could allow the plastics to longer persist in these environments, which can eventually accumulate plastics in their water. Indeed, the relationship between plastic concentration and water retention times has been already hypothesized (Dusaucy *et al.* 2021).

3.5. Conclusions

Results from this study add greatly to the current body of information on plastic contamination in surface water of lakes and reservoirs, especially given the largely claimed need for comparable data in the field. Indeed, we reported data of plastic contamination (>250 µm) in different freshwater ecosystems around the world, obtained through the collection and analysis of samples following a common procedure, and we provided information not only about the concentration but also about the features of these pollutants. This is particularly important as this information will represent a valuable baseline for further comparisons and also help future studies in defining the testing conditions required to better mimic the real environment, thus

conducting experiments with polymers that are more commonly found with color, shape, and dimension that are more likely to be encountered in surface water of lentic systems.

The findings of this study suggest that generally exists a relationship between urban-related attributes of lakes and the plastic contamination, but also highlighted that larger and deeper lakes with higher retention times are accumulating plastic debris at higher concentration. These results are also important as allow targeting systems that may be more impacted by plastic debris, whose accumulation is also expected to increase.

Even if the widespread occurrence of plastic debris in aquatic ecosystems has been firmly established, their concentration and features can vary largely among different systems alongside the impacts that they may exert on aquatic organisms and ecosystem functioning. Therefore, the understanding and the characterization of the plastic debris and the 'cocktail of chemicals' associated within different systems is still necessary especially for understanding and managing the linked risk.

Supplementary materials

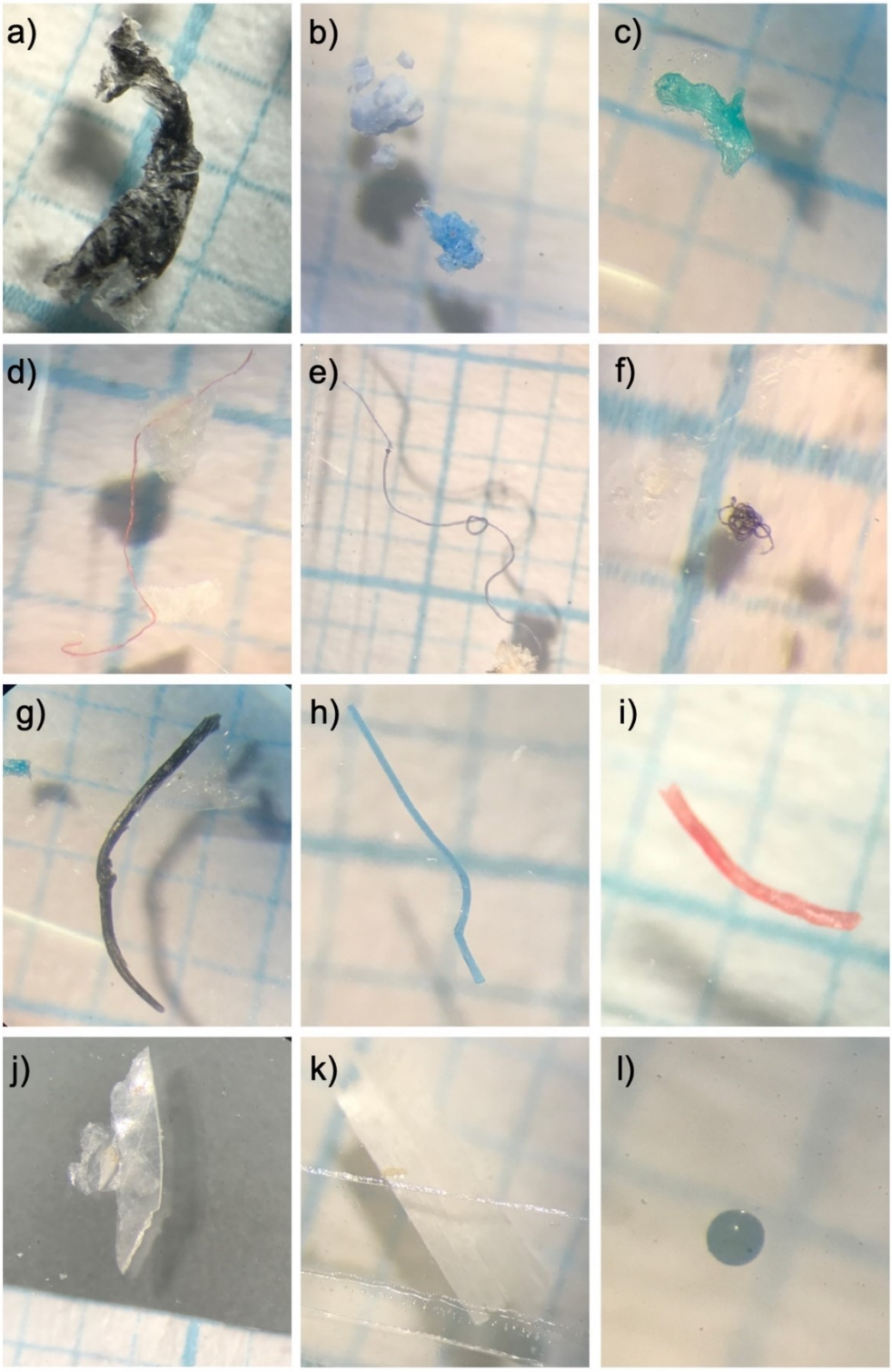

Figure S3.1. Images of different shapes of plastic fragments collected in water samples: a, b, c) fragment; d, e, f) fiber; g, h, i) filament; j, k) film; l) sphere/pellet.

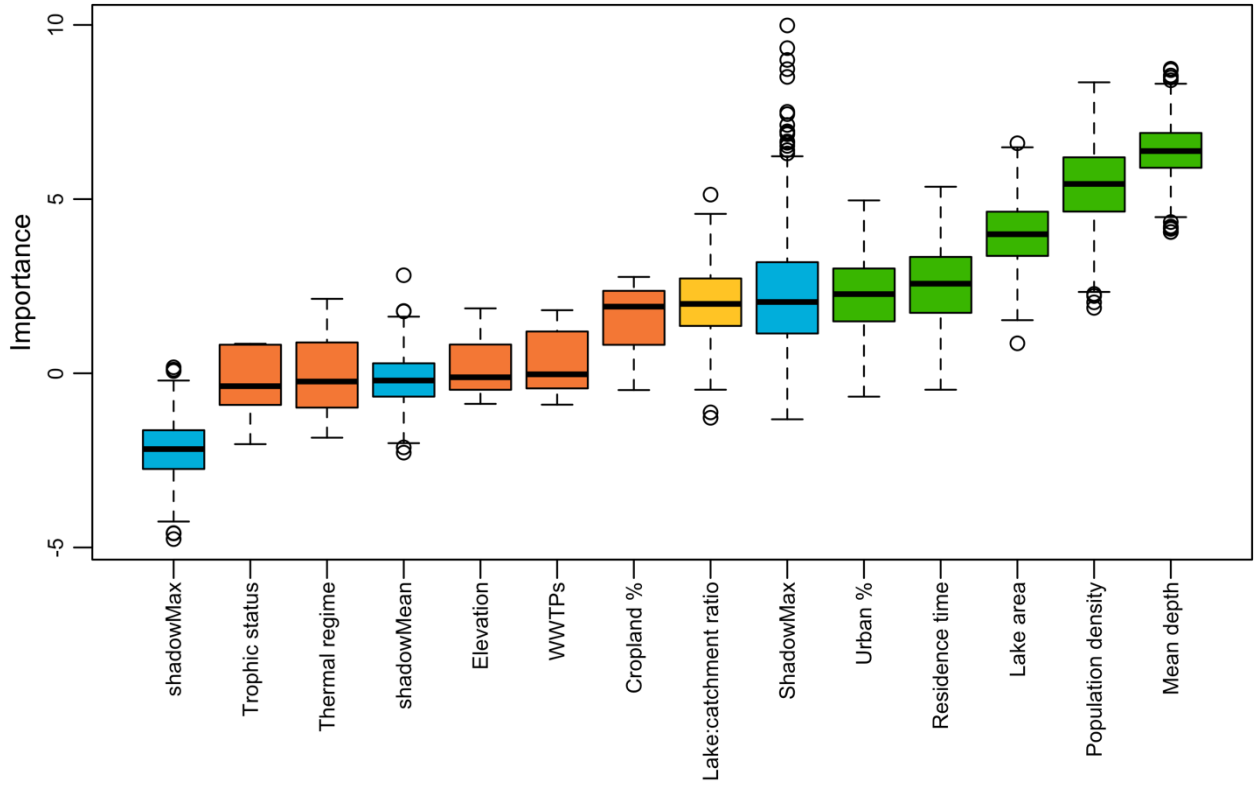

Figure S3.2. Results of Boruta algorithm for feature selection. Important variables are reported in green, while unimportant in orange. Yellow indicates variables for which the algorithm was indecisive, while blue indicates the 'shadow' attributes.

Table S3.1. Hydro-morphological features and attributes of the 38 lakes included in the study.

Lake	Country	Elevation (m a.s.l.)	Mean depth (m)	Maximum depth (m)	Lake area (km^2)	Lake volume (km^3)	Residence time (y)	Shoreline (km)	Watershed area (km^2)	Ratio Catchment: Lake area	Trophic status	Thermal regime
Alqueva	Portugal	152	17	92	250	4.15	0.03	1100	55000	220	Eutrophic	Monomictic
Avery	USA	270	2.0	2.3	0.06	0.000128	1.86	1.1	1.50	23.4	Oligotrophic	Polymictic
Azul	Portugal	260	7.1	25	3.25	0.039764	3.00	9.9	15.35	4.70	Mesotrophic	Monomictic
Bourget	Bourget	231	85	145	44.5	3.6	9.00	44	560	12.6	Oligotrophic	Monomictic
Bueng Boraphet	Thailand	22	50	1.0	1.61	0.09	1.54	64	4400	2733	Eutrophic	Polymictic
Cecebre	Spain	35	5.9	18	3.55	0.02085	0.17	20	245	69.0	Mesotrophic	Monomictic
Clinton	USA	267	2.1	12	28.0	0.1362	1.95	79	950	33.9	Eutrophic	Polymictic
Dickie	Canada	354	5.0	12	0.94	0.00468	1.57	8.2	5.00	5.30	Oligotrophic	Dimictic
Dongqian	China	4.0	2.2	3.3	22.0	0.0339	1.59	46	130	5.90	Mesotrophic	NA
Doninos	Spain	4.4	4.7	11	0.26	0.00146817	0.11	2.06	6.44	24.9	Mesotrophic	Monomictic
Erken	Sweden	11	9.4	21	23.7	0.2135	7.99	62	137	5.80	Mesotrophic	Dimictic
EsrumSo	Denmark	9.4	13.5	22	17.3	0.233	12.70	27	62	3.60	Mesotrophic	Monomictic
Forest	Australia	40	2.5	3.2	0.11	0.000272	0.40	2.7	2.80	25.9	Oligotrophic	Polymictic
Iseo	Italy	186	124	258	62.0	7.6	11.00	63	1840	29.7	Eutrophic	Meromictic
Kasumigaura	Japan	0.0	3.4	7.3	171	0.6	0.57	122	1426	8.40	Hyper-eutrophic	Polymictic
Lugano	Switzerland/ Italy	271	134	288	566	6.5	12.30	77	296	0.50	Eutrophic	Meromictic
Lunz	Austria	608	20	34	0.68	0.0136	0.30	4.0	27	39.7	Oligotrophic	Dimictic
Maggiore	Italy	193	178	370	213	37.5	4.10	170	6599	31.0	Mesotrophic	Meromictic
McNearny	USA	261	7.0	9.5	0.49	0.003	11.40	2.9	0.88	1.80	Ultra-oligotrophic	Dimictic

Meirama	Spain	171	86	205	1.71	0.147	7.00	7.5	33	19.3	Oligotrophic	Meromictic
Mogan	Turkey	972	2.4	4.0	5.40	14	6.90	14	925	171	Mesotrophic	Polymictic
Mozhaysz	Russia	183	7.7	23	30.7	0.235	0.56	87	1360	44.3	Eutrophic	Dimictic
Neagh	Ireland	10	8.9	30	383	3.45	1.25	234	4453	11.6	Hyper-eutrophic	Polymictic
North	USA	184	0.5	2.0	0.04	0.000018	NA	1.2	0.15	4.20	Oligotrophic	Polymictic
Peipsi	Estonia/ Russia	30	7.1	15	3555	25	2.00	520	47800	13.4	Eutrophic	Polymictic
Plastic	Canada	376	7.90	16	0.32	0.002539	3.30	3.1	1.28	4.00	Oligotrophic	Dimictic
Rivadavia	Argentina	527	104	147	21.7	2.25	3.60	32	1900	87.6	Oligotrophic	Monomictic
Rotorua	New Zealand	280	10	45	79.8	0.8	1.60	44	482	6.00	Eutrophic	Polymictic
Sau	Portugal	558	343	427	5.72	0.1513	0.22	0.78	1522	266.1	Eutrophic	Monomictic
Sibaya	South Africa	19	13	41	64.0	0.7	6.17	35	530	8.30	Mesotrophic	NA
Stechlin	Germany	60	23	70	4.12	0.0996487	55.00	16	6.31	1.50	Mesotrophic	Dimictic
Taharoa	New Zealand	70	16	39	2.11	0.02518	10.60	7.4	4.23	2.00	Ultra-oligotrophic	Monomictic
Tahoe	USA	1897	304	501	490	150	650.00	114	1310	2.70	Ultra-oligotrophic	Monomictic
Tanganyka	Burundi	774	580	1470	32600	18980	5500.00	1828	231000	7.10	Oligotrophic	Meromictic
Vortstjarv	Estonia	34	2.8	6.0	269	0.75	1.00	130	3380	12.6	Eutrophic	Polymictic
Wdzydze	Poland	133	19	68	14.6	0.2208	1.30	28	538	37.0	Mesotrophic	Dimictic
Windemere	UK	39	17	42	6.72	0.113	0.74	32	249	37.0	Mesotrophic	Monomictic
Wiritoa	New Zealand	51	5.0	18	0.26	0.00140579	3.77	4.8	6.96	26.5	Eutrophic	Monomictic

Table S3.2. Blank level for laboratory-based QA/QC.

Lake	Laboratory-based QA/QC		
	Replicate 1	Replicate 2	Replicate 3
Alqueva	3	5	5
Avery	0	0	0
Azul	3	2	4
Bourget	0	3	2
Bueng Boraphet	6	4	4
Cecebre	1	0	0
Clinton	1	0	1
Dickie	3	3	1
Dongqian	5	8	0
Doninos	0	2	0
Erken	1	1	0
EsrumSo	1	0	0
Forest	5	2	3
Iseo	0	1	1
Kasumigaura	6	1	1
Lugano	5	11	3
Lunz	3	0	3
Maggiore	2	5	4
McNearny	1	0	0
Meirama	0	1	0
Mogan	5	6	1
Mozhaysz	5	5	6
Neagh	1	2	1
North	1	0	0
Peipsi	5	0	2
Plastic	0	0	0
Rivadavia	1	0	4
Rotorua	1	0	0
Sau	7	1	2
Sibaya	1	1	0
Stechlin	9	7	5
Taharoa	3	6	0
Tahoe	5	5	6
Tanganyka	5	7	4
Vortstjarv	1	3	1
Wdzydze	0	0	0
Windemere	1	2	0
Wiritoa	0	0	0

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Chapter 4

A critical review of interactions between microplastics, microalgae and aquatic ecosystem function

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Water Research **188**, 116476 (2021)
doi: 10.1016/j.watres.2020.11647

Abstract

With the widespread occurrence of microplastics in aquatic ecosystems having been firmly established, the focus of research has shifted towards the assessments of their influence on ecosystem functions and food webs. This includes interactions between microplastics and microalgae, as fundamental components at the base of aquatic food webs and pivotal organisms in a wide range of ecosystem functions. In this review, we present the current state of knowledge on microalgae–microplastic interactions and summarize the potential effect on their respective fate. Microplastics can and do interact with microalgae and the available literature has suggested that the epiplastic community of microalgae differs consistently from the surrounding aquatic communities; however, it is still not clear whether this different colonization is linked to the composition of the surface or more to the availability of a "hard" substrate on which organisms can attach and grow. Further studies are needed to understand to what extent the properties of different plastic materials and different environmental factors may affect the growth of microalgae on plastic debris. Biofouling may alter microplastic properties, especially increasing their density, consequently affecting the vertical fluxes of plastics. Moreover, microplastics may have toxic effects on microalgae, which could be physical or related to chemical interactions with plasticizers or other chemicals associated with plastics, with consequences for algal growth, photosynthetic activity, and morphology. Microplastics seems to have the potential to affect not only the quality (e.g., fatty acids and lipids composition, food dilution effect) but also the quantity of algal production, both positively and negatively. This may have consequences for energy fluxes, which may propagate throughout the whole food web and alter aquatic productivity. Even though experimental results have indicated reciprocal impacts between plastics and microalgae, it is currently difficult to predict how these impacts may manifest themselves at the ecosystem level. Therefore, further studies are needed to address this important topic.

4.1. Introduction

Plastic has become an inherent part of daily life and millions of tons of plastic material are manufactured globally every year. Due to the broad application of plastic in many different sectors and its long-lasting characteristics, the amount of plastic litter has increased dramatically over the last few decades in both aquatic and terrestrial environments (Galafassi *et al.* 2019). The slow breakdown of plastic items produces successively smaller pieces, called microplastics (MPs) – a term that is now commonly used to define particles whose larger dimension is lower than 5 mm (Van Cauwenberghe *et al.* 2015). Agreement on this higher limit of the microplastic range (5 mm) is quite consistent throughout the literature; however, some researchers have recently suggested different thresholds (e.g., 1 mm) (Hartmann *et al.* 2019). Besides the microplastics derived from the degradation of larger plastic items (i.e., secondary microplastics), microplastics can also be specifically manufactured in the micrometer size range (i.e., primary microplastics); for instance, those used in industrial abrasives for sandblasting, plastic pre-production pellets ('nurdles'), or 'microbeads' in personal care products (Horton *et al.* 2017).

The term 'plastic' includes many different polymers, but the most abundant classes of polymer detected in aquatic environments are polyethylene (PE), polypropylene (PP), polystyrene (PS), polyester (PEST), polyamide (PA), and acrylic. This is not surprising, given the fact that these materials have constituted a major proportion of global plastic production and are widely used in short life-cycle products (Erni-Cassola *et al.* 2019).

Different plastic polymers have different chemical and physical properties and, depending on their composition, density, and shape, can be buoyant, neutrally-buoyant, or sink in aquatic systems (Cole *et al.* 2011). In particular, the density of most common plastic polymers ranges from 0.85 to 1.41 g/cm^3 and, as this range includes materials of lower, equal, or higher density than sea and freshwater, microplastics can be easily distributed throughout the

water column. The density can determine whether a particle occupies a pelagic versus benthic transport route: microplastics with a density greater than that of water sink into sediments where they accumulate, while those with low density float on surfaces (Cole *et al.* 2011; Woodall *et al.* 2014). An increase in density, through biofouling by organisms, can eventually result in sinking of microplastics (Auta *et al.* 2017).

Microplastic occurrence in the marine environment has been documented in almost every open and enclosed sea habitat, extending from surface water to deep-sea sediments, and from the equator to the polar regions (Peng *et al.* 2017). Recent estimates suggest that 4.85 trillion microplastic particles are floating in the global ocean (Eriksen *et al.* 2014). The majority of the research about microplastics has focused on seawater environments, while less than 4% of microplastic-related studies concern freshwater environments (Li *et al.* 2018). This limited information, however, has revealed that the abundance of microplastics in freshwater is comparable to that in marine environments (Peng *et al.* 2017; Li *et al.* 2018).

With the widespread occurrence of microplastics in aquatic ecosystems now firmly established, focus has been shifting towards the assessment of their influences on ecosystem functions and food webs. Despite growing research efforts, understanding of the ecological implications that the presence of microplastics may have on aquatic ecosystems, particularly regarding lower trophic levels (e.g., phytoplankton), are still largely unknown (Bryant *et al.* 2016). Different studies have already reported that microplastics can and do interact with aquatic microalgae and this has impacts on their respective fates (Long *et al.* 2015; Yokota *et al.* 2017). Microplastic surfaces constitute suitable substrates for the formation of biofilms (McCormick *et al.* 2014; Amaral-Zettler *et al.* 2020), and microalgae are an important constituent of colonizing biotic communities (Yokota *et al.* 2017). The growth of microalgae on plastic surfaces is important for the plastic degradation process, either having potential for biodegradation or, on the contrary, protecting plastics from ultraviolet radiation and photo-catalysis (Carson *et al.* 2013). Moreover,

microplastics could potentially be incorporated along with microalgae into hetero aggregates with associated changes in buoyance, thus resulting in settling and influencing the fate and bioavailability of microplastics (Carson *et al.* 2013; Long *et al.* 2015). At the same time, microalgae may suffer toxic effects as inhabitants of pelagic areas contaminated with microplastics, with consequences for their growth, photosynthetic activity, and changes in morphology (Mao *et al.* 2018; Zhao *et al.* 2019; Fu *et al.* 2019). In addition, transport via rafting of the microalgae themselves is also of concern, as this community is distinct from that of the surrounding seawater, with potential impacts such as the introduction of harmful or non-native algae into new environments (Carson *et al.* 2013; Masó *et al.* 2016). Thus, the interactions between microplastics and microalgae may also be relevant at the ecosystem level, with possible implications for the productivity of aquatic ecosystems, which have already been affected by other anthropogenic impacts such as climate change, eutrophication, and food web alterations (Yokota *et al.* 2017; Troost *et al.* 2018).

Given the importance of microalgae and the global occurrence of microplastic fragments in aquatic systems, a review on the topic is required, in order to identify the potential mechanisms of interaction as well as to guide further inquiries. For this reason, we conducted a broad and wide-ranging literature review, analyzing around 80 peer-reviewed papers, from 1972 to 2020, most of which (67%) were published from 2018 onwards, indicating the growing interest for this topic. We synthesize the current state of knowledge on microalgae-microplastic relationships, addressing the different aspects of these interactions. The specific objectives are: i) to analyze whether some taxa of microalgae may preferentially colonize microplastic surfaces and to identify the features and extent of such colonization; ii) to synthesize the environmental factors affecting microplastic colonization by microalgae; iii) to discuss the consequences of colonization by microalgae on the fate and characteristics of microplastics; iv) to summarise the effects that microplastics may exert on microalgae; and v) to

evaluate the effects of the interaction between microplastics and microalgae at the ecosystem level.
Moreover, we comment on potential future questions and research directions needed to further define the implications of the relationships between microalgae and these concerning pollutants.

4.2. Microplastic colonization

4.2.1. Plastic as a surface for colonization

There now exists a large body of evidence that microplastics are abundant and widespread in both marine and freshwater environments (Van Cauwenberghe *et al.* 2015; Horton *et al.* 2017; Jiang 2018), and different studies have reported that microplastics can be colonized by a wide range of organisms (biofouling) (Carson *et al.* 2013; Reisser *et al.* 2014). Microplastics harbor a distinct biota and represent a new habitat for rafting organisms to the point that the term "plastisphere" was coined by Zettler *et al.* (2013), to define the diverse community of heterotrophs, autotrophs, predators, and symbionts on the surface of plastic debris.
The first study on plastic colonization dated back to the beginning of the 1970, when an investigation in the Sargasso sea surface revealed the presence of microalgae (in particular diatoms) attached to the surface of plastic (Carpenter and Smith 1972). This early study relied primarily on microscopy but, at present, the application of modern molecular methods, especially high-throughput DNA sequencing, is used to increase our understanding of the diverse micro-organisms inhabiting the plastisphere (Amaral-Zettler *et al.* 2020). While heterotrophic bacteria tend to be the focus of plastisphere research, the presence of both prokaryotic and eukaryotic autotrophs within the biofilm has been documented (Yokota *et al.* 2017). There is a growing field of research focused on the colonization process of

microplastics by microalgae, but many questions are yet to be addressed (Carson *et al.* 2013).

It has been widely reported that the community growing on plastic debris differs consistently from the surrounding free-living organisms (Zettler *et al.* 2013; Oberbeckmann *et al.* 2014; Bryant *et al.* 2016; Dussud *et al.* 2018a; Kesy *et al.* 2019). In general, the dichotomy between particle-attached (PA) and free-living (FL) micro-organisms has been widely documented in several studies across different aquatic biomes and, thus, the discrepancies among organisms attached to plastic debris and planktonic communities may be not strictly linked to the composition of the surface, but more to the availability of a substrate on which organisms can attach to and grow (Oberbeckmann *et al.* 2016). It is likely that many taxa use plastic opportunistically as a niche but can also attach to other substrates (Oberbeckmann *et al.* 2016). However, experimental studies have reported differences in the communities found on plastic surfaces compared to other substrates, such as glass, which is usually used as a control substrate since it is inert (Vosshage *et al.* 2018). During a short-term (two-week) experiment in which plastic particles (polyethylene, polypropylene, and polystyrene) and glass beads were exposed to brackish water from a coastal bay under controlled conditions, Ogonowski et al. (2018) found that the plastic-associated communities were distinctly different from those of the non-plastic substrates, suggesting substrate-driven selection. The same result was reported for a 6-week exposure experiment in the North Sea (U.K.) with polyethylene terephthalate (PET) bottles and glass slides as reference samples in which marked differences were reported in the community isolated from the two substrates with at least 57% divergence and where several biofilm members were detected solely on one of the surfaces, suggesting a preference for plastic or glass (Oberbeckmann *et al.* 2014). Additional studies in the same location reported no significant difference between glass and PET-attached communities (Oberbeckmann *et al.* 2016), thus highlighting that this topic remains controversial.

Vosshage *et al.* (2018) exposed different materials (28 × 48 mm) for 49 days in a shallow, highly productive lake and found a significant difference in the volume of algae and biofilm height, compared to glass substrate, only for polymethyl methacrylate (PMMA) and not for polycarbonate (PC). Several cohort studies have indicated that the colonization process can vary, according to the plastic polymer used. For instance, Lagarde *et al.* (2016) highlighted differences in long-term colonization between high-density polyethylene (HDPE) and polypropylene (PP); Zettler *et al.* (2013) reported slight differences in organism richness associated with polyethylene (PE) and polypropylene (PP) on a wide range of plastic marine debris collected at multiple locations after long residence times in the environment; and Li *et al.* (2019) found significantly higher biomass on polystyrene (PS) than on other plastic polymers, indicating an influence of polymer composition on the biofilm formation. Despite these research efforts, a systematic examination of the colonization process occurring on different plastic polymers is still lacking. Indeed, the term 'plastics' include a wide variety of polymers with different chemical composition and properties. Even for the same type of polymer, the chemical composition may vary considerably, depending on the chemical additives (Lagarde *et al.* 2016). As the surface chemistry of the substrate is often not established, the influence of the substrate chemistry on the colonization process generally cannot be determined (Cooksey and Wigglesworth-Cooksey 1995). Differences highlighted in plastic colonization processes may be partially explained by bearing in mind that different polymers, even if falling into the umbrella term 'plastic', are materials with distinct features. A fundamental role in the process of colonization of plastic substrate by microalgae is played by the Extracellular Polymeric Substances (EPS), which provides an attractive force maintaining cells together and attaching aggregates and cells to biotic or abiotic surfaces. It has been argued that different polymers stimulate the production of EPS in different quantities and/or with different composition, thus determining variability in

the cohesiveness of biofilms and, ultimately, in the biomass of colonizing organisms (Lagarde *et al.* 2016).

It is worth mentioning that the studies reported above described experiments performed in both freshwater and marine environments. However, different hydrological, hydrodynamic, physico-chemical, and species compositions characterize these two systems. Therefore, biofilm attachment on the surface of microplastics in freshwater may show different features (e.g., biomass, type, and quantity of EPS), compared to those in the sea (Chen *et al.* 2019), such that the results obtained for marine systems should be extended to freshwater environments (and vice-versa) with caution.

Moreover, it should also be noted that the colonization experiments listed encompassed studies of different durations, whose variation may influence the process and the conclusions drawn. The major part of the studies was conducted over short time scales; however, considering that synthetic polymers can persist over long periods in natural aquatic environments, incubation over longer timescales may allow for mimicking more realistic conditions (Kirstein *et al.* 2018). In addition, a distinction in the analysis of the different phases of biofilm development is needed, since only the initial recruits have direct contact with the polymer surface; in contrast, later recruits are more likely to interact with existing biofilm members and the abiotic components of the surrounding environment (Oberbeckmann *et al.* 2016; Dudek *et al.* 2020). Therefore, when the different phases of development are studied collectively, only generalized insights about the plastic-associated community can be obtained.

Besides, many other factors likely influence the colonization process. Among others, the age and the time that plastic material spends in the environment play an important role. Aging produces alterations of the plastic surface, and where wrinkled, rough, and fractured surface textures can be observed on aged microplastics (Fu *et al.* 2019). This has consequences for colonization. Indeed, it has been reported that the surface “roughness” of particles is

positively related to the density of attached microalgae (Carson *et al.* 2013). Aging processes can also vary the hydrophobicity of the different particles, which may have consequences on biofouling processes as well, as several articles have acknowledged that high-energy surfaces ("hydrophilic surfaces") tend to favor biofilm growth (Dussud *et al.* 2018a).

Finally, environmental factors and seasonal variations, which exert effects on primary producers in general, may influence colonizing processes (see Section 4.2.2).

4.2.2. Environmental factors and seasonal variations

As the growth and development of primary producers are dependent on several environmental factors, the colonization process on plastic substrates is influenced by their variations over time. Furthermore, biofilms are shaped to adapt to local conditions and environmental factors determine a cell's 'decision' to form or leave a biofilm (Toyofuku *et al.* 2016). Several studies have shown that biogeography plays an important role in the composition of microplastic-colonizing communities (Oberbeckmann *et al.* 2014; Amaral-Zettler *et al.* 2015), but there is still debate among researchers as to whether substrate-specific properties or environmental factors prevail in shaping microorganism assemblages on plastic materials. It is not clear whether the plastic surface 'environment' may exert a strong enough selection to drive species sorting, in order to overcome other niche-defining factors driven by seasonal and spatial patterns. Thus, it is important to study which environmental factors exert the strongest selective pressure and how their synergic relationships can shape plastic-colonizing communities.

Despite inter- and intra-site variability, there is a clear dearth of consensus among researchers that some factors with greater influence on plastic colonization can be identified. Among these, temperature plays a role in the settlement and growth of the colonizing community, as higher temperatures (within the optimum range) increase cell metabolism, resulting in the rapid

development of the attached organisms. Indeed, seasonal differences, driven by temperature variations, have been observed in the plastic colonization processes. Oberbeckmann *et al.* (2014) highlighted the highest and the lowest overall mean diversity of PET plastisphere communities in a marine environment in summer and winter, respectively. Conceptually similar work has also been carried out in a freshwater lake by Chen *et al.* (2019) who, studying the biofilm development on polypropylene sheets in four seasons, pointed out that biofilm developed at a different rate in different seasons, with the highest biofilm biomass per unit area in summer and the lowest in winter.

A pivotal role of salinity has also been recognized, which is known to shape communities in aquatic environments (Dussud *et al.* 2018b; Oberbeckmann *et al.* 2018; Kesy *et al.* 2019). Exposure of five types of plastic debris (polyvinyl chloride, polypropylene, polyethylene, polystyrene, and polyurethane) in the Haihe Estuary highlighted that salinity had a negative correlation with the average growth rate of the biofilm and a positive correlation with the diversity of the colonizing community (Li *et al.* 2019), confirming the results reported in previous studies (Oberbeckmann *et al.* 2018; Kesy *et al.* 2019).

Besides temperature and salinity, nutrients are likely to influence plastic colonization, where an increase in nutrients is usually associated with greater biodiversity on the plastic-attached community (Oberbeckmann *et al.* 2018; Li *et al.* 2019). Different environmental parameters and their variation over time can shape and select the community which is able to colonize the plastic surface; for instance, photosynthetic organisms cannot be found where there is no light irradiance (Chen *et al.* 2019). Moreover, it should be taken into account that there is a constant interplay of different environmental parameters, the combinations of which affect biofilm development.

However, the previous research has obtained mixed results, in terms of the impact of environmental factors. For instance, no effects of geographical location or environmental factors were highlighted on the community assemblages developed on plastics sampled in the western Mediterranean

basin (Dussud *et al.* 2018b). We postulate that these contradictory results may likely be caused by different experimental designs (e.g., polymer considered or analytical methodologies), differences in temporal and spatial scale considered, and the intrinsic diversity in the systems studied. To the best of our knowledge, factors such as hydrodynamic features, physical disturbance, or solar radiation, have not been taken into account in the studies performed to date.

4.2.3. Microalgae taxa colonizing plastic debris

Bacteria usually represent the most-studied organisms in the colonization process of plastic materials. Among them, organisms belonging to the photosynthetic phylum of Cyanobacteria have been widely reported as a group with the capability to colonize plastic debris (Oberbeckmann *et al.* 2014; Bryant *et al.* 2016; Chen *et al.* 2019). They have been shown to dominate in many plastisphere communities (Table 4.1) and may play an important role in the ecological processes occurring in biofilms on plastic (Oberbeckmann *et al.* 2014). Their occurrence and importance on plastic surfaces have also been reported in freshwater systems (Yokota *et al.* 2017). As colonial and filamentous cyanobacteria are able to produce cyanotoxins and are frequently the cause of harmful algal bloom (HAB) in fresh and marine waters (Yokota *et al.* 2017), their interactions with microplastics can have consequences and implications at the ecosystem level (see Section 4.5). Among the most frequent taxa detected, cyanobacteria of the filamentous genus *Phormidium* are commonly found as part of plastic-colonizing communities, with studies attesting to their occurrence in the North Atlantic (Zettler *et al.* 2013; Debroas *et al.* 2017), North Pacific (Bryant *et al.* 2016), and North Sea (Oberbeckmann *et al.* 2014; Oberbeckmann *et al.* 2016). Other marine and freshwater cyanobacteria that have been reported to colonize plastic surfaces belong to the following orders: Chroococcales (genus *Microcystis*), Oscillatoriales (genus *Rivularia*), Nostocales (genera *Calothrix* and

Scytonema), Pleurocapsales (genus *Pleurocapsa*), and Synechococcales (genera *Synechococcus*, *Prochlorothrix* and *Leptolyngbya*) (Bryant *et al.* 2016; Debroas *et al.* 2017; Dussud *et al.* 2018b; Muthukrishnan *et al.* 2019). Due to their high ecological plasticity, Cyanobacteria are capable of adapting to several conditions and changes occurring in the environment (Leoni *et al.* 2014b; Marti *et al.* 2015; Nava *et al.* 2017). Therefore, their ability to colonize plastic substrates in a wide range of different environments is not surprising. Diatoms typically join Cyanobacteria among the photosynthetic representatives that are able to colonize plastic surfaces (Amaral-Zettler *et al.* 2020). Most studies have shown that diatoms are common and omnipresent residents of the plastisphere (Table 4.1) – at least on plastics that are exposed to sunlight – being able to firmly attach to plastic and resist water turbulence and wave action (Reisser *et al.* 2014). For instance, Carson *et al.* (2013), who studied small plastic items from the surface of the North Pacific Gyre, reported that pennate diatoms were among the most abundant organisms (around one thousand individuals mm^{-2}). The same result was highlighted for marine plastic debris collected from pelagic and benthic habitats across the Mediterranean coastal waters of Greece, Italy, and Spain, reporting that diatoms appeared on almost 100% of the sampled plastic debris (Masó *et al.* 2016). A study examining the types of organisms inhabiting the surfaces of 68 small marine plastics (median size equal to 3.2 mm) from inshore and offshore waters around the Australian continent (tropical to temperate areas) reported that diatoms were the most diverse group of plastic colonizers, growing both flat on the surface of plastic materials and erect, being attached by mucous pads or stalks (Reisser *et al.* 2014). All of these studies have employed SEM analysis to identify the colonizing organisms. Additional studies performing DNA metabarcoding on plastic communities highlighted the presence of diatoms on plastic surfaces (Oberbeckmann *et al.* 2014; Oberbeckmann *et al.* 2016; Debroas *et al.* 2017; Kettner *et al.* 2019), despite that, in some cases, diatom clades did not make up more than 1% of the eukaryotic rRNA genes due to their low biomass (as opposed to the

number of individuals), compared to the other eukaryotes (Bryant *et al.* 2016). A high density of diatoms is typically reported on plastic surfaces and, even if their biomass contribution may be low, the presence of many different species of diatoms is often recognized; thus, they importantly contribute to the overall biodiversity of the plastic-associated community. Among the most recurring and abundant taxa of diatoms in marine and freshwater systems are species of the genera *Achnantes*, *Amphora, Cocconeis, Navicula*, and *Nitzschia* (Zettler *et al.* 2013; Reisser *et al.* 2014; Oberbeckmann *et al.* 2014; Masó *et al.* 2016; Lacerda *et al.* 2019). Many of these are known biofilm-forming taxa in aquatic environments. In general, diatoms are reported to be among the first recruits in the colonization of different substrata (Eich *et al.* 2015; Oberbeckmann *et al.* 2016; Debroas *et al.* 2017): after the formation of a 'conditioning layer' by bacteria, surfaces are usually colonized by diatoms; however, it has been reported that diatoms may also directly attach to virgin surfaces (Khandeparker *et al.* 2014). However, it still remains unclear whether an obligate succession of organisms can be determined on the plastic surface or if it is an artifact due to the method of analysis used (Cooksey and Wigglesworth-Cooksey 1995). Regardless of the sequence, it is generally agreed that diatoms represent a fundamental step in biofouling, influencing the subsequent colonization process (Khandeparker *et al.* 2014; Oberbeckmann *et al.* 2016).

Several studies have reported the presence of thecate and athecate dinoflagellates, such as *Alexandrium* sp., *Ceratium* sp., and *Prorocentrum* sp., on plastic surface, with the occurrence of harmful species (Zettler *et al.* 2013; Reisser *et al.* 2014; Masó *et al.* 2016; Kettner *et al.* 2019). The presence of organisms belonging to Chlorophyta, Cryptophyta, and Chrysophyta have also been sporadically reported (Masó *et al.* 2016; Debroas *et al.* 2017; Lacerda *et al.* 2019; Chen *et al.* 2019).

Table 4.1. Species of primary producers colonizing plastic debris surface in several studies performed in different environments, with specification of the methodology adopted.

Group and genus	Environment	Methodology	References
• Bacillariophyta: *Cyclotella, Mastogloia, Pleurosigma*	Marine: Sargasso Sea	Light microscope	(Carpenter and Smith 1972)
• Bacillariophyta • Dinoflagellata	Marine: North Pacific Gyre	Scanning Electron Microscopy (SEM)	(Carson *et al.* 2013)
• Bacillariophyta: *Chaetoceros, Navicula, Nitzschia, Sellaphora, Stauroneis* • Cyanobacteria: *Plectonema*-like, *Phormidium*, *Rivularia*	Marine: North Atlantic	Scanning Electron Microscopy (SEM); DNA sequencing	(Zettler *et al.* 2013)
• Bacillariophyta: *Amphora, Asterionella, Psammodictyon, Synedra* • Cyanobacteria: *Pseudophormidium Phormidium*, *Stanieria, Synechococcus*	Marine: North Sea (UK)	Scanning Electron Microscopy (SEM); Denaturing gradient gel electrophoresis (DGGE); Sequencing analysis	(Oberbeckmann *et al.* 2014)
• Bacillariophyta: *Achnanthes*, *Amphora*, *Cocconeis*, *Cymbella*, *Grammatophora*, *Haslea*, *Licmophora*, *Mastogloia*, *Microtabella, Minidiscus, Navicula*, *Nitzschia, Thalassionema*, *Thalassiosira* • Dinoflagellata: *Ceratium*	Marine: Australian-wide coastal and oceanic	Scanning Electron Microscopy (SEM)	(Reisser *et al.* 2014)
• Bacillariophyta: *Amphora, Asterionellopsis, Cylindrotheca, Diploneis, Gyrosigma, Licmophora, Navicula, Nitzschia, Pleurosigma, Striatella*	Marine: Mediterranean Sea	Light microscope	(Eich *et al.* 2015)

• Bacillariophyta • Cyanobacteria: *Leptolyngbya, Phormidium, Prochlorotrix, Rivularia* • Chlorophyta • Dinoflagellata: *Symbiodinium* • Filosa-Chlorarachnea • Ochrophyta • Stylonematophyceae • Pelagophyceae • Pinguiophyceae • Prasinophyceae	Marine: North Pacific Subtropical Gyre	Scanning Electron Microscopy (SEM); DNA sequencing	(Bryant *et al.* 2016)
• Bacillariophyta: *Achnanthes, Amphora, Ceratoneis, Cyclotella, Cocconeis, Diploneis, Entomoneis, Fragilariopsis, Licmophora, Mastogloia, Navicula, Pleurosigma, Striatella, Thalassionema, Thalassiosira, Thalassiothrix* • Cyanobacteria • Dinoflagellata: *Coolia, Dinophysis, Heterocapsa, Pentapharsodinium, Prorocentrum*	Marine: Mediterranean coastal waters of Greece, Italy and Spain (benthic and pelagic)	Scanning Electron Microscopy (SEM)	(Masó *et al.* 2016)
• Bacillariophyta • Cyanobacteria: *Phormidium, Synechococcus* • Chlorophyta	Marine: North Sea (UK)	Scanning Electron Microscopy (SEM); DNA sequencing	(Oberbeckmann *et al.* 2016)
• Bacillariophyta: *Mastogloia*, *Navicula* • Cyanobacteria: *Leptolyngbya*, *Phormidium*, *Rivularia* • Chlorophyta: *Eremosphaera* • Cryptophyta • Chrysophyta • Dinoflagellata: *Gymnodinium*	Marine: North Atlantic	DNA sequencing	(Debroas *et al.* 2017)

• Bacillariophyta • Cyanobacteria: *Calothrix, Leptolyngbya, [Oscillatoriales], Pleurocapsa, Scytonema, Synechococcus* • Dinoflagellata	Marine: Mediterranean Sea	Scanning Electron Microscopy (SEM); DNA sequencing	(Dussud *et al.* 2018a)
• Bacillariophyta • Cyanobacteria • Chlorophyta • Cryptophyta • Euglenophyta • Pyrrophyta	Freshwater: East Lake (China)	Light microscope	(Chen *et al.* 2019)
• Bacillariophyta: *Chaetoceros, Eucampia, Melosira, Navicula, Pseudogomphonema, Synedropsis, Thalassiosira* • Chrysophyta	Marine: Antarctic peninsula	Scanning Electron Microscopy (SEM)	(Lacerda *et al.* 2019)
• Bacillariophyta: *Amphora, Cocconeis, Diploneis, Fragilara, Mastogloia, Navicula, Nitzschia, Pseudo-nitzschia, Striatella* • Cyanobacteria • Chlorophyta • Dinoflagellata: *Alexandrium, Amphidinium, Prorocentrum*	Marine: Caribbean Sea	Scanning Electron Microscopy (SEM); DNA sequencing	(Dudek *et al.* 2020)

4.3. Effects of microalgae on microplastics

Interactions between microalgae and plastic debris can significantly alter the properties of these polymers, with consequences on their fate in aquatic environments (Yokota *et al.* 2017). The different processes that have been reported in literature can be summarized in two main categories: the alteration and/or biodegradation of the plastic polymer; and the alteration of the polymer density and sinking behavior (Fig. 4.1A).

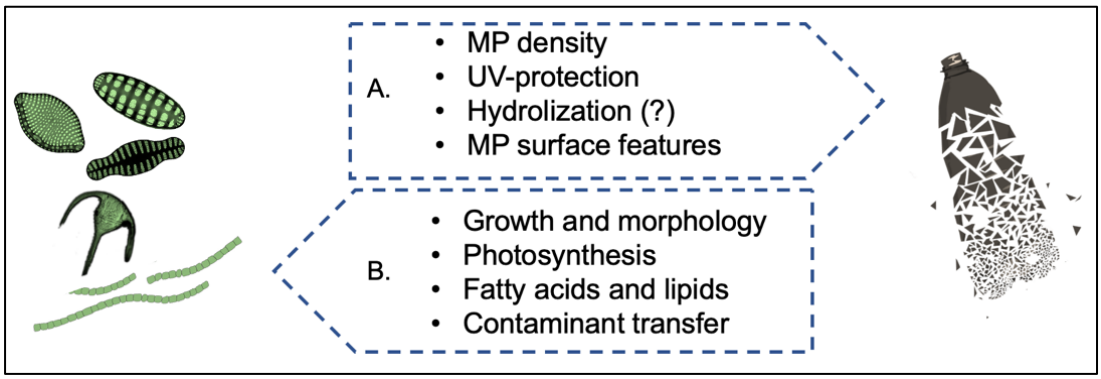

Figure 4.1. Effects that (A) microalgae may have on microplastic particles; (B) microplastics may have on microalgae.

Different studies have reported that the biofouling processes of microplastics may significantly alter their properties and, in particular, their adsorption capability seems to generally be enhanced (Kalčíková *et al.* 2020). For instance, Wang *et al.* (2020) reported that the physical and chemical surface properties of PE microplastics were changed with the development of biofilm, which resulted in different adsorption properties of microplastics for copper and tetracycline. Holmes *et al.* (2014) observed that metal adsorption was considerably greater in aged pellets than new polyethylene pellets. Furthermore, it has been observed that ions adsorbed from water onto biofilms were less intensively bound than to ion-exchange polymers and, thus, the ions are more easily desorbed (leached) from the biofilm (Kurniawan *et al.* 2012; Kalčíková *et al.* 2020). Therefore, the adhesion of microalgae on microplastic surfaces is of critical importance in the adsorption and desorption of pollutants from microplastics.

The capability of micro-organisms to biodegrade plastic (using plastic as a carbon source) has been reported for numerous bacterial strains, although most of the studies performed were based on the selection and testing of single strains in laboratory, which is far from environmental conditions (Jacquin *et al.* 2019). Among primary producers, the filamentous cyanobacteria of the genus *Phormidium* are known to degrade hydrocarbons (Oberbeckmann *et al.* 2016). The occurrence of species belonging to this genus on plastic surfaces has been widely documented (see section 4.2.3). This raises an interesting possibility that *Phormidium* in the plastisphere may be actively hydrolyzing the plastic (Yokota *et al.* 2017). However, as cyanobacteria are photosynthetic organisms, the advantage of higher exposure to sunlight on floating plastic pieces may be the actual explanation for their enrichment on plastic debris (Roager and Sonnenschein 2019). Even if microalgae may not have a direct effect on the degradation of plastic, their presence can be important in determining the presence of other hydrocarbon-degrading bacteria, and it has been argued that diatoms may function as an important habitat for such micro-organisms (Dudek *et al.* 2020). At the same time, the biofouling of extensive surfaces may have an opposite effect, protecting the plastic from UV radiation and thus retarding photo-degradation processes (Andrady 2011).

Another important role played by microalgae concerning plastic materials is linked to their capacity to alter the density of the colonized polymer which, consequently, affects the vertical fluxes of plastics. Chen *et al.* (2019) performed an experiment in a freshwater system and highlighted the changes in the buoyancy of polypropylene sheets (squares with a side length of 5 and 10 mm) following the development of microalgae biofilms. It has been reported that microplastics could potentially be incorporated into hetero-aggregates, composed of algae and small plastic materials, but this process remains little studied and, thus, is largely unpredictable. Several studies on microalgae have demonstrated significant interactions and the rapid formation of hetero-aggregates when microalgae were exposed to 400-

1000 μm diameter polypropylene and high-density polyethylene microplastics at a concentration of 1 g L^{-1} (Lagarde *et al.* 2016); 2 mm polystyrene at 3.96 μg L^{-1} (Long *et al.* 2017); and microbeads from cosmetic products at around 4000 microbeads L^{-1} (Möhlenkamp *et al.* 2018).

Long *et al.* (2015) performed a lab experiment to study this interaction using three types of aggregates formed from two different algae species (the diatom *Chaetoceros neogracile*, the cryptophyte *Rhodomonas salina,* and a mix of them) and 2 μm polystyrene microbeads. The experiment highlighted that all three types of aggregates concentrated the microbeads. Once incorporated, the microbeads impacted aggregate sinking rates reaching several hundred meters per day, a high value compared to the sinking rate of free beads (less than 4 mm day^{-1}). These results support the idea that phytoplankton aggregates act as a potential microplastic sink. However, the extent to which different species form such aggregates was not the same, and dissimilarities were also reported for different plastic polymers in other studies (Lagarde *et al.* 2016). Many factors play a role in the hetero-aggregation process. For instance, major aggregate permeability increases the encounter chance between small particles and aggregates, as the small particles are not moved away from the aggregate but can go through aggregate macropores and be caught. Moreover, when an aggregate breaks, new surfaces, and macropores become available for microbeads to adhere to, where a succession of fragmentation and coagulation allows microbeads to be incorporated not only at the aggregate surface or in macropores but also into the entire aggregate (Long *et al.* 2015).

Resuspension processes can also occur, where several factors of the aggregates, such as size, density, porosity, shape, and stickiness play roles in determining the resuspension behavior of aggregates following settling (Möhlenkamp *et al.* 2018). Moreover, hetero-aggregates can also include inorganic material which both substantially enhance the settling velocity and determine lesser mobility once deposited in bottom layers (Möhlenkamp *et al.* 2018). The studies reported have provided interesting insight about the

hetero-aggregation and the subsequent effects of microalgae on plastic debris, but they were all performed at a lab-scale and environmental conditions may widely differ from those tested. For instance, monospecific aggregates are unlikely to exist in nature and turbulence can quickly breaks up the hetero-aggregates (Lagarde *et al.* 2016).

4.4. Effects of microplastics on microalgae

Microalgae may experience toxic effects as inhabitants of pelagic areas contaminated with plastic debris. Furthermore, as primary producers essential to the functioning of aquatic ecosystems, small disruptions of microalgae populations may contribute to serious impacts on food webs (Fig. 4.1B). However, the effects and toxicity of microplastics have seldom been determined in microalgae and the current experimental results offer no consensus (Table 4.2).

One of the endpoints frequently measured is the effect on microalgal growth, on which microplastics seem to have a negligible impact (Lagarde *et al.* 2016; Sjollema *et al.* 2016; Long *et al.* 2017), even if some studies have reported effects after exposure to high concentrations of microplastics or small-sized particles (Sjollema *et al.* 2016; Zhang *et al.* 2017; Gambardella *et al.* 2018; Zhao *et al.* 2019; Venâncio *et al.* 2019). These findings point out two important aspects that should be taken into account: the relevance of the concentration and the features of the plastic particles tested. The characteristics of the microplastic used can play a fundamental role in determining the toxicity exerted. Besides the wide variety of polymers and additives that can be tested, other features such as the size and the charge of the plastic particles may be of paramount importance in their effect on organisms. The relationship among particle dimension and toxicity has been widely observed and it is generally agreed that smaller dimensions lead to higher toxicity in microalgae (Garrido *et al.* 2019; Chae *et al.* 2019).

Table 4.2. Toxic effects of microplastics exposure to microalgae. Only studies investigating microplastics (~1-5000 μm) were reported.

Study	Polymer	Polymer features	Size (μm)	Concentration (mg L^{-1})	Test species	Toxic endpoint	Test duration (h)	Results
(Lagarde *et al.* 2016)	HDPE		400-1000	400	*Chlamydomas reinhardtii*	Growth; Chloroplastic/stress response/apoptosis genes	1872	• Non-significant decrease of growth • Non-significant change in expression of chloroplastic genes • No effect on stress response/apoptosis genes
	PP							• Growth decrease (18%) • Non-significant change in expression of chloroplastic genes • No effect on stress response/apoptosis genes
(Sjollema *et al.* 2016)	PS	Uncharged	0.05	25; 250	*Dunaliella tertiolecta*	Growth; Photosynthesis	72	• No effect on photosynthesis • Growth inhibition at 250 mg L^{-1} (57% for 0.05 μm; 13% for 0.5 μm)
			0.5					
			6					
		Negatively charged	0.5	25; 250	*Dunaliella tertiolecta*	Growth; Photosynthesis	72	• No effect on photosynthesis • Growth inhibition (13%) at 250 mg L^{-1}
			0.5		*Thalassiosira pseudonana*	Photosynthesis		No effect
			0.5		*Chlorella vulgaris*	Photosynthesis		No effect
(Long *et al.* 2017)	PS		2	$3.96 \cdot 10^{-3}$	*Tisochrysis lutea; Heterocapsa triquetra; Chaetoceros neogracile*	Growth; Chlorophyll fluorescence	840	No effect

(Yokota *et al.* 2017)	Unknown (microbeads from body wash product)		20-350 (mean 60)	66.7	*Microcystis aeruginosa; Dolichospermum flos-aquae.*	Cell counts; Biomass; Growth; Cell morphology	504	• Increased algal particle counts • No effect on algal biomass and growth • Smaller algal particle size
(Zhang *et al.* 2017)	PVC		1	1; 5; 10; 50	*Skeletonema costatum*	Growth	96	Growth inhibition (40%)
				5; 50		Photosynthesis		Decrease of chlorophyll content and photosynthetic efficiency
			1000	50; 500; 1000; 2000		Growth		No effect
(Mao *et al.* 2018)	PS		1	10; 50; 100	*Chlorella pyrenoidosa*	Growth; Photosynthesis; Cell morphology	720	• Growth inhibition until 528h • Photosynthesis inhibition until day 144-192h • Unclear pyrenoid, damaged membrane, distorted and unclear thylakoid, cell wall thickening until 312h
(Prata *et al.* 2018)	PE		1-5	0.75; 1.5; 3; 6; 12; 24; 48	*Tetraselmis chuii*	Growth	96	No effect
						Chlorophyll concentration		Significant reduction of chlorophyll at 0.9 (46%) and 2.1 (37%) mg L^{-1}
(Chae *et al.* 2019)	PE		180-212 (mean 204)	50; 100; 150; 200; 250; 300; 350	*Dunaliella salina*	Growth; Photosynthesis; Cell morphology	144	• Significant increase in growth (125-140%) and photosynthetic activity • Few effects on cell morphology
(Davarpanah and Guilhermino 2019)	Unknown		1–5	0.3; 0.9; 4	*Tetraselmis chuii*	Growth	96	Non-significant decrease of growth
(Fu *et al.* 2019)	PVC	Virgin	~97-197	10; 100; 1000	*Chlorella vulgaris*	Growth; Biomass productivity	240	Growth and biomass inhibition, esp. at 10 mg L^{-1}

		Aged				Growth; Biomass productivity; Antioxidative enzymes (SOD)		• Growth and biomass inhibition, esp. at 10 mg L^{-1} • Stronger effect on biomass of aged than virgin microplastics • No effect on SOD
(Garrido *et al.* 2019)	PE		1.4-42 (mean: 2-6)	0.5; 1; 10; 25	*Isochrysis galbana*	Growth	72	No effect
(Seoane *et al.* 2019)	PS	Amino-modified	2	2.5	*Chaetoceros neogracile*	Growth; Photosynthesis; Cell morphology; Esterase activity; Reactive Oxygen Species (ROS); Cytoplasmic membrane potential; Neutral lipid content	72	• Slight but significant decrease in growth rate • No effect on cell morphology or photosynthesis • Decrease in esterase activity at 24-48h • No significant effects on ROS • No significant alterations in cytoplasmic membrane potential; • Significant decrease in the cellular neutral lipid content
(Zhao *et al.* 2019)	PVC		1	5; 25; 50; 100	*Karenia mikimotoi*	Growth; Photosynthesis	96	• Significant effect on growth: inhibition increased firstly and then decreased with the exposure time, and had a significant negative response with the increasing dose • Significant inhibition of photosynthetic activity

Very small particles may be more likely to inhibit the growth of microalgae through adsorption on the surface of the algal cell; for instance, inducing shading, blocking algal pores or gas exchanges, and embedding in microalgae cells (Zhang *et al.* 2017; Fu *et al.* 2019). Additionally, the surface ionic charge of microplastics seems to affect their toxicity. Weathering and degradation processes such as photo-oxidation, which can lead to carbonyl group formation (Bellingeri *et al.* 2019), can change the ionic charge of the microplastic surface. Furthermore, it has been shown that detrimental effects on microalgae growth were found only when exposing micro-organisms to positively charged particles, while, to the contrary, no impacts were reported in the same experimental conditions for uncharged particles (e.g., Feng *et al.* 2019). The effects may differ not only due to the types of polymers but also from the various responses of different microalgal species. It is likely that interactions between microplastics and microalgae may vary with cell characteristics, such as size and shape, as algal cell walls act as barriers to particle penetration and different cell wall characteristics may consequently influence particle sorption (Chae *et al.* 2019; Fu *et al.* 2019).

Toxic effects on microalgae may not only be physical but also related to interactions with the chemicals associated with plastics. Indeed, microplastics can potentially transfer contaminants adsorbed onto their surface, residual (unpolymerized) monomers deriving from incomplete polymerization reactions, or additives, which may represent a high percentage of the final plastic materials (e.g., Hermabessiere *et al.* 2017; Maity and Pramanick 2020). Most additives are not covalently bound to the plastic polymer and, thus, they can migrate to the material surface, potentially being released into the environment. Capolupo *et al.* (2020) investigated the effects of plastic leachates on the microalgae *Raphidocelis subcapitata* (freshwater) and *Skeletonema costatum* (marine), reporting that quite all of the leachates (i.e*.,* benzothiazole, phthalide, acetophenone, cobalt, zinc, lead) inhibited algal growth. However, it has been hypothesized that the leaching of some additives, which are usually released at low

concentration, may also stimulate the growth of microalgae due to a "hormesis" phenomenon (Chae *et al.* 2019; Song *et al.* 2020).

Besides the effects on microalgae growth, studies have found that microplastics seem to affect algal photosynthesis, as both chlorophyll content (Zhang *et al.* 2017; Prata *et al.* 2018; Fu *et al.* 2019) and photosynthetic efficiency (Zhang *et al.* 2017; Mao *et al.* 2018) decreased under microplastic exposure. Moreover, microplastics may induce morphological changes in microalgae (Mao *et al.* 2018), modulate the energy metabolism by decreasing the oil bodies that could serve as energy sources (Seoane *et al.* 2019), and may be ingested and internalized by mixotrophic algal species through phagocytosis processes (Long *et al.* 2017). However, many of the effects experienced by microalgae appear to be temporary, with an initial period of vulnerability followed by adaptative responses leading to recovery (Prata *et al.* 2019 and references therein). Different mechanisms of detoxification have been hypothesized to influence the recovery reported in microalgae activities, such as membrane thickening, reduction of surface exposure through homo-aggregation, and hetero-aggregation (Mao *et al.* 2018; Prata *et al.* 2019). With respect to the latter point, it has been reported that the bioavailability of plastic particles changed during the different experiments, due to adsorption to experimental containers, or embedding in organic aggregates. This underlines the need to quantify the bioavailability and distribution of microplastics in the experimental systems, in order to obtain accurate values of the actual microplastic concentration to which the microalgae are truly exposed (Long *et al.* 2017).

A recent laboratory study has suggested that microplastics may also affect microalgae lipid and fatty acid composition, which are important dietary components for primary consumers as a source of energy and essential nutrients (Guschina *et al.* 2020). Indeed, the exposure of *Chlorella sorokiniana* to polystyrene microplastics (<70 μm, 60 mg/L) resulted in the alteration of essential fatty acids-major structural compounds in algal cell membranes-and chloroplast galactolipids-which have important functions in

photosynthesis. These findings raise questions about the impact of microplastics on algal productivity and the transfer of important lipid compounds through food webs, thus requiring further investigation.

It is important to stress that the ecological relevance of laboratory observations is likely to be low, as they are far from reflecting the complexity of the aquatic environment, in which we must consider non-equilibrium conditions, large volumes, lower algal cell concentrations, the co-existence of different microalgal species, different polymers, sizes, doses, and so on (Sjollema *et al.* 2016; Long *et al.* 2017). Moreover, in nature, mixtures of microplastic with different contaminants can also occur (e.g., Davarpanah and Guilhermino 2019; Johansen *et al.* 2019; Zhu *et al.* 2019); however, studies regarding this topic are still scarce.

4.5. Ecosystem implications

Many of the effects obtained through lab-scale experiments, as discussed in the previous sections, may potentially have consequences on the ecosystem functioning. However, it is difficult to predict how specific results obtained through laboratory experiments can manifest in real aquatic systems. As microalgae are keystone organisms, impacts and alterations of such communities can have substantial consequences for the whole aquatic ecosystem (Wright *et al.* 2013). Increasing levels of plastic pollution may not only exert pressure at the individual or population level but, additionally, may cause cascading secondary effects on the functioning and services of other communities and, ultimately, on the ecosystem as a whole (Leoni *et al.* 2018; Kong and Koelmans 2019).

One of the key mechanisms that has been claimed to be crucial is the perturbation effect that the interaction between plastics and microalgae may have on the aquatic food web. There are substantial issues regarding whether microplastics affect the quality and quantity of algal production and

whether this may propagate through food webs. Microalgae mixed with plastics may exhibit morphological changes that affect their detectability, palatability, and ease of handling by grazers (Yokota *et al.* 2017; Lacerda *et al.* 2019). Due to biofouling, microplastics may become more available for interaction with animals within the water body. Although many species are able to discriminate between inert and edible particles, the developed biofilm "camouflages" plastic particles, thus allowing them to potentially become attractive food items to grazers, such as zooplanktonic organisms (Vroom *et al.* 2017). Consequently, biofilms may alter the interactions between microplastics and primary consumers, increasing ingestion rates of 'flavored' microplastics. Ingestion of microplastics aggregated with microalgae leads to the dilution of food due to the co-ingestion of inert plastic together with regular food or prey (Kong and Koelmans 2019). Ecological implications are expected to be triggered especially by the responses of zooplanktonic species, which represent the natural predators of algae (Kong and Koelmans 2019). Kong and Koelmans (2019) applied a theoretical model to investigate the negative impacts of microplastics on food webs. The research reported that reduced assimilation rates due to the ingestion of microplastics resulted in a lower population density of the corresponding organisms. Considering zooplankton, decreased population density lessens the grazing pressure on phytoplankton, which leads to increased population sizes of diatoms and green algae. In response to the loss of zooplankton, planktivorous fish feeding on zooplankton become largely limited, thereby restricting the piscivorous fish population. Consequently, benthivores fish start to dominate the fish community, reducing the abundance of zoobenthos through predation. This results in stronger perturbation on the sediment and increased water turbidity due to resuspension (Kong and Koelmans 2019). Despite the model being applied to shallow lakes and using high concentrations of microplastics (40 and 4000 particles L^{-1}), the results provided valuable insights about how the impact of microplastics, whose

concentration is expected to increase in the near future, can propagate through whole aquatic systems.

The decreased quality of algae ingested by zooplanktonic organisms is not the result of the dilution of food with inert particles, but also by the direct effects of microplastics on algal quality. Recent evidence has suggested that microplastics may also affect the lipid and fatty acid composition of microalgae (see Section 4.4), which are critical regulators of the survival, reproduction, and population growth in invertebrates and fish. As polyunsaturated fatty acids are highly retained during transfer through aquatic food webs, any factors affecting their quantity and quality in phytoplankton may affect the growth, reproductive capacity, and fitness of aquatic invertebrates and fish (Leoni *et al.* 2014a; Guschina *et al.* 2020).

Furthermore, microplastics may exert potential effects on the quantity of algal production. As plastic debris provides an abundant growth matrix and better floating conditions for microalgae, while exerting adverse effects (i.e., toxicity or "food dilution") on phytoplankton consumers (i.e., zooplankton), it has been argued that plastic pollution can promote the multiplication of microalgae in large quantities, with consequent detrimental effects for aquatic ecosystems already disturbed by eutrophication processes (Zhang *et al.* 2020); for instance, it has been reported that plastic surface represents a net autotrophic "hot spot" in the oligotrophic ocean, with high density of chlorophyll a and high oxygen production (Bryant *et al.* 2016). Thus, plastic pollution might affect ecosystem productivity, with substantial consequences for those ecosystems that are characterized by oligotrophic conditions. To date, studies that have attempted to calculate the increase of primary productivity due to the presence of plastic debris are still missing and, so, quantitative data are not available to determine whether the occurrence of plastic is relevant to the perturbation of primary productivity. However, an opposite mechanism may be observed: microplastics may exert toxic effects on microalgae (see Section 4.4), causing a decrease in phytoplankton species, and eventually leading to losses in ecosystem productivity. This can induce

cascading impacts on biodiversity and ecosystem services (e.g., fish provisioning), which are also influenced by the productivity at lower levels of the trophic chain and cultural services (e.g., tourism and recreation). Evidence supporting one mechanism over the other is still missing, and, to the best of our knowledge, field and experimental approaches regarding this topic are lacking. There is no consensus even about the primary effects that microplastics may have on organisms and, so, scaling up results to the ecosystem level is complex and it is only possible to postulate some hypotheses about the possible mechanisms.

Moreover, indirect effects can also occur. Microplastic-associated bacteria have the ability to alter the nutrient cycling processes (e.g., increasing the denitrification capability, or transforming phosphorus through microbe-mediated processes). Nutrients can be assimilated by plastic materials and then released into the surrounding water, altering the nutrient concentration and thus affecting primary producers (Chen *et al.* 2020). Therefore, microplastics seem to be able to interact with several biotic and abiotic factors, creating new "micro-ecosystems" in which both nutrients and primary producers can be concentrated and not dispersed into the pelagic environment.

As previously discussed, the colonization of microplastics by microalgae adds additional weight to the plastic particles facilitating the sedimentation and burial of these pollutants. Thus, microplastics can influence energy fluxes not only in the pelagic compartments but also in benthonic environments, where they may negatively affect the general fitness of benthonic organisms (Bellasi *et al.* 2020)

Another aspect that should be considered is the threat that microplastics may pose to aquatic biodiversity. Plastic debris offers a new colonization substrate for organisms and a durable dispersal medium for several organisms, raising their dispersion capacity. As plastics can be transported by winds and currents over long distances, the dispersion of debris containing epiplastic organisms may cause the transport of alien species and

changes in aquatic biogeographical patterns (Kettner *et al.* 2019; Dudek *et al.* 2020). Barnes (2002) has estimated that human litter, the majority being plastic, more than doubles the rafting opportunities for biota, which could endanger aquatic biodiversity. Considering microalgae, concerns have been stated regarding harmful bloom-forming organisms, such as some species of dinoflagellates or cyanobacteria (Masó *et al.* 2016; Amaral-Zettler *et al.* 2020). The increasing colonization opportunities provided by the presence of microplastics in aquatic ecosystems can allow the colonization of microalgae able to produce toxins (synthesized for defense from predation), which can lead to harmful algal blooms (HABs), with consequent negative impacts on aquatic ecosystem services and, thus, representing a pressing environmental and human health problem. For instance, the presence of the cyanobacterium of the genus *Phormidium* has been widely reported on microplastic surfaces, which is a cosmopolitan genus found in diverse substrates and habitats in both oligotrophic and eutrophic conditions. Under favorable hydrological and environmental conditions, *Phormidium* forms cohesive mats that can cover large areas and species belonging to this genus can produce a range of cyanotoxins, such as the neuromuscular-blocking anatoxin-a (ATX) and homoanatoxin-a (HTX) and their structural derivatives, causing HABs (McAllister *et al.* 2016).

4.6. Conclusions and recommendations for future research

Different studies have reported that plastics can and do interact with microalgae, which represent the base of the aquatic food web and, thus, play a key role in marine and freshwater ecosystems. However, studies investigating the relationships between plastic debris and microalgae are still scarce, especially in freshwater systems. Therefore, many aspects (as

described in previous sections) require further research and fundamental gaps exist in the current state of knowledge regarding this subject.

The available literature has suggested that the “epiplastic” community of microalgae consistently differs from the surrounding aquatic communities; however, it is still not clear whether such differences in colonization are linked to the compositions of the surfaces or more to the availability of a “hard” substrate on which organisms can attach and grow; thus, further studies are required to address this question. Furthermore, we need to better understand to what extent the properties of different plastic materials and different environmental factors-which can also act synergistically- may affect the growth of microalgae on plastic debris. In particular, field-studies on microplastic colonization by microalgae are especially missing for freshwater environments. Therefore, future studies, taking as an indication the information gathered from previous studies performed in marine systems, should address this topic. The colonization and hetero-aggregation between microplastics and microalgae lead to changes in particle buoyance, thus resulting in settling and influencing the fate and bioavailability of plastics in surface waters. At the same time, microplastics can harm microalgae, by inhibiting their growth, reducing chlorophyll and photosynthesis, and causing changes in morphology. As reported, previous research regarding the toxic effects that plastic can exert on microalgae is controversial and, thus, it is not clear whether these effects are of concern for microalgae. The difficulty in obtaining a clear answer is linked to the several confounding factors (e.g., microplastic dose, features, and size) in laboratory experiments, resulting in conflicting results on plastic toxicity. Thus, future studies need to clarify the differences in effects of microplastics on primary producers due to microalgae species and the properties of plastic materials (e.g., polymer type, chemical composition, weathering condition, surface charge, and size). Most of these studies have tested high concentrations of plastic particles, thus not accurately reproducing the current environmental concentrations reported in studies investigating the occurrence of plastic in marine and freshwater

systems. However, the concentrations of these pollutants are expected to increase in the near future and, so, testing higher concentrations can provide important insight into the future situation. Furthermore, the transport via the rafting of the microalgae themselves is also of concern, as this community is distinct from that of the surrounding water, with potential impacts such as the introduction of harmful or non-native algae into new environments, potentially impairing local aquatic biodiversity. The effects that microplastics can have on the biodiversity of microalgae, with subsequent cascading effects on entire aquatic ecosystems, have not yet been fully considered; therefore, future studies should attempt to include and evaluate this theme. As highlighted in this review, the largest gap in the current knowledge is our understanding of the ecosystem implications of the microplastic-microalgae relationships. Although some attempts have been made to address this issue, the effects that plastic debris can have on aquatic ecosystems – which are already affected by other anthropogenic impacts such as climate change, eutrophication, and food web alteration – remain unclear. When experimental results indicate reciprocal impacts between plastics and microalgae, it is difficult to predict how these impacts manifest themselves at the ecosystem level. For instance, the effects that microplastics have on algal photosynthesis, the hormesis phenomenon linked to the leachates of additives, or the alteration of microalgae lipid and fatty acid composition could trigger subsequent effects throughout the whole aquatic ecosystem. Further investigations are urgently needed, as this theme is of pivotal importance for aquatic environments and their ecosystem services. Future studies should take into account the results reported from the different laboratory studies, in order to prioritize research questions and test the same hypotheses in real systems. In particular, the effect that microplastic-microalgae interactions can have on the primary productivity of aquatic ecosystems, which may be negatively or positively affected, should be studied; in particular, quantitative estimations are needed. Modeling approaches can be useful to test the broad-scale consequences of different

scenarios of plastic pollution. However, it is important to “validate” these models and, so, realistic in-field observations are needed to improve their performance. However, the results of in-field studies may be difficult to interpret, due to the many possible confounding factors. Thus, a useful tool that can be used for testing the consequences of microplastic-microalgae interactions for aquatic ecosystems can be represented by mesocosms, including artificial ponds or enclosures in natural environments. These are physical models of natural systems which allow for the controlling of experimental conditions for research purposes, while also providing some level of realism.

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Chapter 5

Microalgae colonization of different microplastic polymers in experimental mesocosms across an environmental gradient

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Global Change Biology (2021)
doi: 10.1111/gcb.15989

Abstract

A variety of organisms can colonize microplastic surfaces through biofouling processes. Heterotrophic bacteria tend to be the focus of plastisphere research; however, the presence of epiplastic microalgae within the biofilm has been repeatedly documented. Despite the relevance of biofouling in determining the fate and effects of microplastics in aquatic systems, data about this process are still scarce, especially for freshwater ecosystems. Here, our goal was to evaluate the biomass development and species composition of biofilms on different plastic polymers and to investigate whether plastic substrates exert a strong enough selection to drive species sorting, overcoming other niche-defining factors. We added microplastic pellets of high-density polyethylene (HDPE), polyethylene terephthalate (PET) and a mix of the two polymers in 15 lentic mesocosms in 5 different locations of the Iberian Peninsula and after one month we evaluated species composition and biomass of microalgae developed on plastic surfaces. Our results, based on 45 samples, showed that colonization of plastic surfaces occurred in a range of lentic ecosystems covering a wide geographical gradient and different environmental conditions (e.g., nutrient concentration, conductivity, macrophyte coverage). We highlighted that total biomass differed based on the polymer considered, with higher biomass developed on PET substrate compared to HDPE. Microplastics supported the growth of a rich and diversified community of microalgae (242 species), with some cosmopolite species. However, we did not observe species-specificity in the colonization of the different plastic polymers. Local species pool and nutrient concentration rather than polymeric composition seemed to be the determinant factor defying the community diversity. Regardless of specific environmental conditions, we showed that many species could coexist on the surface of relatively small plastic items, highlighting how microplastics may have considerable carrying capacity, with possible consequences on the wider ecological context.

5.1. Introduction

While the benefits of plastics are undeniable, their widespread use as well as their inherent resistance to (bio)degradation, ultimately leads to their accumulation in the environment (Thompson *et al.* 2009). In 2019, global plastics production almost reached 370 million tons and approximately 50% of plastic objects manufactured are intended for single use (Geyer *et al.* 2017; PlasticsEurope 2020). As the production and use of plastic materials have intensified, the quantity of waste generated has also increased (Kedzierski *et al.* 2020). Just a small fraction of plastic waste is recycled (according to PlasticsEurope (2020) the percentage worldwide was equal to 32.5% in 2018), while the remaining is incinerated or accumulates in landfills, eventually ending up in natural environments, including marine and freshwater aquatic systems (Geyer *et al.* 2017). Previous studies estimated that ~8 million metric tons of plastic waste enter the ocean annually (Jambeck *et al.* 2015). Once in the aquatic environment, plastic debris undergoes mechanical, chemical, and biological modifications, which lead to the weathering and fragmentation of macroplastics into smaller and more abundant particles, forming the so-called 'microplastics' (MPs, <5 mm) (Julienne *et al.* 2019). Besides these degradation products (i.e., secondary microplastics), MPs can also be specifically manufactured within the millimetric size (i.e., primary MPs), like, for instance, those used as resin pellets or as an ingredient of personal care products (Horton *et al.* 2017). Beyond their effects as waste or pollutants, very little is known about the role of these particles when eventually colonized and incorporated as substrate by aquatic organisms.

Studies have shown that a wide variety of organisms can colonize microplastic surfaces through biofouling processes. Indeed, floating plastics represent a new habitat for rafting organisms to the point that the term "plastisphere" was coined to define the diverse community of heterotrophs, autotrophs, predators, and symbionts growing on the surface of plastic debris (Zettler *et al.* 2013). Even if heterotrophic bacteria tend to be the focus

of plastisphere research, the presence of epiplastic microalgae (i.e., algae growing on plastic surfaces) within the biofilm has been repeatedly documented (Carpenter and Smith 1972; Yokota *et al.* 2017). While it has been shown that the communities differ between biofilms and the ambient environment, there is no consensus on whether biofilms differ between substrates (Rogers *et al.* 2020). Experimental studies have reported differences in the communities found on plastic surfaces compared to other inert substrates, like for instance glass, suggesting substrate-driven selection (e.g., Oberbeckmann *et al.* 2014; Ogonowski *et al.* 2018). Additionally, several lines of evidence indicate that the colonization process can vary depending on the plastic polymer used (Zettler *et al.* 2013; Lagarde *et al.* 2016; Vosshage *et al.* 2018). However, different research reported opposite results and it has been hypothesized that many taxa may use plastic opportunistically as a niche but can also attach to other substrates (Oberbeckmann *et al.* 2016; Smith *et al.* 2021).

The adhesion of microalgae on microplastics increases the density of the colonized polymer and, consequently, affects the vertical fluxes of plastics (Long *et al.* 2015). Therefore, biofouling processes are of critical importance for the fate of microplastics in aquatic systems, influencing their distribution along the water column and determining whether a particle occupies a pelagic versus benthic transport route. Moreover, it is reported that biofouling processes can alter the polymer features, influencing their capability to adsorb/desorb pollutants from the environments, with consequences for the toxic effects exerted by MPs (Wang *et al.* 2020; Kalčíková *et al.* 2020). Additionally, the interaction between microplastics and microalgae may have effects at the ecosystem level, as it is argued that MPs may affect algal productivity. Indeed, plastic debris provides a growth matrix and better floating conditions for microalgae and therefore it has been hypothesized that plastic pollution can promote the development of microalgae, with consequent detrimental effects for aquatic ecosystems already disturbed by eutrophication processes (Zhang *et al.* 2020).

Local conditions and environmental factors (e.g., temperature, nutrient concentration, salinity) influence the community composition of biofilm, and thus these variables play an important role in determining the development and diversity of MP-colonizing communities (Oberbeckmann *et al.* 2014; Amaral-Zettler *et al.* 2015). Indeed, the environmental context influences the development of periphyton, both with regard to their architectural structure and their taxonomic diversity and function (Villeneuve *et al.* 2010). However, there is still controversy as to whether substrate-specific properties or environmental factors prevail in shaping microalgal assemblages on plastic debris. Indeed, it is not clear if the plastic surface 'environment' may exert a strong enough selection to drive species sorting, overcoming other niche-defining factors driven by seasonal and spatial patterns (Nava and Leoni 2021).

Despite the importance of biofouling for microplastics in aquatic systems, data about this process are still scarce, especially for freshwater ecosystems, and mixed in terms of results due to different confounding factors that arise from in-field experiments. To better understand the process of colonization by microalgae of different microplastic polymers and to identify possible shaping factors, we performed an experiment using a multi-site mesocosm experimental system distributed across an environmental gradient in five different locations of the Iberian Peninsula (Spain, Portugal). These systems are characterized by a naturalized microalgae community with over 4 years of colonization. In each site, we deployed microplastic pellets of high-density polyethylene (a floating plastic), polyethylene terephthalate (a polymer denser than water), or a mix of the two polymers. Following one month of colonization, we determined species composition and quantified biomass of microalgae developed on plastic pellets. The resulting dataset allowed us to: i) evaluate whether different plastic polymers constituted suitable substrates for the development of microalgal communities; ii) quantify the microalgae biomass developed on microplastics with different density and polymeric composition and determine whether biomass vary significantly among

substrates; iii) identify which algal species were able to colonize different plastic polymers; iv) determine whether substrate-driven or environmental factors prevail in shaping the species diversity of epiplastic community.

5.2. Methods

5.2.1. Study area

The Iberian Pond Network (IPN, https://www.aquacosm.eu/mesocosm/iberian-pond-network-ipn/; Pereira *et al.* 2021) is a multi-region mesocosm system built in 2014 to investigate ecological responses to climate change and anthropogenic impacts across bioclimatic regions (Fig. 5.1a). This infrastructure includes a range of environments from semi-arid conditions to mountain tops. A total of 192 mesocosms, each 0.70 m deep and 1.85 m in diameter, are deployed across 6 regions on the Iberian Peninsula. Locations include semi-arid, Mediterranean, temperate, and alpine environments (Pereira *et al.* 2021). At each location, 32 mesocosms with a volume of 1000 L each are placed at ~3-5 m distance from one another (Fig. 5.1b). The mesocosms were initiated by adding 100 kg of locally collected topsoil, then filled with local water. All mesocosms have been left untouched until 2019 to allow the establishment of aquatic food webs. For the present study, we selected five sites (i.e., Murcia, 'MR'; Toledo, 'TL'; Evora, 'EV'; Porto, 'PT'; Jaca, 'JC') and three mesocosms for each site; furthermore, three enclosures were deployed in each mesocosm (Fig. 5.1c).

Murcia has a Mediterranean climate with semi-arid features. The average annual temperature ranges from 15.0 ºC to 19.0 ºC and the annual rainfall is less than 350 mm (Alonso-Sarría *et al.* 2016). The climate in Toledo is continental semiarid with an annual rainfall of 487 mm and an average annual temperature of 14.0°C (Hernández *et al.* 2007). Evora has a typical Mediterranean climate, with hot and dry summers. More than 80% of annual

precipitation occurs between October and April. The long-term mean annual temperature is 15.0-16.0°C, with 669 mm of precipitation on average (Pereira *et al.* 2007). Due to the maritime influence, Porto has mild temperatures with an annual average of 14.4°C. No cold season can be found in Porto, with January being the coldest month, with an average temperature of 9.3°C. The mean summer temperature is about 18.1°C, although very high temperatures can be reached between May and September. The most significant feature of the Porto climate is the annual rainfall level (1236 mm) which has an irregular distribution throughout the year, mainly concentrated in winter and spring (Abreu *et al.* 2003). In Jaca, climate conditions are typically alpine, with average annual temperatures that range between -0.7°C and 5.0°C and high-mean annual precipitation values well distributed throughout the year (Garcia-Pausas *et al.* 2007).

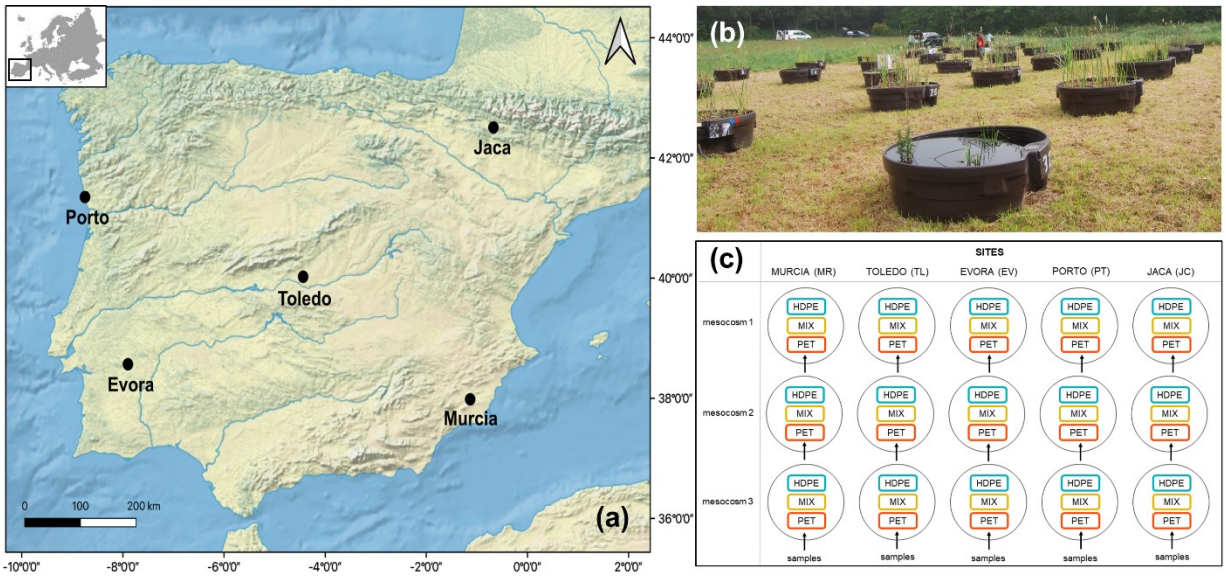

Figure 5.1. a) Study area with location of the sites in which mesocosms are deployed; b) Example picture of freshwater mesocosms (1000 L tanks); c) Schematic representation of the 5 sites selected for our experiment, the 15 mesocosms (with three enclosures each), and the resulting 45 samples (of which 15 with HDPE, 15 with MIX, and 15 with PET).

5.2.2. Field experiment

The experiment was carried out in spring and summer 2019. We employed virgin plastic pellets, provided by Serioplast Global Services. We used high-density polyethylene (HDPE) and polyethylene terephthalate (PET), which are among the polymers more commonly found in freshwater systems (Li *et al.* 2020). Before use, plastic polymers were characterized through micro-Raman spectroscopy (Raman Horiba Jobin Yvon LabRAM HR Evolution; Nava *et al.* 2021). The Raman system is equipped with an Nd laser (532 nm) and a cooled charge-coupled device (CCD, 1024 x 256 px, -60°C) detector. A grating with 600 grooves/µm was used. Raman spectra were recorded with a 50× objective (Olympus BXFM) with an integration time of 30 seconds. The spectral range was set to 223–3177 cm^{-1} and the spectral resolution was equal to 1.47 counts/points (Fig. S5.1).

To prevent contamination of the ongoing experiments in the mesocosms, smaller enclosures were prepared. These consisted of containers with two openings of 15x5 cm (75 cm^2 area), covered by a net with a mesh size of 100 µm. Three experimental treatments were implemented in each mesocosm: 6 g of high-density polyethylene (HDPE), 6 g of polyethylene terephthalate (PET) or a mixture of the two polymers (MIX, 3 g each). Overall, we evaluated 5 locations, 15 mesocosms and 45 samples, of which 15 HDPE, 15 PET, and 15 MIX (Fig. 5.1c).

Several physical and chemical parameters were measured in each mesocosm. Water temperature, pH, turbidity, and conductivity were measured using a multi-parameter probe (Hach HQ40D). Chlorophyll-a was measured using an AquaFluor (Turner Designs) portable fluorometer. Nutrients (i.e., nitrate, NO_3^-; ammonium, NH_4^+; phosphate, PO_4^{3-}; silicate, SiO_4^{4-}) were measured following standard methods (APHA/AWWA/WEF 2012). After one month, microplastics from each site and mesocosm were collected and placed in a 50 mL sterile Falcon tube with a known volume of mesocosm water (filtered before use); then, plastic pellets were scraped off

using a small sterile conical brush. Then, samples have been gently mixed, and preserved in Lugol solution. Before the identification in the laboratory, microplastics were visually inspected under optical microscope to ensure that the surfaces were properly cleaned, and no remaining microalgae were left attached to the polymer surfaces.

5.2.3. Laboratory analyses

Algae were counted and identified with an optical microscope at 400X magnifications in a Utermöhl chamber following the Utermöhl technique (EN 15204:2006). To identify diatoms, permanent slides were prepared using standard procedures: the samples were heated with 30% hydrogen peroxide (H_2O_2) for at least four hours to oxidize organic material, then we added concentrated hydrochloride acid (HCl, 1 M) to remove carbonates and finally we rinsed the processed material with distilled water in four centrifugation steps (Battarbee 1986). Cleaned material was transferred on a 24 × 24 mm cover slip and a drop of Naphrax (R.I. = 1.7) was used to mount the slides. Diatoms were identified with the optical microscope with 1000x magnification under oil immersion. Identification of microalgal species was based on the microscopic analysis of their morphological features, according to specific identification keys (Komárek and Anagnostidis 2007; Lange-Bertalot *et al.* 2017), updated to recent taxonomic nomenclature using internet databases (Gury and Gury 2021). The biovolume of each species was determined through a volumetric analysis of cells using geometric approximation and expressed as a weight following Wetzel & Likens (2000). Algal density (cell cm^{-2}) and biomass (µg cm^{-2}) were estimated.

5.2.4. Data analyses

Samples collected from the three mesocosms within the same site and with the same treatment of plastic polymer were considered as replicates (Fig. 5.1c). Relationships between biomass across the different samples and sites

have been evaluated through linear mixed-effect models (LME, Zuur *et al.* 2009). As fixed effects, we entered site and plastic type (with interaction term) into the model. As random effects, we included the different mesocosms nested in site. *P* values were obtained by F test on fixed effects using Satterthwaite approximation.

Alpha diversity (i.e., the number of taxa or number of functional characteristics within a location, cf. Rolls *et al.* 2018) was evaluated using the Shannon index, the inverse of Simpson index, and Pielou evenness index. To measure the association between species and the different levels tested (i.e., polymer type and site), we used the composite index called 'IndVal' (indicator value) by Dufrêne and Legendre (1997), which ranges from 0, no association, to 1, maximum association. Significant differences in sample diversity were assessed through Kruskal-Wallis test.

Differences in microalgal communities among samples (Beta diversity, cf. Rolls *et al.* 2018) were analyzed by non-metric multidimensional scaling (NMDS), based on Bray & Curtis' dissimilarity distances (Legendre and Legendre 1998) calculated from the biomass of the different species. Before NMDS computation, the data were transformed by double square root to reduce the importance of the more abundant taxa (Salmaso 2010). As an indicator of fitness, a stress function that measures the fit between NMDS distance and actual dissimilarities was calculated. A stress value (STR) > 0.20 provides a representation not different from random, STR < 0.15 a good representation, and STR < 0.10 an ideal representation (Clarke 1993). The significance of main effects (based on "site" and "polymer type" groups) was tested using permutational multivariate analyses of variances (PERMANOVA) applied to the distance matrix used as input for the NMDS ordination of samples with 999 permutations. Physical and chemical variables were related to the strongest gradients in species composition by fitting environmental vectors to the NMDS configurations. The significance of vectors was based on 999 random permutations of the data. To further evaluate the type of relationship between configurations and environment, a few selected

variables were related to the gradients in species composition by surface fitting. All the analyses were carried out in R 4.0.3 (R Core Team, 2020), using the following packages: 'lmerTest' (Kuznetsova *et al.* 2017), 'vegan' (Oksanen *et al.* 2012), 'ggplot2' (Wickham 2016).

5.3. Results

5.3.1. Physical and chemical parameters

Mesocosm waters over the five locations differ based on meteorological data and chemical and physical parameters (Table 5.1 and Table S5.1). Mean air temperature, over the experimental period, ranged between 17.0 to 21.0°C across the different locations, while cumulative rainfall varied between 0 and 115 mm (Table S5.1). High values of electrical conductivity (EC) were recorded in Murcia, as a result of dry conditions and small rainfall amount, reaching values wide above the usual range in freshwater systems. Slightly acid conditions were highlighted in Porto, while in the remaining locations pH is almost neutral (Jaca, Evora) or alkaline (Toledo, Murcia). Phosphate (PO_4^{3-}) reached high values in Toledo and, especially, in Evora, with a concentration above 3 mg L^{-1}; differently, Murcia, Porto and Jaca showed lower concentration with values spanning from 3 to 50 µg L^{-1}. Nitrate (NO_3^-) concentrations were quite similar across locations, ranging from values around 0.003±0.003 mg L^{-1} in Toledo to a concentration of 0.17±0.02 mg L^{-1} in Jaca. Ammonium ranges from 0.2 to 0.7 mg L^{-1} in all the locations, except for Toledo where the concentration reached 2.25±0.84 mg L^{-1}. The highest values of silicate (SiO_4^{4-}) were highlighted in Toledo and Evora, even if with marked intra-site differences. Macrophytes are absent in Toledo and low coverage was observed in Jaca (~38%); instead, a mean coverage above 60% was recorded in Porto (~63%, *Typha* sp.), Evora (~88%, *Typha* sp. and *Lemna*

sp.) and especially in Murcia, with almost full coverage (~99%) dominated by *Zannichelia* sp.

Table 5.1. Mean (±standard error) of physical and chemical parameters measured in the three mesocosms selected for each site.

	Murcia (MR)	Toledo (TL)	Evora (EV)	Porto (PT)	Jaca (JC)
Water temperature (°C)	19.0±0.4	9.3±0.6	15.7±0.5	15.5±0.1	15.5±0.6
EC (µS cm^{-1})	22046±460	2840±46	893±109	69±16	276±45
Turbidity (NTU)	2.19±1.67	27.35±21.21	8.88±1.33	3.37±0.77	3.27±0.84
pH	10.37±0.13	9.78±0.40	7.91±0.32	6.40±0.26	7.73±0.16
NO_3^- (mg L^{-1})	0.097±0.031	0.003±0.003	0.134±0.035	0.012±0.003	0.170±0.023
PO_4^{3-} (mg L^{-1})	0.046±0.044	1.960±1.168	3.219±0.918	0.003±0.003	0.016±0.013
NH_4^+ (mg L^{-1})	0.706±0.078	2.253±0.836	0.625±0.086	0.194±0.061	0.245±0.058
SiO_4^{4-} (mg L^{-1})	4.777±3.099	62.207±11.940	17.277±14.787	1.134±0.333	5.379±4.955
Macrophyte coverage (%)	99±1	0±0	88±13	63±7	38±22

5.3.2. Density and biomass distribution

Regardless of sites or plastic polymers, all the 45 samples analyzed have been colonized by microalgae. The mean microalgal density developed on HDPE substrate was equal to $1.6 \cdot 10^5 \pm 2.2 \cdot 10^4$ (mean±standard error) cell cm^{-2}, presenting on average less pronounced colonization compared to MIX, where the mean density was of $2.0 \cdot 10^5 \pm 4.2 \cdot 10^4$ cell cm^{-2}, and especially to PET with a value of $3.2 \cdot 10^5 \pm 1.3 \cdot 10^5$ cell cm^{-2} (Fig. 5.2a). Considering the different sites, the highest density has been highlighted in Toledo with a mean value of $5.4 \cdot 10^5 \pm 1.9 \cdot 10^5$ cell cm^{-2}, followed by Evora ($2.4 \cdot 10^5 \pm 2.7 \cdot 10^4$ cell cm^{-2}), Murcia ($1.4 \cdot 10^5 \pm 2.9 \cdot 10^4$ cell cm^{-2}), Porto ($1.3 \cdot 10^5 \pm 1.5 \cdot 10^4$ cell cm^{-2}), and Jaca ($9.8 \cdot 10^4 \pm 1.9 \cdot 10^4$ cell cm^{-2}; Fig. 5.2a).

Considering the biomass values, the average value for the samples collected on HDPE substrate is 219.7±46.4 µg cm^{-2}, while average value for MIX and PET is equal to 315.1±67.3 µg cm^{-2} and 329.1±70.5 µg cm^{-2}, respectively (Fig.

5.2b). Biomass values across the several locations are equal to 410.8±145.3 μg cm^{-2} in Murcia, 357.5±74.7 μg cm^{-2} in Evora, 265.4±53.8 μg cm^{-2} in Toledo, 215.6±31.5 μg cm^{-2} in Jaca and 190.5± 22.2 μg cm^{-2} in Porto (Fig. 5.2b).

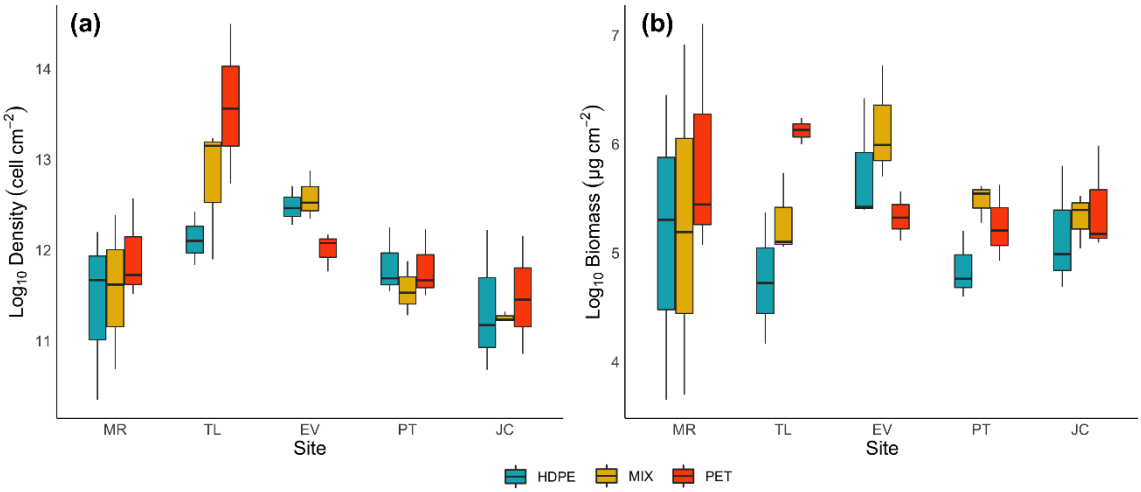

Figure 5.2. Tukey boxplot of (a) density (cell cm^{-2}), and (b) biomass (μg cm^{-2}) of microalgae for different sites on the two plastic polymers and the 'MIX' treatment. MR: Murcia, TL: Toledo, EV: Evora, PT: Porto, JC: Jaca.

Generally, within the sites, higher values of biomass were detected on PET compared to HDPE. This is verified for all the sites except for Evora, in which, in contrast, PET samples showed the lowest microalgal biomass. This difference observed in Evora was mainly linked to one mesocosm (i.e., EV-2), in which the biomass developed on PET substrate was much lower than the biomass on HDPE (Fig. S5.2). Biomass developed on MIX samples is generally higher than the biomass of HDPE samples; the evidence however is controversial when comparing results of MIX with PET samples, since for Murcia and Toledo mean biomass on MIX is lower than PET, while for the Evora, Porto, and Jaca the opposite is true.

Results of the linear mixed-effect model, reported in Table 5.2a, indicate that there are significant differences in total biomass colonizing different plastic types ($P < 0.05$), although the magnitude of these differences varies across sites ('plastic × site' interaction; $P < 0.05$); while site is not a significant factor. Across all samples, the taxa that gave the major contribution to total biomass were Bacillariophyta (diatoms) and Miozoa (Dinophyceae), with an average

relative abundance of 28.7±3.2% and 27.5±3.4% (mean±standard error), respectively. Considering the different polymers, HDPE had on average a higher abundance of diatoms, with a mean value of 59.2±13.5 μg cm^{-2}; instead, Miozoa was the taxon with the greatest abundance in MIX and PET samples (107.7±40.5 μg cm^{-2} for MIX; 111.0±54.0 μg cm^{-2} for PET; Fig. S5.2). Porto and Jaca were mainly dominated by Miozoa, representing on average 37.8±6.4% and 50.6±3.4% of the biomass respectively. Bacillariophyta provided the major contribution to microalgae biomass in Evora (46.4±8.1%) and Murcia (47.7±5.0%); while in Toledo, Ochrophyta represented 40.1±5.2% of the biomass (Fig. S5.1.).

Significant differences of the linear mixed model performed on the biomass of the different taxa (phyla=9) were only found for Bacillariophyta and Ochrophyta (Table 5.2b). For Bacillariophyta, significant differences were found for different polymers ($P < 0.05$), with higher biomass of diatoms being found on PET samples when compared to HDPE. For Ochrophyta, significant differences in biomass were found only among sites ($P < 0.01$), and the post hoc pairwise comparison highlighted that the site that significantly differed from the others is Toledo, where we observed a high abundance of microalgae belonging to this phylum.

Table 5.2. Results of linear mixed-effect model (LME) testing effect of plastic type ('Plastic', three levels: HDPE, MIX, PET), site ('Site', five levels: MR, TL, EV, PT, JC) and their interaction ('Plastic × site') on (a) total biomass; (b) biomass of different taxa.

		Plastic			**Site**			**Plastic × site**		
		df	**F**	***P***	**df**	**F**	***P***	**df**	**F**	***P***
a)	Total biomass	2	3.881	**0.038***	4	0.514	0.727	8	2.886	**0.026***
b)	Diatom biomass	2	4.049	**0.028***	4	2.467	0.113	-	-	-
	Ochrophyta biomass	2	1.911	0.174	4	8.091	**0.003****	8	2.988	**0.022***

Note: Bold indicates significant values.

Significance level: * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

5.3.3. Alpha diversity and community composition

The average number of species among all samples is equal to 35±1, with a maximum value of 47 (identified in MIX sample in Evora) and a minimum of 27 (highlighted in MIX sample in Murcia). Considering the different polymers, alpha diversity (expressed by the Shannon index, inverse Simpson index, and Pielou's evenness) displayed slightly higher values on HDPE compared to PET samples; however, these differences are not remarkable, and no significant differences were highlighted by Kruskal-Wallis test among groups (Fig. 5.3).

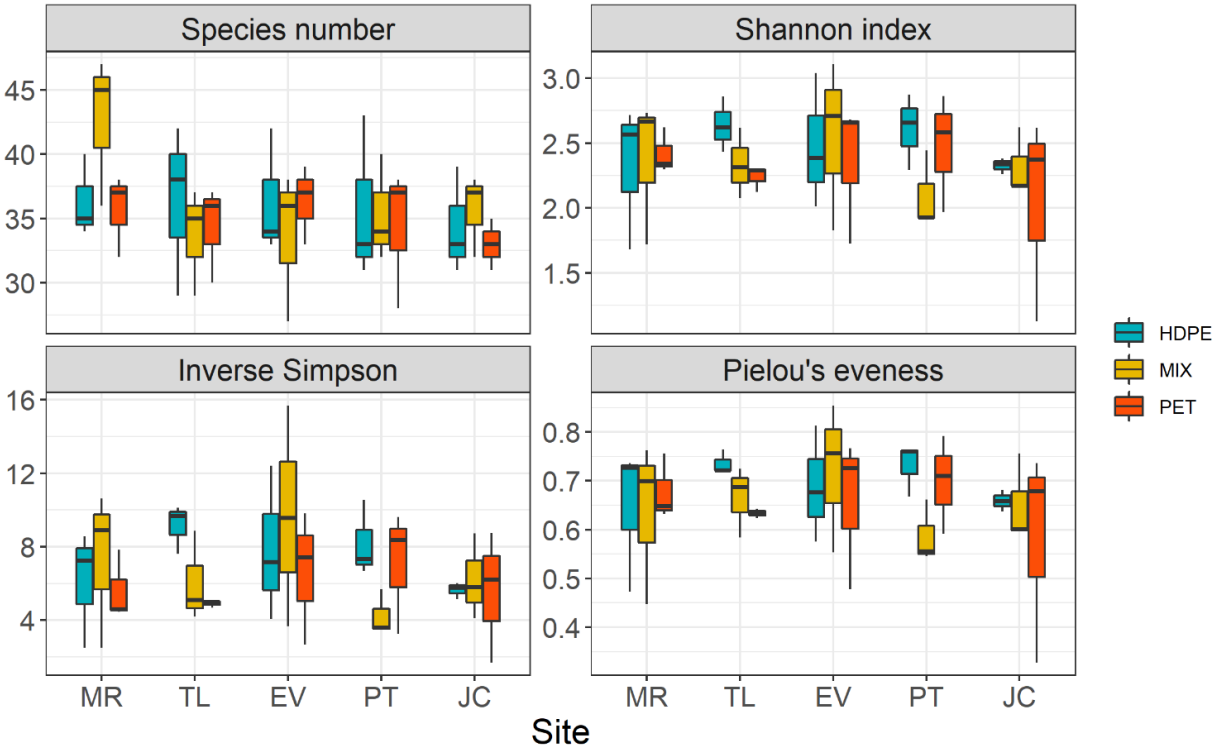

Figure 5.3. Alpha-diversity for different plastic types expressed as number of species ('Species number'), Shannon index ('Shannon index'), inverse of Simpson index ('Inverse Simpson'), and Pielou's evenness index ('Pielou's evenness').

Over the 45 samples analyzed, we found 242 different species distributed in 144 genera. The 33.5% belong to the phylum Bacillariophyta, followed by 26.4% Chlorophyta, 11.6% Cyanobacteria, 8.3% Charophyta, 6.6% Euglenozoa, 6.6% Ochrophyta, 4.1% Miozoa, 2.5% Cryptophyta, and 0.4% Haptophyta. We found some cosmopolite species, which were recorded in

almost all the samples, like *Aphanocapsa incerta* (Lemm.) Cronberg & Komárek identified in 44 over 45 samples. Besides this, the most frequent species were *Cocconeis placentula* Ehr. identified in 39 samples*; Peridiniopsis elpatiewskyi* (Ostenf.) Bourrelly in 37 samples; *Achnanthidium minutissimum* (Kütz.) Czarnecki in 36 samples; *Cocconeis pediculus* Ehr. in 35 samples; and *Planktolyngbya limnetica* (Lemm.) Kom.-Legn. & Cronberg in 33 samples. 48 species were present only in one sample and 41 in just two samples. Overall, 20 species from 19 genera, with a mean relative abundance of 1.4±0.1%, occurred only in HDPE samples, and 17 species from 16 genera only in PET samples, with an average relative abundance of 0.9±0.1%. However, in all the cases these species had very low recurrence, being identified at most in four different samples.

The biomass distribution of the most recurrent genera across the samples is reported in Fig. 5.4. The highest contribution to biomass was provided by the following species: *Gymnodinium discoidale* Harris (33.3±20.0 µg cm^{-2}); *Peridiniopsis cunningtonii* Lemm. (22.8±5.9 µg cm^{-2}); *Dictyosphaerium ehrenbergianum* Nägeli (20.1±14.0 µg cm^{-2}), *Chromulina pseudonebulosa* Pascher (18.1±9.3 µg cm^{-2}); *Cocconeis pediculus* (17.1±3.0 µg cm^{-2}). In particular, the genera *Cocconeis* and *Peridiniopsis*, besides having high abundance, also had high recurrence. The most species-rich genera were *Gomphonema* and *Navicula*, with a maximum of 7 and 6 taxa, respectively.

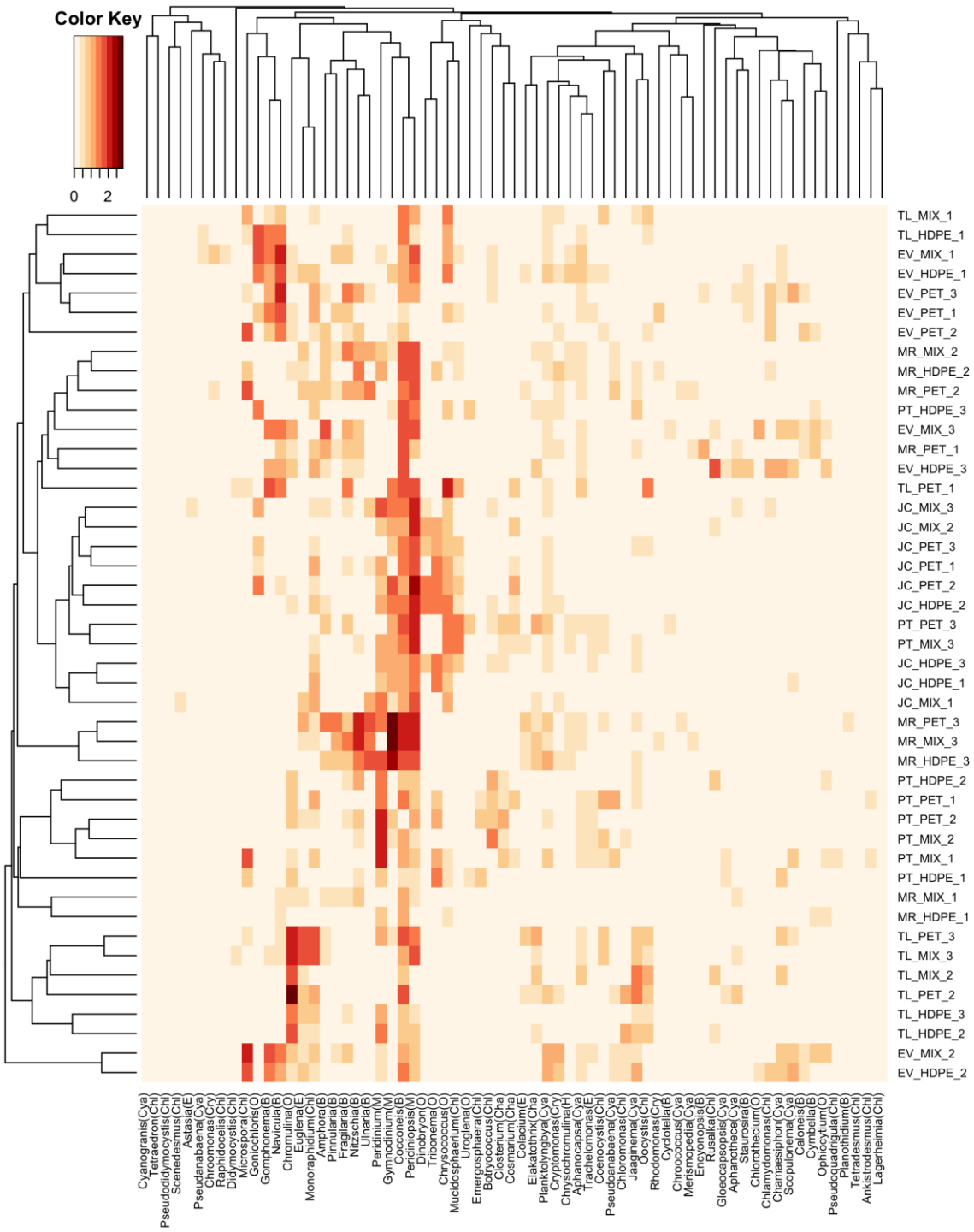

Figure 5.4. Heatmap visualizing the biomass distribution of genera of microalgae across the samples. Only genera that were identified in at least 10% of the samples (n=5) are reported. Biomass data were $\log_{10}(x+1)$ transformed before plotting. Clusters have been calculated based on Bray-Curtis distance. Clusters of samples (row cluster) have been calculated considering all the genera identified. The corresponding phylum for each genus is given in brackets: 'Cya' Cyanobacteria, 'B' Bacillariophyta, 'Chl' Chlorophyta, 'Cha' Charophyta, 'E' Euglenozoa, 'O' Ochrophyta. 'Cry' Cryptophyta, 'M' Miozoa.

5.3.4. Beta diversity and relationship with environmental variables

Cluster analysis based on Bray-Curtis similarity index calculated on community composition (Fig. 5.4) did not discriminate samples based on the different polymers colonized (HDPE, PET or MIX), but rather by the different sites, which seems to be a more influential factor affecting community composition. This is confirmed by the IndVal index since no significant indicator species were identified based on the different polymers. Indicator species were in turns highlighted for the different sites, showing the highest values (P = 0.001) for the genera *Nitzschia*, *Pinnularia, Ulnaria,* and *Merismopedia* in Murcia, *Oocystis* in Toledo, *Navicula*, *Caloneis*, *Staurosira,* and *Gomphonema* in Evora, *Closterium* in Porto, and *Dinobryon* in Jaca (Table S5.2). The ordination of the samples through NMDS analysis (three-dimensional solution, STR = 0.14) based on the species-specific biomass composition allowed separating the samples mostly based on the geographic position (Fig. 5.5a). In particular, a noticeable difference in community composition is evident for samples collected in Jaca, with a clear separation compared to the other sites. The separation of the groups based on the geographic position was confirmed by the PERMANOVA analysis (P = 0.001). Significant differences were not highlighted, instead, considering the polymer colonized. The variation in species composition along the first axis was positively linked to phosphate (PO_4^{3-}) concentration, which had the highest importance in the ordination space. This result suggests that phosphate concentration may be the dominant driver of microalgae community composition. The high association of species composition with the gradient of phosphate concentration is further illustrated by the pattern of PO_4^{3-} in the NMDS configuration (Fig. 5.5b). Other significant environmental variables included silicate (SiO_4^{4-}) and ammonium (NH_4^+), which also contributed to explaining the variation along the second NMDS axis. The macrophyte coverage (% macrophyte) was associated with both the first and the second axis, but its

importance is also limited. Instead, conductivity and pH were not significant variables.

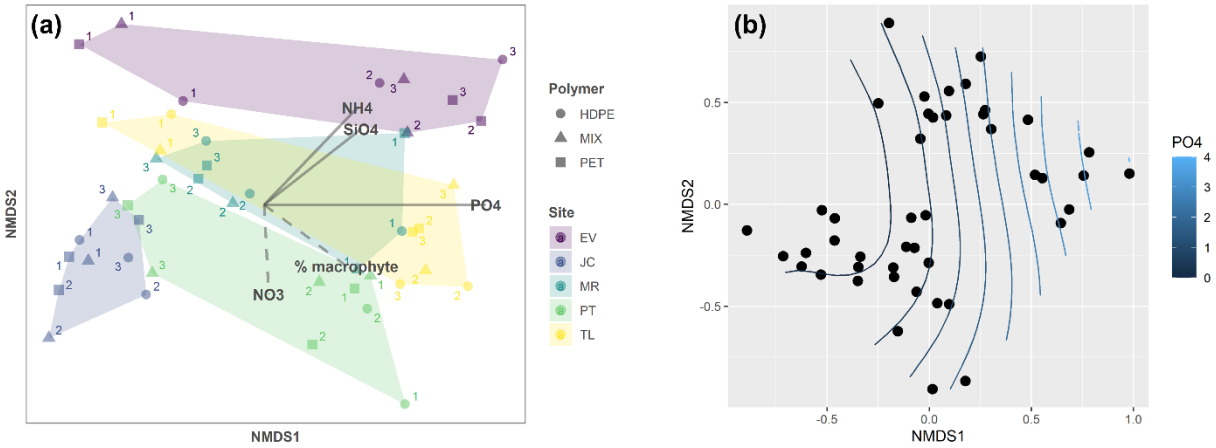

Figure 5.5. (a) Non-metric multidimensional scaling (NMDS) run on species-level microalgae biomass with vectors of significant environmental variables. Vectors are significant at $P < 0.05$ (solid arrows) and $P < 0.10$ (dashed arrows). 'PO4': phosphate concentration; 'NH4': ammonium concentration; 'SiO4': silicate concentration; 'NO3': nitrate concentration; '% macrophyte': coverage of macrophyte in percentage. (b) Surface fitting for the PO_4^{3-} concentration (bin width=0.5).

5.4. Discussion

In a mesocosm investigation across a geographical gradient, this study has highlighted that different microplastic polymers represent a substrate that can be widely colonized by a diverse community of microalgae. Indeed, biofouling of the surfaces of HDPE and PET microplastics occurred in all conditions, regardless of the sites and the plastic polymer considered. This is in line with the available literature in which it is reported that microplastics provide new niches in the aquatic environment and, thus, represent available and long-lasting substrates for a diverse microbial community (Zettler *et al.* 2013; Rummel *et al.* 2017).

Our results showed that colonization occurred in a range of lentic ecosystems since mesocosms used for our experiment cover a wide geographical gradient and different environmental conditions. Colonization of plastic

surfaces by microorganisms has been reported for microplastics collected in a variety of aquatic systems (Oberbeckmann *et al.* 2014; e.g., Debroas *et al.* 2017; Dussud *et al.* 2018). However, to date, studies in freshwater systems are numerically less abundant than the ones in marine environments, and there is a need to increase the knowledge of plastisphere consortia in freshwater systems (Harrison *et al.* 2018). Besides, the majority of the studies have focused on the bacterial community, neglecting or marginally considering microalgae community in epiplastic assemblages, which are fundamental components at the base of aquatic food webs and pivotal organisms in a broad variety of ecosystem functions (Nava and Leoni 2021). In our studies, we performed a thorough microscopic (phenotypic) investigation of the microalgae community in the epiplastic assemblage, which allowed evaluating the community diversity and the abundance of the different microalgae species. We highlighted that total biomass differed based on the polymer considered, with higher biomass developed on PET substrate compared to HDPE. This result seemed to be linked especially to diatoms since the outcome of the linear mixed model highlighted differences of total biomass only for this taxon, which constitute one of the most diverse and numerically abundant groups. The different amounts of biomass developed on the two polymers may be linked to the differential position of HDPE (i.e., floating) and PET (i.e., sinking) on the water column. Indeed, it is possible that microalgae community developed on floating plastics were more exposed to UV radiation with subsequent photoinhibition effect, which could have limited algae growth, or physical abrasion of biofilm of floating particles could have occurred (Raven and Waite 2004; Arias-Andres *et al.* 2018).

It seems now clear that microbial communities on plastic debris differ consistently from the surrounding aquatic communities, as the presence of an additional substrate constitutes a new niche with the possibility of development for a distinct community (Yang *et al.* 2020; Wright *et al.* 2020). The discussion is now moved towards whether different substrates could

allow the growth of distinct species. Our results highlighted a rich and diversified community of microalgae developed on both HDPE and PET substrate, but we did not observe species-specificity in the colonization of the different plastic polymers. Indeed, local species pool rather than polymeric composition seems to be the determinant factor defying the community diversity. We hypothesize that the existing communities in the different mesocosms may be responsible for some of the trends in species assemblages and future studies should address this relationship. Indeed, previous research, investigating periphyton assemblages in temperate lakes, reported as many of the algae identified in the periphyton were common components of phytoplankton community that had likely settled out of the water column (Wood *et al.* 2012) and other studies have also shown that regional microalgae species richness have a strong influence on richness of periphyton algae communities (Algarte *et al.* 2017). But still additional factors contribute in determining species diversity of phytobenthos, like abiotic and biotic factors such as nutrient levels, temperature, light, grazing, but also habitat heterogeneity, and hydrological factors (Algarte *et al.* 2017).

Previous research from marine environments showed similar results to those observed in this study, reporting that geography is likely to be a stronger predictor of plastisphere community composition at the scale of ocean basins (Harrison *et al.* 2018; Oberbeckmann *et al.* 2021). However, this is still under debate with several studies suggesting substrate-driven selection, with differences reported not only when comparing inert control material to plastic substrates (Ogonowski *et al.* 2018; e.g., Miao *et al.* 2019) but also when colonization on different plastic polymers was evaluated (e.g., Li *et al.* 2019; Pinto *et al.* 2019). However, since the term plastics includes a plethora of different polymers, with different chemical and physical features and different additives, results should be extended and compared with caution (Yang *et al.* 2020). Another variable that should be taken into account is the study duration, whose variation may influence the process and the conclusions drawn (Nava and Leoni 2021). Indeed, previous studies have

shown that there are strong shifts and distinct communities during early stages of colonization. Over time, however, communities converge and remain stable in mature biofilms (Pinto *et al.* 2019; Wright *et al.* 2020). This may be explained as only the initial recruits have direct contact with the polymer surface; in contrast, later recruits are more likely to interact with existing biofilm members and the abiotic components of the surrounding environment (Oberbeckmann *et al.* 2016; Dudek *et al.* 2020). It is reported as a stable and consistent epiplastic bacterial community can be achieved within days to over one week, while the establishment of a mature eukaryotic community may take longer (Lobelle and Cunliffe 2011; Erni-Cassola *et al.* 2020). The one-month duration for our study may have allowed observing a more developed microalgal community with different recruits not directly attached to the polymer surfaces and hence it could be hypothesized that this may constitute the cause of the absence of clear divergence in community composition on HDPE compared to PET. However, it is difficult to define the time frame in which the community becomes mature as this can also vary depending on different factors and environmental conditions. For instance, Smith *et al.* (2021), studying the evolution of algal biofilm assemblages on plastic polymers over time, reported significant differences between diatom assemblages also between week 4 and week 6, demonstrating as differences in community composition can be also observed in longer period of colonization. At the same time, it is reported as differences between materials are usually driven by rare taxa (Pinto *et al.* 2019). We identified 17 species developed exclusively on PET and 20 species exclusively on HDPE with a low relative abundance. However, we cannot exclude stochastic processes determining the presence of distinct species on the different substrates.

Species belonging to the phylum Bacillariophyta were among the most abundant, and the most diverse in almost all the sites. Most studies have shown that diatoms are common and omnipresent residents of the plastisphere, at least on plastics that are exposed to sunlight (Amaral-Zettler

et al. 2020). Common diatoms reported in previous plastisphere research include species belonging, for instance, to genera *Cocconeis*, *Amphora*, *Fragilaria*, *Navicula*, *Nitzschia* (Reisser *et al.* 2014; Oberbeckmann *et al.* 2014; e.g., Dudek *et al.* 2020), which were all observed in our study. Besides diatoms, Cyanobacteria and Chlorophyta were the only taxa for which we identified species in 100% of samples analyzed. Indeed, diatoms, cyanobacteria, and green algae have been reported as pioneering microbes that colonize plastic debris and their presence is also widely reported (Wright *et al.* 2020). However, it is difficult to compare results about community composition with previous studies since different "location-specific" factors (i.e., microbial community, environmental condition, macrophyte coverage) determine the development and growth of the species on plastic surfaces (Yang *et al.* 2020). For instance, different macrophytes, which have different architecture and constitute an important parameter for periphytic algal community organization (Messyasz *et al.* 2009; dos Santos *et al.* 2013), might represent a source of microalgae species that can later develop on microplastic. However, our analyses showed that macrophyte coverage did not strongly affect community composition. Among the different environmental conditions, nutrient concentration has been reported as one of the most influential factors influencing microplastic biofilm structure (Oberbeckmann *et al.* 2018; Li *et al.* 2019). Our results are consistent with these findings, as we showed that nutrient concentration, and in particular phosphate concentration, was pivotal in determining the species assemblages on plastic surfaces, as highlighted by the NMDS analysis. In sites where the concentration of phosphate was low, like Porto and Jaca, we observed a slightly lower microalgae growth, even if we did not find any relationship between phosphate concentration and total biomass (see Fig. S5.4); however, we identified a high number of species in Porto and Jaca, with almost identical values to those of sites and mesocosms with higher nutrient concentrations. This showed that the presence of microplastics, offering a new substrate on which microalgae can grow, may promote the development

of a high number of diverse species of microalgae with values comparable to those observed in environments with higher nutrient concentration. It has been already highlighted as floating plastics, which also increases the entrapment of nutrients from the surrounding environment, may constitute net autotrophic "hot spots" in the oligotrophic ocean, with high density of chlorophyll and high oxygen production (Bryant *et al.* 2016; Chen *et al.* 2020). This may also happen in freshwater ecosystems with consistent development of epiplastic algae also in oligotrophic environments. Regardless of specific environmental conditions, we showed that many species can coexist on the surface of relatively small plastic items, highlighting as microplastics may have considerable carrying capacity, with possible consequences on the wider ecological context, for both aquatic food webs and ecosystem functioning (Wright *et al.* 2020; Nava and Leoni 2021).

5.5. Conclusions

Outcomes of this study highlighted that microplastics represent an available substrate for the colonization by a variety of phytobenthic organisms in freshwater ecosystems. Small surfaces of plastics may host many different species of microalgae, but we did not observe a dissimilarity in community composition based on distinct polymeric composition, corroborating findings from previous research conducted mainly in marine ecosystems. Local species pool and nutrient concentration seem to be the most crucial factors in driven species sorting of epiplastic community. Future studies should look at both the existing microalgal assemblages in water and the new assemblages that form on plastics polymer samples in order to understand the relationship between them.

Differences based on the polymer types were highlighted, instead, for the total biomass of microalgae, which was, however, high in all the samples in both eutrophic and oligotrophic systems. In a broader context, the

considerable growth of primary producers on microplastic particles, whose presence is argued to be relevant in freshwater systems and expected to increase in the future, may have important consequences on food-web functioning and aquatic ecosystems productivity. These effects are currently overlooked and need to be thoroughly considered in future studies. The use of a mesocosm infrastructure in the present study allowed testing our hypotheses among many different systems in an environmental gradient, but future studies in real aquatic ecosystems are needed. Starting from the knowledge acquired from this study and growing body of research about plastisphere, future research should investigate the time development of biofouling of different plastic polymers by microalgae investigating at the same time the interaction with other components of aquatic food webs.

Supplementary materials

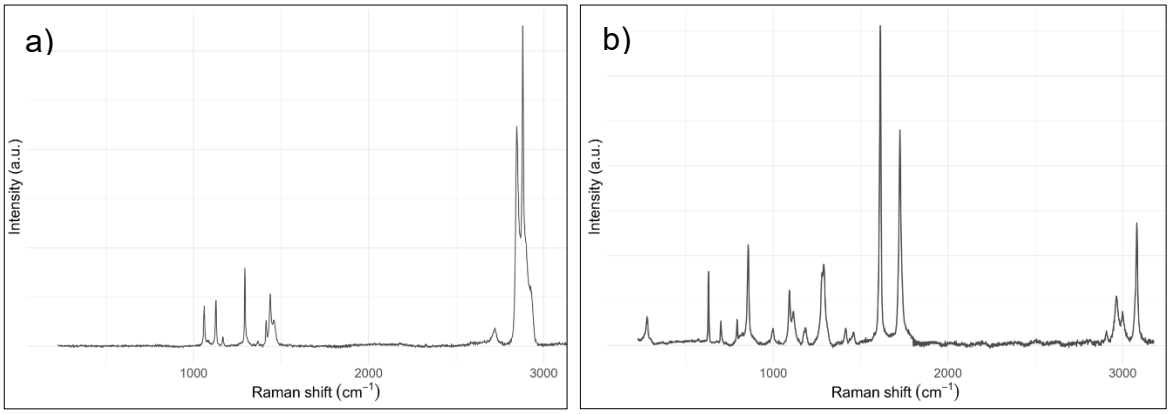

Figure S5.1. Raman spectra of the two virgin polymers employed in the study: (a) high-density polyethylene (HDPE); (b) polyethylene terephthalate (PET).

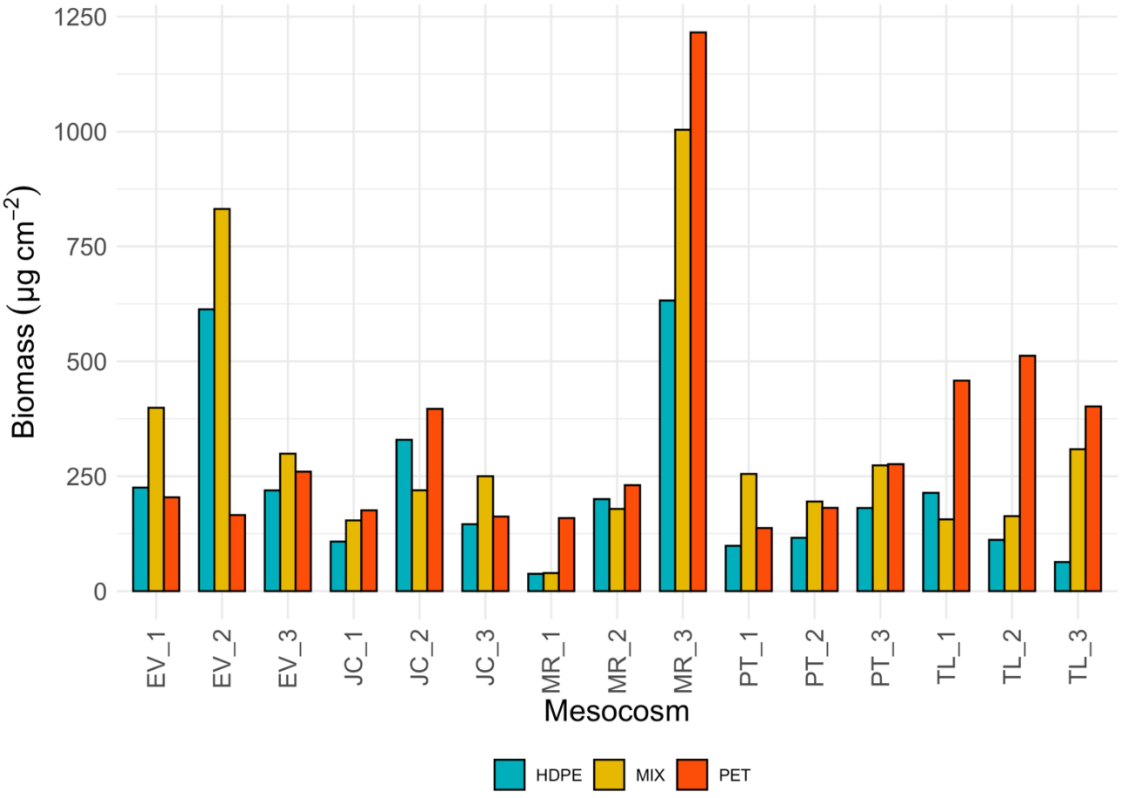

Figure S5.2. Biomass of microalgae developed on the different plastic polymers in the different mesocosms.

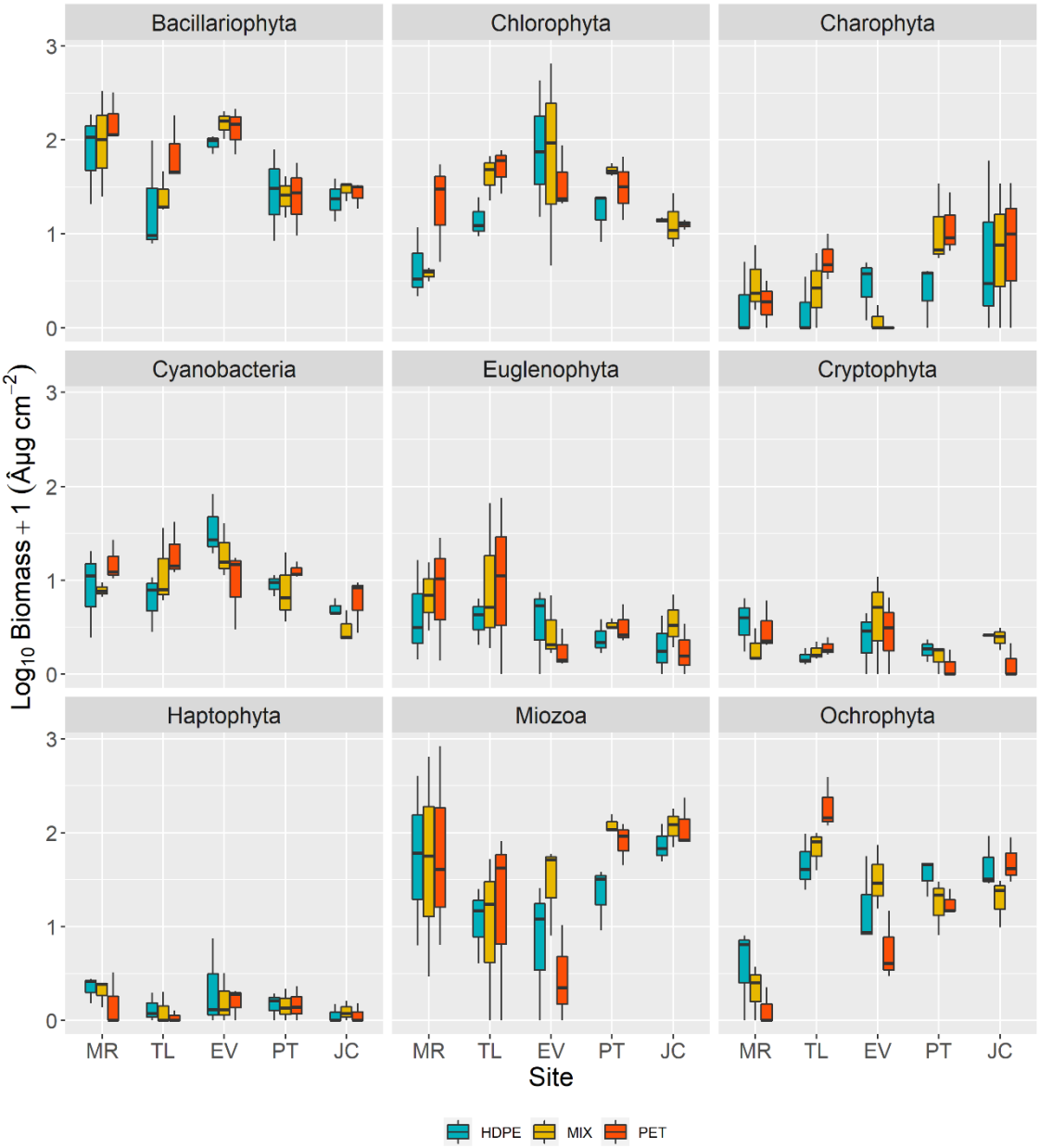

Figure S5.3. Tukey boxplot of biomass (µg cm^{-2}) of microalgae for different sites on the two plastic polymers and the 'MIX' treatment for the different phyla.

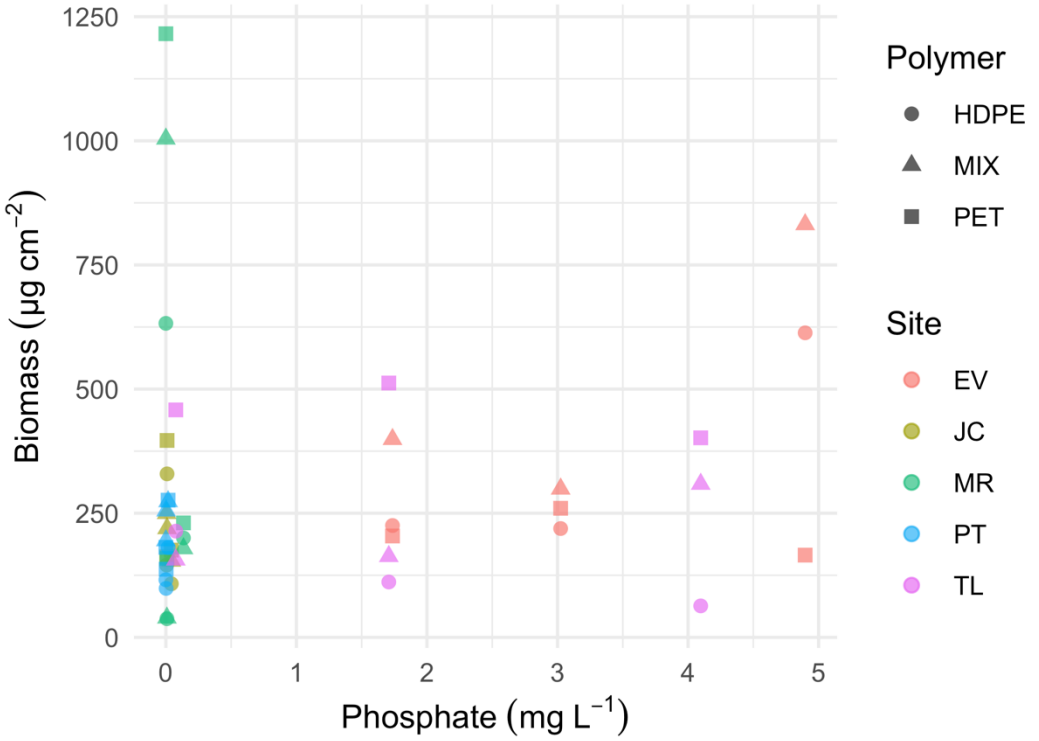

Figure S5.4. Scatterplot of phosphate concentration (PO_4^-, mg L^{-1}) and total biomass (µg cm^{-2}) for different sites ('Site') and different polymer types ('Polymer').

Table S5.1. Mean air temperature (°C) ± standard error and cumulative rainfall (mm) over the 30 days of duration of the experiments for the different sites.

Site	Temperature (°C)	Rainfall (mm)
Murcia	18.28 ± 0.57	115.20
Toledo	17.03 ± 0.70	64.20
Evora	18.39 ± 0.10	0.00
Porto	17.63 ± 0.60	7.60
Jaca	21.00 ± 0.99	66.40

Table S5.2. Results of indicator value (IndVal) index for the different sites.

Genus	site	IndVal	*P*-value
Nitzschia	MR	0.7463	0.001***
Pinnularia	MR	0.6426	0.001***
Ulnaria	MR	0.5724	0.001***
Merismopedia	MR	0.5351	0.001***
Chroococcus	MR	0.5300	0.002**
\Cocconeis	MR	0.4046	0.004**
Colacium	MR	0.3430	0.047*
Oocystis	TL	0.9381	0.001***
Chromulina	TL	0.7150	0.009**
Jaaginema	TL	0.5256	0.007**
Didymocystis	TL	0.5222	0.003**
Euglena	TL	0.4924	0.031*
Carteria	TL	0.4444	0.003**
Monomorphina	TL	0.3333	0.033*
Navicula	EV	0.8852	0.001***
Caloneis	EV	0.7758	0.001***
Gomphonema	EV	0.7296	0.001***
Staurosira	EV	0.5513	0.001***
Chlamydomonas	EV	0.5305	0.012*
Chamaesiphon	EV	0.4531	0.004**
Scopulonema	EV	0.4448	0.003**
Uroglenopsis	EV	0.3284	0.035*
Platessa	EV	0.3195	0.037*
Aphanocapsa	EV	0.3117	0.041*
Geminella	EV	0.2919	0.033*
Craticula	EV	0.2784	0.041*
Closterium	PT	0.6517	0.001***
Botryococcus	PT	0.4626	0.018**
Peridinium	PT	0.4497	0.013**
Coenocystis	PT	0.4482	0.005**
Synura	PT	0.4212	0.007**
Emergosphaera	PT	0.3976	0.011*
Trachelomonas	PT	0.3454	0.009**
Lyngbya	PT	0.3239	0.025*
Tetraplektron	PT	0.2584	0.044*
Dinobryon	JC	0.7615	0.001***
Tribonema	JC	0.5435	0.002**
Peridiniopsis	JC	0.4200	0.006**
Spirogyra	JC	0.3333	0.031*
Staurastrum	JC	0.3333	0.040*
Mougeotia	JC	0.2858	0.021*

Significance level: * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

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Chapter 6

Conclusions

Synthesis and future perspectives

Plastic pollution has attracted the widespread attention of the public and policy makers, galvanized by reports of the ubiquity of these pollutants. However, data about the characteristics and distribution of plastics and microplastics in the aquatic systems is still not comprehensive and, thus, their effects and fate, and interaction with the biota are far from being clearly understood. This is especially true for freshwater environments, for which studies about plastic debris are still limited compared to those performed in the ocean, even if evidence highlighted that freshwaters represent not only transport routes for land-based sources of plastics but also can accumulate these contaminants, with possible detrimental effects. This thesis contributed to the field of microplastic research, with a particular focus on freshwater ecosystems, filling gaps about the polymeric characterization, the occurrence, and the interaction of these pollutants with aquatic organisms.

To be able to answer relevant and overreaching questions such as about the quantities and features of plastics in the aquatic systems, the quality of the data is pivotal and thus the analytical procedures aimed at detecting these pollutants cannot leave polymeric identification out of consideration. Indeed, this step is fundamental for both confirming the synthetic nature of the particles identified, minimizing the occurrence of false positives, and also for providing information about their features. Indeed, the term plastic includes a plethora of different polymers, and their characterization in the environment cannot disregard the identification of the chemical composition. Spectroscopic analyses, such as FT-IR and Raman spectroscopy, are the most common techniques for identifying plastic particles in microplastic studies. However, microplastic pollution research has suffered from inadequate data and tools for spectral analysis especially regarding Raman spectroscopy, whose potential in plastic research is not yet fully exploited. Therefore, through the analysis and elaboration of data about Raman spectroscopy for the identification of plastic polymers, this thesis

contributed to providing guidance for future studies which can take full advantage of this technique. Indeed, we developed and provided a free database and an R package for the identification of plastic polymers and additives through this spectroscopic technique. A key contribution of this work regarded plastic additives, especially colorants, whose presence in plastic fragments is widespread but still poorly investigated and may influence the impacts of these contaminants in the environment. Moreover, their presence may complicate the polymer identification. Therefore, by analyzing a large number of plastic polymers with colorants, the results presented provided valuable information for their spectroscopic analysis that will help future investigations in characterizing also these components.

As widely mentioned, the occurrence of plastics in freshwater systems is still largely unexplored and this constitutes a critical gap in our understanding of the presence and distribution of these pollutants in the environment. It is largely claimed that inherent difficulties exist in comparing data across different studies and systems. Indeed, to date, researchers have failed to articulate and standardize a common protocol widely adopted, and this has resulted in scattered information about the scale of the problem in inland waters. This thesis contributed to the advancement of the knowledge in the field by analyzing samples from 38 lakes located in 28 different countries around the world, that were collected following a common protocol and processed with a standardized analytical procedure. This was possible due to the establishment of an international collaboration with more than 70 scientists within the framework of 'GLEON – Global Lake Ecological Observatory Network' which promotes networked lake science.

We provided fundamental information about the concentration of plastics in these systems, whose values in some cases outpaced those identified for instance in the oceanic accumulation areas (e.g., North and South Pacific gyres), while in others resulted to be extremely low. This showed as concentrations can vary widely among the systems considered, thus

highlighting the presence of distinct causes for their accumulation. Information was not limited to concentration, but we also included data about the features of plastics and microplastics, like shape, dimension, color, and polymeric composition. This knowledge is particularly relevant as a baseline for future investigation and to guide studies aimed at investigating the effects of these particles. In addition to this critical data, we highlighted the existence of a relationship between the abundance of plastic debris and urban-related attributes of the watershed. We also observed as larger and deeper lakes with higher retention times were accumulating plastic debris at higher concentrations, highlighting as different systems can be exposed to a different risk of plastic accumulation. Even in the most optimistic future scenarios of plastic waste reduction, emissions of plastic are increasing and will continue to do so. According to a business-as-usual scenario, the emission rates of 2016 will be approximately doubled by 2025. Also considering scenarios that include coordinated global actions and mitigation strategies, plastic emissions are expected to continue to rise yearly. Given this and considering that plastics are classified as “poorly reversible pollutants”, it would be easy to understand that the accumulation of plastic fragments in freshwater systems is likely to increase in the future alongside the possible effects that they could exert.

When released in the environment, these particles interact with the surrounding and with aquatic biota, starting from organisms at the base of food webs (i.e., microalgae). This thesis explored the relevant interaction between microalgae and microplastics, which often result in two-way effects, with consequences for the fate of microplastics in the environment but also for the organisms which may be affected by these particles. By combining a thorough and critical investigation of the published literature with mesocosm experiments performed across a wide environmental gradient (five locations across the Iberian Peninsula), we showed that small plastic particles supported the growth of a rich and diversified community of microalgae. This

highlighted as many species could coexist on the surface of relatively small plastic items, thus showing as plastics represent new substrates that offer favorable conditions for the development of microalgal communities. Moreover, we showed as plastic particles did not exert a strong enough selection to drive species sorting, overcoming other niche-defining factors, since we did not observe species-specificity in the colonization of the different plastic polymers. Local species pool and nutrient concentration rather than polymeric composition seemed to be the determinant factors defying the community diversity. This sets important knowledge in the field and opens up to further investigations aimed at understanding the consequences of this interaction on a larger scale.

In conclusion, the research presented here enhances the knowledge of microplastics in freshwater systems and taken together, these findings trigger and serve as foundations for further research questions, which are now better refined and informed. Some of these future research directions are detailed below:

a) *Need to detect and characterize small microplastics (<250 μm)*: there is a need to improve sampling and analysis capabilities to detect the full dimensional range of plastics and provide a comprehensive characterization of these pollutants. Raman spectroscopy, which we examined in Chapter 2, may be especially relevant in this respect given the high spatial resolution, but there is the need to improve automatic procedures and the efficacy of purification steps during the sample pre-treatment to facilitate the subsequent analyses.

b) *Use realistic data to assess the effects of plastic pollution on aquatic organisms:* controlled laboratory studies are essential for determining the impacts of different plastic materials on various aquatic organisms. However, such studies must be designed to address true, environmental threats. Data about the occurrence of microplastics in the environment,

like those reported in Chapter 3 of the present thesis, should be used to investigate the potential effects of microplastics, testing particles with features and polymeric composition that are more likely to be found in the real environment, thus providing a more reliable assessment of potential risk linked to microplastics.

c) *Discover the complexities of plastic and microplastic interactions with the environment and the linked ecological implications*: the possible interactions of microplastics with different aquatic organisms are varied and complex, and potential effects can reverberate throughout the whole aquatic food web. This makes the analysis of the ecological implications linked to the presence of microplastics extremely difficult. The approach that we adopted (Chapter 5) using experimental mesocosms may constitute a good compromise, enabling experimental controlling but allowing for more realistic conditions than small-scale experiments. Thus, future studies may use these approaches to further examine the questions linked to these complex interactions and the effects at a larger scale. Moreover, this may also help in exploring how plastic pollution can act in concert with other geophysical, biological, and chemical stressors to cause impacts.

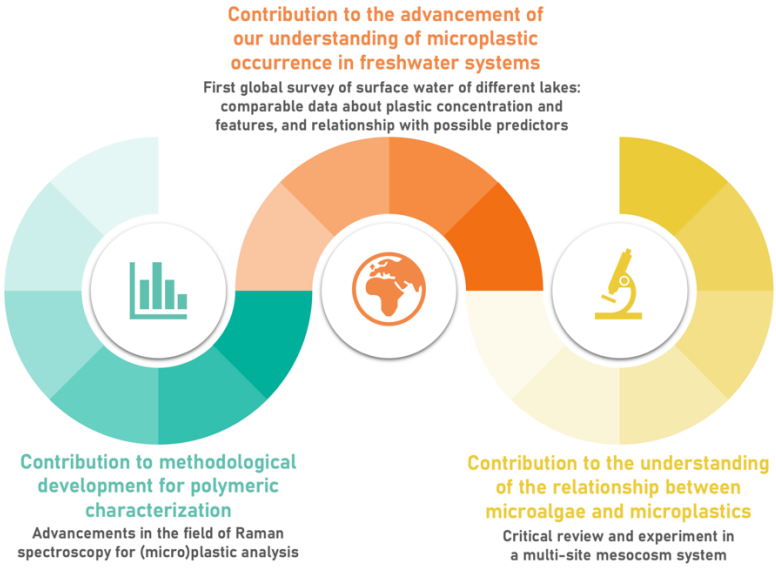

Figure 6.1. Synthesis of the contributions of the present Ph.D. thesis.

Appendix A

A.1. Peer reviewed publications

1. Nava V., Matias M., Castillo-Escrivà A., Messyasz B., Leoni B. (2021). Microalgae colonization of different microplastic polymers in experimental mesocosms across an environmental gradient. *Global Change biology*, Online early. doi:10.1111/gcb.15989
2. Nava V., Frezzotti M.L., Leoni B. (2021). Raman analysis for the analysis of microplastics in aquatic systems. *Applied Spectroscopy.* 75(11), 1341-1357. doi:10.1177/00037028211043119
3. Nava V., Leoni B. (2021). A critical review of interactions between microplastics, microalgae and aquatic ecosystem function. *Water Research* **188**, 116476. doi:10.1016/j.watres.2020.116476
4. Nava V., Leoni B. (2021). Comparison of different procedures for separating microplastics from sediments. *Water* **13**(20), 2854. doi:10.3390/w13202854
5. Leoni B., Zanotti C., Nava V. *et al*. (2021). Freshwater system of coral inhabited island: availability and vulnerability (Magodhoo Island of Faafu Atoll - Maldives). *Science of the Total Environment* **785**,147313. doi:10.1016/j.scitotenv.2021.147313
6. Leoni B., Patelli M., Nava V., Tolotti M. (2021). Cladocera paleocommunity to disentangle the impact of anthropogenic and climatic stressors on a deep subalpine lake ecosystem (Lake Iseo,Italy). *Aquatic Ecology* **55**, 607–621. doi:10.1007/s10452-021-09850-9
7. Jenny J.-P., Anneville O., Arnaud F., Baulaz Y., Bouffard D., Domaizon I., …, Nava V. *et al*. (2020). Scientists' Warning to Humanity: Rapid degradation of the world's large lakes. *Journal of Great Lakes Research* **46**, 686–702. doi:10.1016/j.jglr.2020.05.006

8. Rotiroti M., Bonomi T., Sacchi E., McArthur J.M., Jakobsen R., Sciarra A., …, Nava V. *et al.* (2020). Overlapping redox zones control arsenic pollution in Pleistocene multi-layer aquifers, the Po Plain (Italy). *Science of the Total Environment* **758**, 143646. doi:10.1016/j.scitotenv.2020.143646

9. Nava V., Patelli M., Bonomi T., Stefania G.A., Zanotti C., Fumagalli L. *et al*. (2019). Chloride balance in freshwater system of a highly anthropized sub-alpine area: load and source quantification through a watershed approach. *Water Resources Research* **56**(1), e2019WR026024. doi:10.1029/2019WR026024

10. Nava V., Patelli M., Rotiroti M., Leoni B. (2019). An R package for estimating river compound load using different methods. *Environmental Modelling & Software* **117**, 100-108. doi:10.1016/j.envsoft.2019.03.012

11. Leoni B., Spreafico M., Patelli M., Soler V., Garibaldi L., Nava V. (2019). Long-term studies for evaluating the impacts of natural and anthropic stressors on limnological features and the ecosystem quality of Lake Iseo. *Advances in Oceanography and Limnology* **10**(2).

12. Stefania G.A., Rotiroti M., Buerge I. J., Zanotti C., Nava V. *et al.* (2019). Identification of groundwater pollution sources in a landfill site using artificial sweeteners, multivariate analysis and transport modeling. *Waste Management* **95**, 116-128. doi:10.1016/j.wasman.2019.06.010

13. Rotiroti M., Bonomi T., Sacchi E., McArthur J.M., Stefania G.A., Zanotti C., …, Nava V. *et al*. (2019). The effects of irrigation on groundwater quality and quantity in a human- modified hydro-system: the Oglio River basin, Po Plain, northern Italy. *Science of the Total Environment* **672**, 342-356. doi:10.1016/j.scitotenv.2019.03.427

14. Rotiroti M., Zanotti C., Fumagalli L., Taviani S., Stefania G.A., Patelli M., Nava V. *et al*. (2018). Multivariate statistical analysis supporting the hydrochemical characterization of groundwater and surface water: a case study in northern Italy. *Rendiconti Online Società Geologica Italiana* **47**, 90-96. doi:10.3301/ROL.2019.17

15. Leoni B., Nava V., Patelli M. (2018). Relationships among climate variability, Cladocera phenology and the pelagic food web in deep lakes

in different trophic states. *Marine and Freshwater Research* **69**(10), 1534-1543. doi:10.1071/MF17243

16. Leoni B., Patelli M., Soler V., Nava V. (2018). Ammonium transformation in 14 lakes along a trophic gradient. *Water* **10**, 265. doi:10.3390/w10030265
17. Nava V., Patelli M., Soler V., Leoni B. (2017). Interspecific relationship and ecological requirements of two potentially harmful Cyanobacteria in deep south alpine lake (L. Iseo, I.). *Water* **9**, 993. doi:10.3390/w9120993

A.2. International conferences

1. Nava V., Matias M., Messyasz B., Castillo-Escrivà A., Ersoy Z., Raposeiro P.M. *et al.* (2021). Microalgal colonization of microplastics in experimental mesocosms across a biogeographical gradient. Oral communication: *2021 Virtual Meeting – ASLO*. Online.
2. Nava V., Matias M., Messyasz B., Castillo-Escrivà A., Ersoy Z., Raposeiro P.M. *et al.* (2021). Microalgal colonization of microplastic substrates: results from mesocosm experiment across a biogeographical gradient. Oral communication: *SEFS12, Symposium for European Freshwater Sciences*. Online.
3. Nava V., Matias M., Messyasz B., Castillo-Escrivà A., Leoni B. (2021). Periphyton development on artificial substrates across a biogeographical and trophic gradient. Oral communication: *35th Congress of the International Society of Limnology*. Online.
4. Nava V., Aherne J., Alfonso M.B., *et al.* (2021). Global patterns and predictors of microplastic occurrence and abundance in lentic systems. Poster: *GLEON 2021 All Hands' Meeting*. Online.
5. Nava V., Patelli M., Soler V., Leoni B. (2019). How climate change can influence biogeochemical cycle of Dissolved Silicon (DSi): insights from long-term monitoring (1993-2019) analysis in deep oligomictic lake

(Lake Iseo, Northern Italy). Poster: *GLEON 2019 All Hands' Meeting*, Huntsville (Muskoka), Ontario (Canada).

6. Nava V., Patelli M., Zanotti C., Rotiroti M., Stefania G.A., Soler V. *et al*. (2018). Chloride balance in freshwater system of a highly anthropized subalpine area: load and source quantification through a watershed approach. Oral communication: *ELLS-IAGLR 2018 "Big Lakes, Small World"*, Evians-les-bains (France).
7. Nava V., Patelli M., Zanotti C., Rotiroti M., Stefania G.A., Soler V. *et al.* (2018). Long-term chloride quantification in a mixed-land-use watershed (Iseo-Oglio system). Oral communication: *34th Congress of the International Society of Limnology - SIL*, Nanjing (China).

A.3. National conferences

1. Nava V., Matias M., Messyasz B., Castillo-Escrivà A., Ersoy Z., Raposeiro P.M. *et al.* (2021). Microalgal colonization of microplastics in experimental mesocosms across the Iberian Peninsula. Oral communication: *XXV Congresso A.I.O.L*. Online.
2. Nava V., Matias M., González Sanchidrián H., Pinedo-Troncoso M., Raposeiro P.M., Ribeiro S. *et al.* (2019). Microplastic colonization by primary producers: a mesocosm experiment across a biogeographical gradient. Oral communication: *XXIX Congresso della Società Italiana di Ecologia (S.It.E)* – Ferrara (Italy).
3. Nava V., Patelli M., Zanotti C., Rotiroti M., Stefania A., Soler V. *et al.* (2018). Effect of hydrological variations and land-use evolution on chloride trend: a long-term analysis in the Oglio River-Lake Iseo system (Northern Italy). Oral communication: *XXVIII Congresso della Società Italiana di Ecologia (S.It.E)*, Cagliari (Italy).

A.4. Awards

- Winner of the student travel award for the Virtual Meeting – ASLO (2021).
- Winner of the travel award for the GLEON 2019 – All Hands' Meeting, Huntsville, Ontario, Canada provided by "Cary Institute of Ecosystems Studies", New York, USA (2019).
- Winner of the Transnational Access (TA) grant from AQUACOSM (funded by the European Commission EU H2020-INFRAIA-project No 731065) for the project "MPhyto: Influence of microplastics on primary productivity" (2019).
- Winner of the "Wetzel SIL Congress Travel Award" for the participation at the 34th Congress of the International Society of Limnology – SIL, Nanjing, China (2018).
- Winner of the fellowship for the participation at "XIV Incontro dei Dottorandi e Giovani Ricercatori in Ecologia e Scienze dei Sistemi Acquatici", Genova, Italy (2018).