BIOACCUMULATION OF TRACE METALS IN THE PLASTISPHERE: AWARENESS OF ENVIRONMENTAL RISK FROM A EUROPEAN PERSPECTIVE

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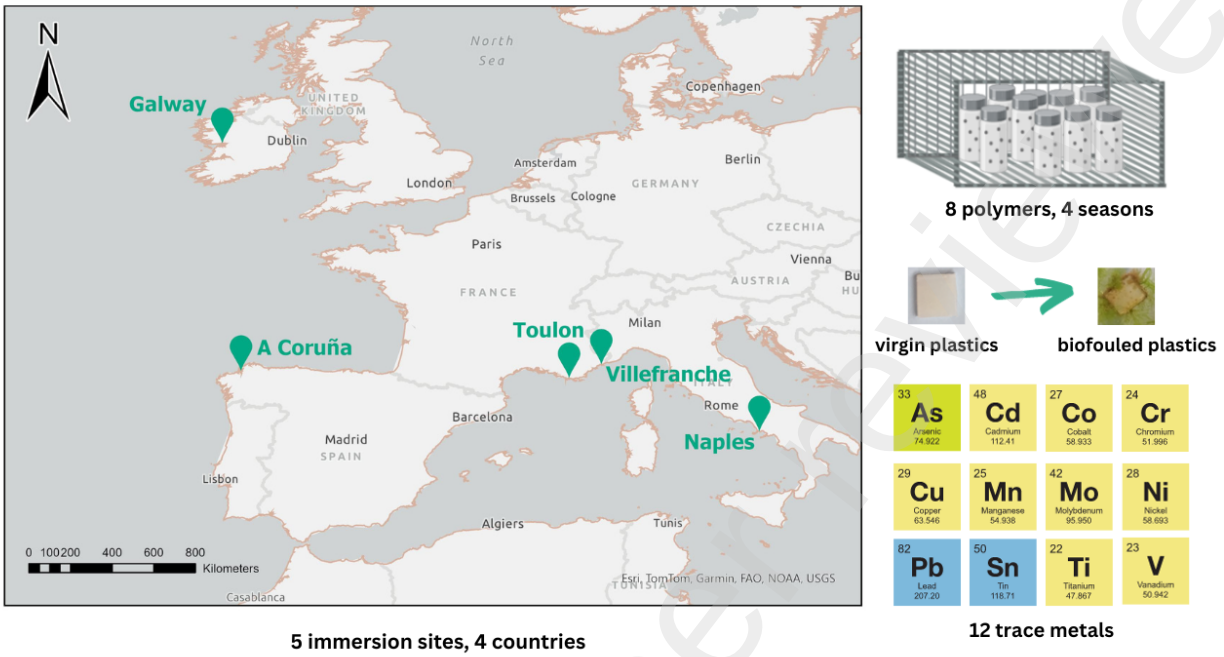
GRAPHICAL ABSTRACT



ABSTRACT

The term "Plastisphere" refers to a biofilm layer naturally formed by microorganisms attaching to plastic surfaces. This layer possesses the capability to adsorb persistent organic and inorganic pollutants, particularly trace metals, which constitutes the central focus of the undertaken research. Immersion experiments were concurrently conducted in five locations spanning four European countries (France, Ireland, Spain, and Italy) utilizing eight distinct polymers. These immersions, repeated every four months over a one-year period, aimed to evaluate the baseline bioaccumulation of 12 trace metals. The study underscores the intricate nature of metal bioaccumulation, influenced by both micro-scale factors (such as polymer composition) and macro-scale factors (including geographical site and seasonal variations). Villefranche Bay in France exhibited the lowest metals bioaccumulation, whereas Naples in Italy emerged as the site where bioaccumulation was often the highest for the considered metals. Environmental risk assessment was also conducted in the study.

The lightweight nature of certain plastics allows them to be transported across significant distances in the ocean. Consequently, evaluating trace metal concentrations in the plastisphere is imperative for assessing potential environmental repercussions that plastics, along with their associated biota, may exert even in locations distant from their point of emission.

KEYWORDS

Plastisphere, metal bioaccumulation, plastic pollution, Environmental risks

1. INTRODUCTION

The unprecedented challenges that the marine environment is facing as a result of human activities, particularly climate change and chemical pollution, have placed humanity outside of the safe operating space (Rockström et al., 2009). Chemical pollution, which includes emerging contaminants such as large plastics (> 5mm), microplastics (1-5 mm) and their chemical additives, is of particular concern (da Costa et al., 2022).

The widespread distribution and accumulation of plastic in aquatic environments has been reported since the early 1970s (Carpenter and Smith, 1972), with an initial focus on baseline concentrations, assessment of sources, pathways and distribution patterns, in both freshwater and marine environments (Galgani et al., 2015; Lambert and Wagner, 2018). Over the last decade, with growing evidence of weathering, degradation and fragmentation of plastics into micro- and nanoplastics, particular attention has focused on ecotoxicology assessments, including leaching of chemicals and additives (Yamashita et al., 2021; Ragusa et al., 2021). In parallel, solution-oriented research into market-based instruments, transition to circular economy models, and mitigation of environmental impacts started to become more prevalent, as the G20 (G20, 2021) and the United Nations (UNEP, 2022) started devising strategies and treaties to minimise impacts caused by plastics.

Plastics are synthetic materials that can be moulded into rigid or mildly elastic forms from a wide range of organic polymers, and can be divided into thermoplastics or thermosets, depending on their elastic properties (Shashoua, 2008). Thermoplastics are known for their ability to soften while heated and for being able to be reshaped and reused. Thermosets, on the other hand, do not soften on heating and cannot be reshaped (Bîrcă et al., 2019). To enhance such physicochemical properties, plastic additives, including pigments, dyes, surfactants, antioxidants, UV stabilisers, flame retardants, plasticisers, lubricants, etc., (Hahladakis et al., 2018; Slama et al,

2021, Wiesinger et al., 2021) are incorporated, which can potentially further leach into aquatic ecosystems (Ngoc Do et al., 2022). Additionally, plastics also have the ability to adsorb hazardous substances such as persistent organic pollutants and trace metals (Liu et al., 2021, Wang et al., 2021). These sorption processes in aquatic conditions are enhanced by the plastisphere, a biofilm layer developing on the surface of immersed plastics, that serves as a hotspot for microorganisms (Pete et al., 2023). Furthermore, the plastisphere harbours fungi (Lacerda et al., 2020) and viruses (Moresco et al., 2022), as well as potentially pathogenic bacteria (Pedrotti et al., 2022; Sababadichetty et al., 2024) hosting antibiotic resistance genes (ARGs) and metal resistance genes (MRGs). In fact, even though the organisms associated with plastics in the ocean had been described in the early 1970s (Carpenter and Smith, 1972), their evaluation gained more attention only in the last decade, after the term 'plastisphere' was created (Zettler et al., 2013).

The co-occurence of plastics and pollutants poses significant risks to marine ecosystems, particularly when combined with degradation and fragmentation processes that increase the plastic surface-to-volume (S:V) ratio (Khalid et al., 2021). Metals and metalloids represent 85% of the elements in the periodic table and are divided into trace metals, which are toxic even at low levels, and oligo-elements, which are essential for physiological processes (Chandrapalan and Kwong, 2021). For example, cadmium (Cd) and arsenic (As) are toxic even at µg/L levels (Rebelo and Caldas, 2016), while trace amounts of copper (Cu), zinc (Zn), selenium (Se) and manganese (Mn) are required for cell growth and reproduction (Chouvelon et al., 2022). Trace metal poisoning can impair growth and development of fish and shrimps, also causing reproductive abnormalities or compromising immune functions (Hossain et al., 2022). As such, the combination of trace metals and persistent organic pollutants in biofouled plastics represents a novel human health concern, due to the toxic potential associated with bioaccumulation, biomagnification, bioleaching, biomineralization, and biotransformation (Syed et al, 2022).

Metals bioaccumulation in the plastisphere is a biofilm-induced process that has been previously described in the literature (Richard et al., 2019; Djaoudi et al., 2022; Sun et al., 2022), with more studies conducted in laboratory settings than *in situ,* and more in freshwater than in seawater (Pan et al., 2023). The work presented in this paper was designed to assess the bioaccumulation of 12 trace metals by the biofilm developing on immersed plastics from five sites across four European countries (France, Ireland, Italy

and Spain). This *in-situ* experiment included eight polymers that were incubated for periods of three months over the course of one year, i.e. repeated every four months, to account for seasonal variations. The main objective of this study was to assess trace metals bioaccumulation in the plastisphere. Therefore, the research addressed the following research questions:

1. Are there seasonal variations influencing trace metals bioaccumulation in the plastisphere?
2. Are there relationships between immersion sites characteristics and metals bioaccumulation?
3. Are there trace metals bioaccumulation differences between fossil-fuel based and bio-based plastics?
4. Are there trace metals bioaccumulation levels that pose a potential risk to the ecosystem?

2. MATERIALS AND METHODS

2.1 Immersion sites

The sampling sites were: Port of Galway, Ireland; Port of Ares (A Coruña), Spain; Toulon Bay, France; Villefranche Bay, France; and Bay of Naples, Italy (Figure 1).

Figure 1 - European *in-situ* cage deployment sites (map produced using ArcGIS Pro, Source: ESRI).

The Port of Galway (53°16’08’’N; 9°02’55’’W) is located in the inner part of Galway Bay, in the west coast of Ireland. The port and the bay are protected from the strong Atlantic Ocean swells by the Aran islands (Frias et al., 2020). Galway city has a population of approximately 83,460 inhabitants (CSO, 2023) and in 2022, about 350 vessels passed through the city’s port carrying approximately 500,260 t of cargo (Port of Galway, 2023). This immersion site was chosen inside the port of Galway, as it is protected from anthropogenic activities at sea.

The Port of Ares (43º25’20’’N; 8º14’22’’W), northwest of Spain, is located about 50 km north from the city of A Coruña, hosting almost 5,800 inhabitants. It is located at the bottom of an inlet 800m deep and 1.6 km wide, protected from the open sea and winds. Its main activity is recreational and sport sailing, so there is a high number of recreational boats that fill the five available

pontoons (Port of Areas, 2023). The immersion site was chosen so as to be protected from any anthropogenic activities.

Toulon Bay (43°04’60’’N; 5°54’00’’W) is located in the southeast of France, within the city of Toulon, a large urban area with a population of approximately 180,000 inhabitants (~600,000 in the metropolitan area). The Toulon Bay hosts various anthropogenic activities (military/civil operations, aquaculture, urban sewage release, industrial shipping), which have been highlighted for their multi contamination ecosystem effects (Dang et al., 2015; Coclet et al., 2018; Layglon et al., 2020). The immersion site was chosen for being a regular monitoring site within the bay.

Villefranche Bay (43°41’47’’N; 7°18’33’’W) is also located in southeastern France, within the city of Villefranche-sur-mer. This city contains a population of approximately 5,000 residents, and is located between the urban centers of Nice and the principality of Monaco. Notably, it serves as a prominent tourist destination, attracting substantial annual visitors. Villefranche Bay experiences maritime traffic mostly by luxury yachts, sailboats, and large cruise vessels, particularly accentuated during the summer months, and it is also a place of shipyards and nautical maintenance (Coglievina et al., 2016). However, distinct from the other sites investigated in this study, fishing and industrial activities are not relevant within this region. This site was chosen as a reference site given its unique conditions.

Naples harbour (40°49’59’’N; 14°15’11’’W), located in the southeastern Tyrrhenian Sea, within the city of Naples (Italy), which has a population of about 4 million people (~2 million in the metropolitan area) and presents diverse urban and industrial activities, which strongly impact its water quality (Cianelli et al., 2012; Esposito et al., 2020). With an annual traffic capacity of around 25 million tons of cargo and 500,000 TEUs capacity, the port of Naples is one of the main ports in Europe. It also serves as a tourist hub, servicing an estimated 10 million people annually transiting through the port. The immersion site was situated in a marina very close to the commercial port, site of three yacht clubs, so as to be protected from the commercial routes and other activities, but still representative of an urban-influenced coastal area.

2.2 Experimental design

Stainless steel cages (81.3L x 27.9W x 33H cm) were used as a holding structure to harbour plastic containers that were drilled to allow water circulation, where plastics were introduced. One cage was deployed in each immersion site, with eight containers, each carrying one polymer. The polymers used in this work were: 1) High-Density PolyEthylene (HDPE), 2) Low Density PolyEthylene (LDPE), 3) Polyethylene Terephthalate (PET), 4) recycled Polyethylene Terephthalate (PET-Post), 5) PolyPropylene (PP), 6) recycled PolyPropylene (PP-Post), 7) PolyStyrene (PS) and the 8) bioplastic PolyLacticAcid (PLA). Polymers were chosen based on their relevance and use in global markets. All polymers were acquired from the same batch and supplier (Carat-lab GmbH, Germany), and were provided to partners in 6 x 6 cm sheets (1 mm thickness, n = 50). Each laboratory cut the sheets into 1 cm^2 squares, to have 100 pieces in each container, for several analyses in the MicroplastiX project. For the trace metals analysis, 5 pieces at the start of the project and 5 pieces after three month incubation were sampled (Figure 2a).

After 3 months of immersion, the bottles were retrieved and replaced by new ones, containing pristine virgin polymer pieces, for another trimester. This was performed 4 times, to cover a period of one-year immersion, reducing the effect of overlapping seasons. Furthermore, throughout the immersion periods, the sea temperature was regularly monitored to assess whether it influenced trace metal accumulation.

At each sampling time, the individual pieces of each polymer were retrieved into sterilised trace metal grade conical tubes (VWR) where the plastisphere was digested using a 5 mL sodium hydroxide (NaOH) 0.1 M solution (Suprapur quality, 99.99%). After 24 h, the plastic pieces were removed from the solution, using plastic forceps under a controlled fume hood. To the remaining solution, 50 µL nitric acid (HNO_3) Suprapur solution was added (to reach final 10% concentration) and kept in the dark until shipping (Figure 2b) and further analysis. Pristine pieces were also digested following the same protocol, to ensure that no metal leaching occurred from the various polymers. All solutions used in processing (including NaOH), were analysed as blanks and controls to quantify the baseline trace metal content.

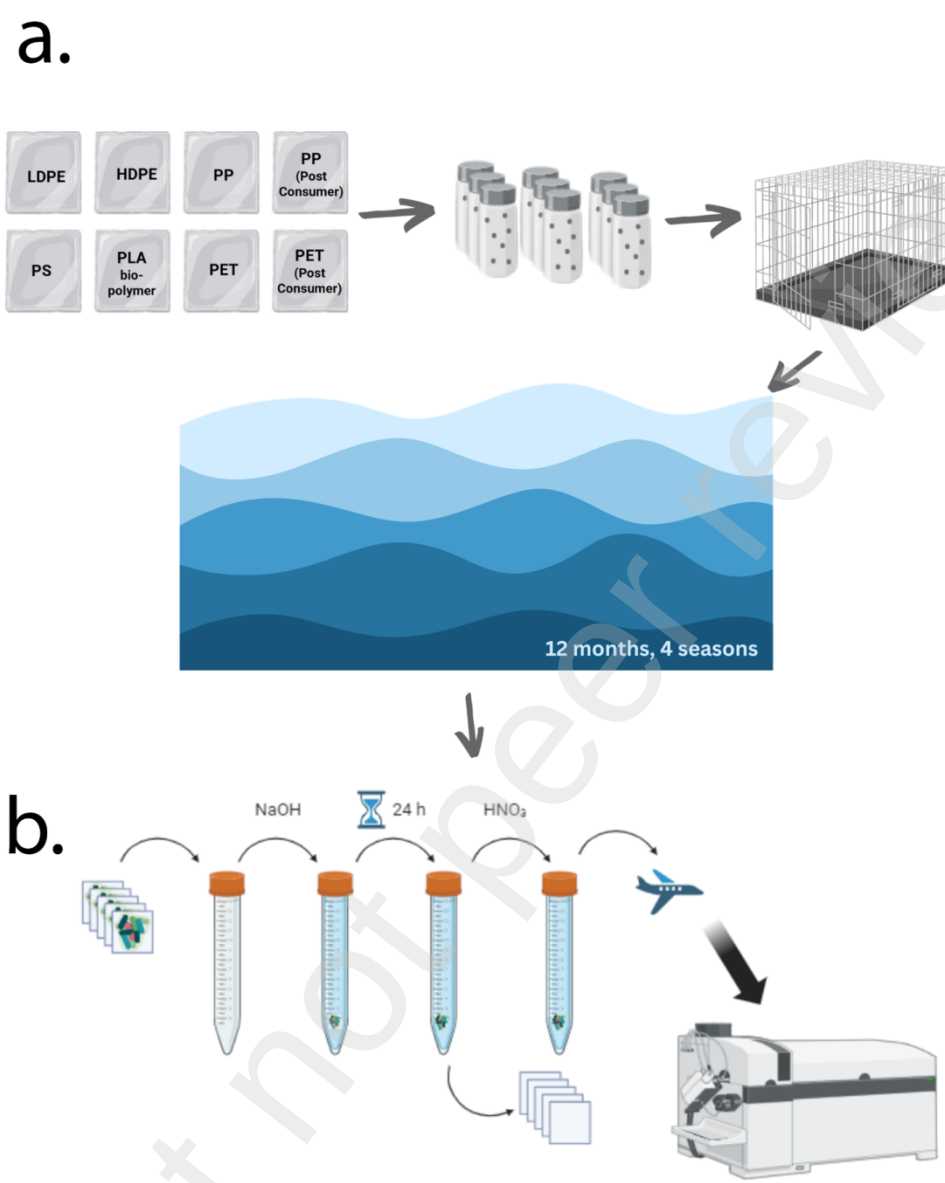


Figure 2 - Experimental design. a) *In-situ* immersion deployment, b) Digestion of plastisphere and ICP-MS processing (image produced in Adobe Photoshop. Source: Biorender and Canva).

2.3 Trace metals analysis

Concentrations of 12 trace metals (arsenic (As), cadmium (Cd), cobalt (Co), chromium (Cr), copper (Cu), manganese (Mn), molybdenum (Mo), nickel (Ni),

lead (Pb), tin (Sn), titanium (Ti) and vanadium (V)) were assessed in two matrices: a) the digested biofilm from the eight polymers and b) the surrounding seawater. Processing was conducted at the Ruđer Bosković Institute (Croatia) using an Inductively Coupled Plasma Mass Spectrometer (ICP-MS, Agilent 8900 qqq), with a 20-fold dilution for seawater samples to reduce salt-matrix effect. All samples were spiked with an Internal Standard (Indium). A certified reference material (CASS-5, Nearshore seawater reference material for metals, National Research Council Canada) was used as a quality control of ICP-MS measurements. Two separate CASS-5 control samples were measured after every 10–15 samples. Matrix matching calibration (in 20-fold diluted CASS-5 sample) was used for concentration quantification. Determined concentrations of trace metals in the CASS-5 sample were within 15% of the certified reference.

The measured concentrations were expressed in µg of metal/L and harmonised according to the volume of NaOH used. The obtained values are corresponding to the metals bioaccumulated in the biofilm which developed around the immersed plastic pieces. Throughout the text, the term "bioaccumulation" refers to this process of metals assimilation by the biofilm.

2.4 Environmental risk assessment

To assess environmental risk, the Risk Quotient (RQ) was calculated. This parameter is obtained by calculating the Measured Environmental Concentration (MEC) divided by the Predicted No-Effect Concentration (PNEC) (Liu et al., 2015, Eq. 2). The MEC values were obtained from the plastisphere analysis and the PNEC concentrations were obtained from the NORMAN Ecotoxicology Database (NORMAN, 2023), which is based on the European Directive (EU, 2013) and on the REACH registration Process (ECHA, 2023). The RQ was calculated for all considered metals, for all immersion sites and all polymers. The risk quotient is classified as follows: when RQ<0.01, there is no environmental risk; when 0.01<RQ< 0.1, the risk is considered low; when 0.1<RQ<1, there is medium risk; when RQ>1, the risk is high to very high (Nika et al., 2020).

2.5 Statistical analysis

All statistical analyses were performed with RStudio 2022.12.0.353 software (RStudio Core Team, 2023) under R 4.2.2 environment. The variability of

metals bioaccumulation according to seasons and polymers for each immersion site was represented through standardized Principal Component Analysis (PCA) with *FactoMiner* and *ggplot2* packages. Significant contribution of the represented variables was verified with the *factoextra* package.

3. Results and Discussion

3.1 Seawater temperature

Seawater temperature at each immersion site was measured to account for seasonal variations within and between sites (Figure S1). Galway was the coldest site of all surveyed, with an average water temperature in summer around 20°C, which is the equivalent temperature experienced in the Mediterranean during late spring and early autumn. The site with the smallest variation is A Coruña, where the range went from 12°C in winter to 18°C in summer.

3.2 Trace metals bioaccumulation

The bioaccumulation of trace metals, expressed in µg/L, were variable across seasons and sites. Villefranche Bay frequently presented the lowest bioaccumulation, while Naples consistently presented amongst the highest bioaccumulation (Figure 3). The higher bioaccumulation levels were particularly apparent for Ti (181-fold increase), Ni (15-fold increase), Cr (10-fold increase), V (8-fold increase), Pb (5-fold increase) and Mn (2-fold increase).

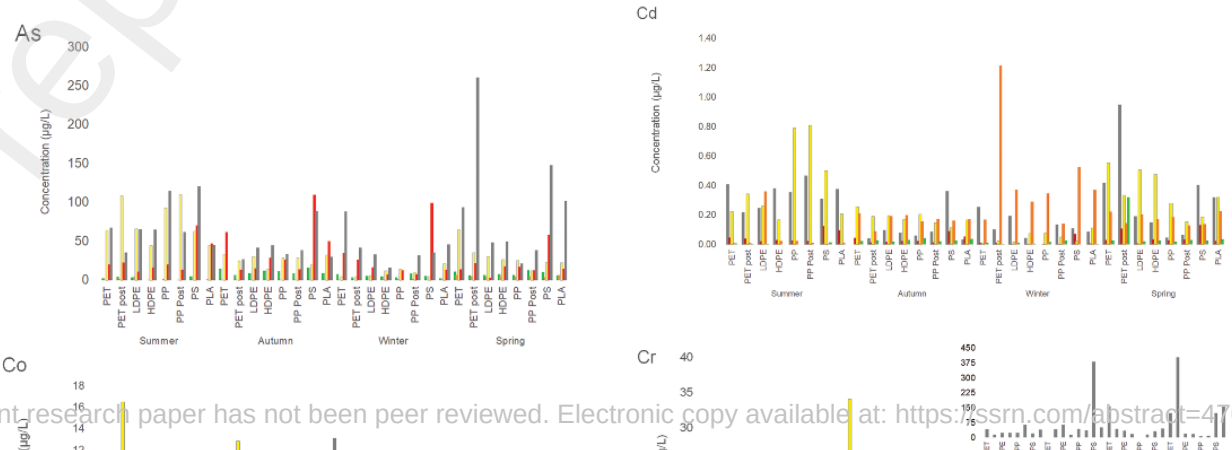
In Villefranche Bay, bioaccumulation was generally low, except for Mo, Ni, Pb, Ti and V (Figure 3). Small bioaccumulation of As, Cd and Cr was found at this site (below 15, 0.04 and 0.3 µg/L, respectively). Bioaccumulation of Ti was relatively high in spring for all polymers (around 45 µg/L), and also in autumn, however, to a lesser extent (around 30 µg/L). High bioaccumulation in winter and spring was shown for V, and Sn had high levels in the biopolymer PLA. For Cu and Mn, both oligo-elements, the bioaccumulation was very low (<10 µg/L for both elements), and for Co, a very toxic element, bioaccumulation was the lowest (< 0.3 µg/L) compared to the other sites.

The Port of Galway presented low levels of As, Co, Cd, Ni and Sn throughout all seasons (Figure 3), except for Sn on PP-post in summer. Bioaccumulation of Mn, Pb and V were low at this site (< 50, 6 and 2.5 µg/L, respectively), except in spring. Galway has low bioaccumulation of Cu (< 25 µg/L) all year around. Similarly, Mo and Ti were also bioaccumulated in low levels throughout the year (< 1 and 15 µg/L) except for summer.

In Toulon, Co, Cu, Mn and Ni were more bioaccumulated in summer (71, 2.1, 250, 241 and 9.5 µg/L, respectively) (Figure 3). As, Cr, Mo, Pb, Ti and V were bioaccumulated all year except in winter, with a maximum in summer for Pb and Ti (200 and 51 µg/L, respectively). Cd and Sn bioaccumulation was very low all year (< 0.1 µg/L for Cd and < 1.5 µg/L for Sn, except for bioaccumulation promoted by PS and PLA which was higher).

In A Coruña, winter was also the season promoting the lowest metals bioaccumulation (Figure 3). As, Cd, Co, Ni and V were bioaccumulated all year except in winter, with a maximum in summer (110, 0.8, 16.5, 53 and 93 µg/L, respectively). Cr, Mn, Pb and Ti were more bioaccumulated in autumn (35, 955, 73 and 103 µg/L, respectively). Cu and Mo were more bioaccumulated in summer (323 and 11 µg/L, respectively). Sn was always low (< 0.8 µg/L , except for bioaccumulation promoted by PLA which was higher).

Among the measured trace metals, 10 of the 12 assessed presented bioaccumulation among the highest in samples immersed in Naples. In this site, 4 trends can be observed. As, Cd and Cu were more accumulated in summer and spring (at maximum 260, 0.95 and 1560 µg/L, respectively) (Figure 3). Cr, Pb and Sn were more accumulated in spring and autumn, with a higher accumulation in spring (402 and 1075 µg/L for Cr and Pb, respectively) or autumn (36 µg/L for Sn). Ni and Ti were mainly accumulated in autumn (603 and 19455 µg/L, respectively). Co, Mn, Mo and V were accumulated all year, except winter, with maximum in summer for Co and Mn (8 and 1980 µg/L, respectively) or spring for V (391 µg/L at maximum).



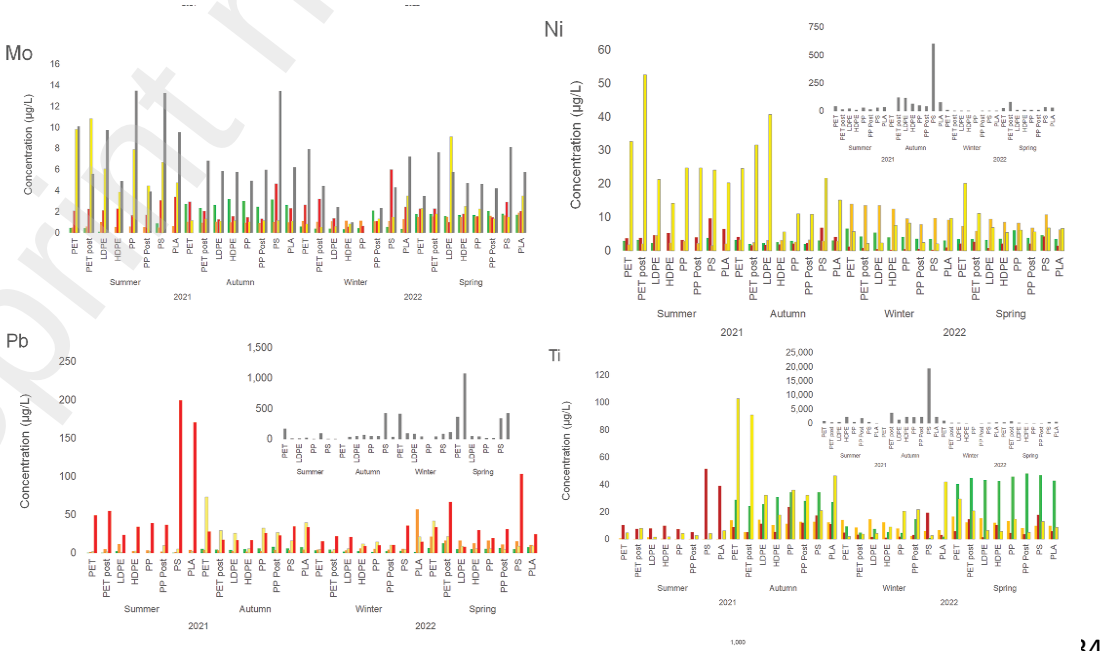
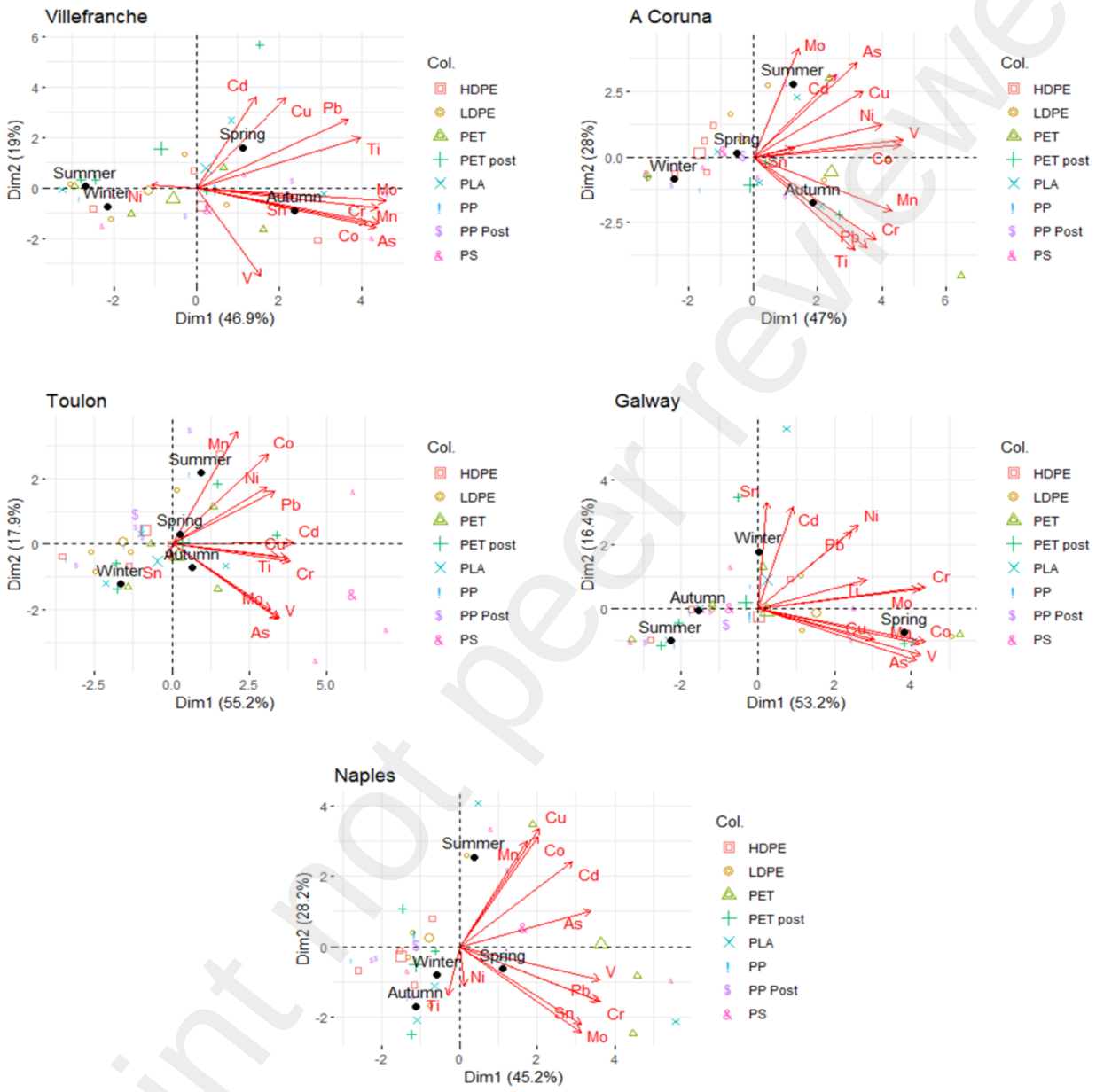


Figure 3: Bioaccumulation (µg/L) of trace metals in the plastisphere. When required, Naples results are shown in separate plots due to changes in bioaccumulation scale.

A Principal Component Analysis (PCA) was performed using the dataset for each site (Figure 4).



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375 Figure 4: Principal Component Analysis of the metals bioaccumulation as a function of season
376 and polymer, in each immersion site.

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The two-dimensions represented between 70 and 80% of the observed variations. Such representation highlighted the concomitant trace metals bioaccumulation as well as seasonal correlations.

Concerning seasonal variations in Villefranche (Figure S2), metals bioaccumulation were correlated to spring and autumn and Ni showed a disparaging behaviour from any other metal for this site. For this site, two subsets of metals were evident: one with Cd, Cu, Pb, Ti on one side, and As, Co, Cr, Mn, Mo and V below, in spring and autumn. This is in agreement with the results shown in Figure 3, where Cd, Cu, Pb and Ti had higher bioaccumulation in spring and in PET-Post. For the second group (As, Co, Cr, Mn, Mo and V), the bioaccumulation was higher in autumn and spring, and PS and PLA were the polymers with the highest bioaccumulation in autumn, while PP-Post had relatively higher bioaccumulation in spring.

In Galway, winter and spring were the seasons promoting the higher bioaccumulation for the considered trace metals, with one main group correlated to PET and PET-Post (As, Co, Cu, Mn and V).

In Toulon, metal bioaccumulation was correlated with summer, autumn and spring. Three metals (As, Mo and V) were higher in spring and autumn, while Cr, Cu and Ti were higher in the spring-summer period. Two polymers (PLA and PS) had systematically higher metals bioaccumulation in summer, across all sites.

In A Coruña, metals bioaccumulation was correlated with summer and autumn (Figure S2). Two groups were observed depending on the season: Cr, Mn, Pb and Ti in autumn, and Co, Cu, Ni, Sn and V in summer. For the first group (Cr, Mn, Pb and Ti) the highest metal bioaccumulation (Figure S2) occurred on PET and/or PET-Post in autumn. For the second group, the highest metals bioaccumulation occurred for PET-Post and PLA in summer.

In Naples, metals bioaccumulation was correlated with summer and spring seasons (Figure S2). The group of Cu, Co, Cd and Mn had higher bioaccumulation in summer, while the group of Cr, Mo, Pb, Sn and V was correlated with spring. The first group showed higher bioaccumulation in PET and PLA for summer, while the second group had higher bioaccumulation in PET and PET-Post in spring.

3.3 Polymer type as a substrate and its influence on metals bioaccumulation in biofilms

When considering metals bioaccumulation as a function of polymer type (Figure 3), in Villefranche, the polymers promoting higher metal bioaccumulation were PS, PLA, PET and PET-Post. In spring, both PET-Post and PP-Post promoted higher metals bioaccumulation, except for As, Co, Cr, Mn on PET-Post, and for Ni in PP-Post. In summer, both recycled plastics promoted an increase in As, Cu, Ni, Pb and Sn loadings. For the other trace metals, the recycled plastics did not seem to have an effect, except for a decrease in V bioaccumulation. In autumn, both recycled plastics promoted a decrease in all metals bioaccumulation, except for Cd and Cu (on PET-Post) and Cr, Cu, Pb Sn on PP-Post. In winter, the trends between recycled plastics were completely opposite: PET-Post promoted a decrease for all metals (except for Pb and Cu) whereas PP-Post promoted an increase (except for Ni).

In Galway, the polymers promoting the higher metals bioaccumulation were PET, PET-Post, LDPE and PLA (Figure 3). Both PET-post and PP-post were mainly promoting a decrease in metals bioaccumulation during the seasons where the highest bioaccumulation was observed (winter and spring). In summer and autumn, recycled plastics presented opposite trends. PET-Post increased metals bioaccumulation (except for Cd, Mn, Ti) in summer and decreased metals bioaccumulation (except for V) in autumn, whereas PP decreased metals bioaccumulation (except for Cd) in summer and increased metals bioaccumulation (except for Sn) in autumn.

In A Coruña, the polymers promoting the higher metals bioaccumulation were PET, PET-Post and PLA (Figure 3). For these polymers, the highest bioaccumulation was measured in summer and autumn. In summer, PET-Post mainly promoted an increase in metals bioaccumulation (except for Mn, Pb), when no significant trend was observed for PP-Post. In autumn, both recycled plastics promoted a decrease in metals bioaccumulation (except for Ni on PET-Post). PP-Post promoted a decrease in metals bioaccumulation in autumn, winter and spring (except for Sn). In winter, PET-Post promoted an increase in metals bioaccumulation (except for Co, Mn Ni) as opposed to spring, where PET-Post promotes a decrease for all metals.

In Toulon, PS and PLA were systematically promoting the higher metals bioaccumulation (Figure 3). In spring, both PET-Post and PP-Post promoted an

increase in metals bioaccumulation (except for Sn and Ti). In summer, no trend could be drawn out. In autumn, both recycled plastics were promoting a decrease in metals bioaccumulation. In winter, the effect was opposite, PP-post promoted an increase (except for As, Cd and Co), whereas PET-Post promoted a decrease (except for Cd, Mo, Pb and V).

In Naples, the polymers promoting the higher metals bioaccumulation were PET-Post, PS and PLA (Figure 3). In spring, both PET-Post and PP-Post promoted an increase in metals bioaccumulation (except for Mn and Ni on PP-Post). In summer and winter, on the contrary, PET-Post promoted a decrease in metals bioaccumulation. In summer and in autumn, PP-Post did not present any trends.

The cumulative trace metals bioaccumulation at all sites in all seasons were plotted to identify which polymers promoted the higher metal bioaccumulation (Figure S2).

Generally, cumulative bioaccumulation of metals was within the same order of magnitude for each polymer type, with some exceptions for PET, PET-Post, PP, PS and PLA. Bioaccumulation of As, Cu, Cr, Mn, Mo, Ni, Pb and Ti, also had a different order of magnitude when compared to Cd, Co or Sn. Nonetheless, the highest bioaccumulation of Sn was recorded from PLA. If focusing on PLA, results here show that this polymer was often promoting a high metals bioaccumulation at all sites (Figure S2). In Villefranche, it promoted high bioaccumulation of Cd, Cr, Cu, Mo and Pb in autumn and spring (Figure 3). In Galway, PLA promoted high bioaccumulation of Pb and Ni in winter, especially Pb. In this site, PLA also promoted the highest Cd, Cr, Sn and Ti bioaccumulation among all polymers. In winter, which was a season promoting metals bioaccumulation in Galway, PLA was often among the polymers reflecting the highest bioaccumulation. In Naples, PLA promoted one of the highest bioaccumulation in spring, for all the measured metals (Figure 3). In Toulon, PLA promoted one of the highest bioaccumulation in summer for all the measured metals, in addition it promoted the highest Sn bioaccumulation in spring. In A Coruña, PLA promoted among the highest bioaccumulation in autumn and winter for all measured metals. It also promoted high bioaccumulation in summer for Cu, Mn, Ni, Sn and V.

3.4 Ecosystem Risk Assessment

Risk Quotients (RQ) were calculated for all metals (Table S2). For all immersion sites, RQ values for Arsenic were high and this will be further discussed. RQ values for three trace metals (Cr, Cu and Pb), were selected according to their toxicity or variations and are presented in Figure 5.

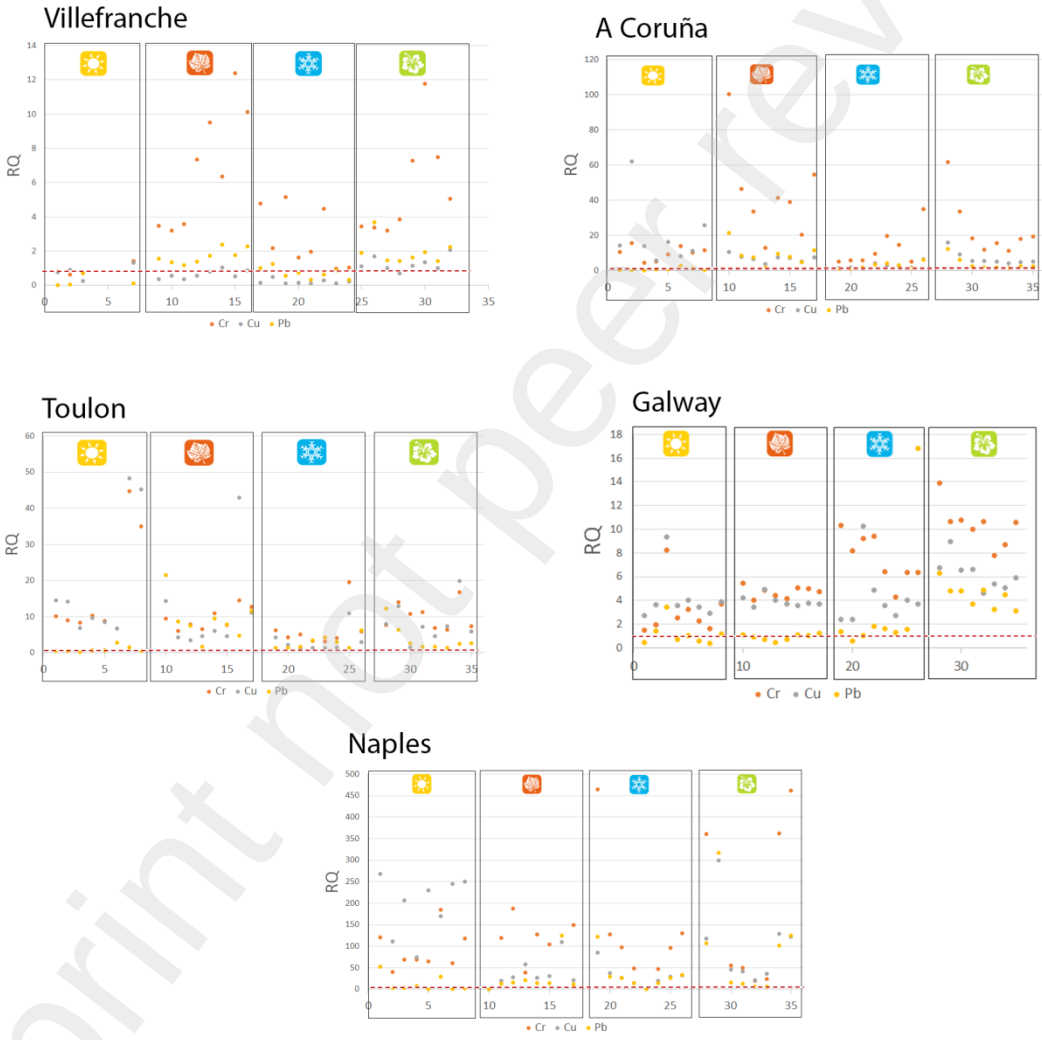

Figure 5: Risk quotients (RQ) for metal bioaccumulation at all immersion sites. The red dotted lines represent RQ value of 1, above which the environment is considered to be at high risk (Nika et al., 2020). On each graph, the season is evidenced by a symbol.

In Villefranche, RQ for Cd, Mo and Ti were always below 0.01 (Table S2), which means that these trace metals did not represent environmental risk. RQ values for Co, Cu, Ni, Sn and Pb were always below 1 (except on some occasions), showing medium risk for these metals (Figure 5). RQ values for Mn were above 1 in autumn and spring only. RQ values for Cr were below 1 in summer only. Finally, RQ values for V were always above 1 (reaching 13 at maximum in autumn), demonstrating high risk for this contaminant. RQ for As in Villefranche ranged between 55 and 315, indicating a high risk for this element.

In Galway, RQ values for Mo and Ti were always below or close to 0.01 (Table S2), meaning that these trace metals did not represent an environmental risk. RQ values for Cd, Co, Ni, Sn and V were below 1, representing a medium risk, except in winter (and spring for V; Figure 5). RQ values for Cr, Cu and Pb were always above 1 (maximal value around 16), demonstrating a high risk for these trace metals, especially in winter and spring (Figure 5). RQ values for Mn and As were even higher, reaching values as high as 130 (in spring), showing that these contaminants are linked with high environmental risk, especially in winter and spring.

In A Coruña, RQ values for Mo, Sn and Ti were always below or close to 0.01 (Table S2), meaning that these trace metals did not represent an environmental risk. RQ values for Cd and Ni were always around 1, whereas those for Co, Cr, Cu, Pb and V were much higher, with a general decrease in winter (Figure 5). RQ values for Mn and As were really high.

In Toulon, RQ values for Mo and Ti were always below or close to 0.01 (Table S2), meaning that these trace metals did not represent an environmental risk. RQ values for Cd, Co, Ni and Sn were always low, proving that these trace metals did not impact on the environment. RQ values for Cr, Cu, Mn, Pb and V corresponded to medium to high environmental risk (Figure 5), with a general decrease in winter for all, especially for Mn, which turned to low environmental risk at that season. Like all other immersion sites, RQ values for As were particularly high.

In Naples, all RQ values were above the range of low environmental risk, except for Mo. The RQ values for Cd, Sn and Ti were in the medium environmental risk, except for summer and spring in the case of Cd, and in autumn in the case of Ti (Figure 5 and Table S2). RQ values for Co and Ni were

always above 1, demonstrating the risk associated with these trace metals. RQ values for Cr, Cu, Mn, Pb and V were really high, though not reaching RQ values for As (Table S2).

4. Discussion

This work explores the bioaccumulation of trace metals in biofilm growing on immersed plastic pieces from eight polymers, at five European coastal sites over a one-year period. The findings presented herein significantly contribute to the understanding of bioaccumulation of trace metals within the plastisphere, a rather understudied research topic, as they cover both Atlantic and Mediterranean sites. Our results showed a high variability depending on polymer type and associated trace metals. In this section, we advance hypotheses for the high metals bioaccumulation observed at certain sites, when possible.

As previously mentioned, Villefranche Bay is a protected area with low fishing, industrial and anthropogenic impacts, where we hypothesised that bioaccumulation of trace metals would be generally low, and this was confirmed by our results. In contrast, due to its proximity to many anthropogenic activities, Toulon harbour is known for a wide-range metallic contamination in water and seafloor sediments (Tessier et al., 2011; Layglon et al., 2021). This range of contamination is linked to high bioaccumulation of trace metals in the biofilm developing on the plastic pieces immersed at this site. Similarly, Naples, the site with the larger volume of cargo and anthropogenic activities, has a multiple range of contamination sources, which are thought to contribute to the highest metals bioaccumulation observed. The results of this study show that the scale of nearby anthropogenic activities might be the factor that has the highest influence on the metal bioaccumulation by the plastisphere. In A Coruña, despite the main activities being recreational and sport sailing, bioaccumulation of trace metals were relatively high, potentially reflecting historical and present anthropogenic activities still reflected in high bioaccumulation.

As already mentioned, a wide range of polymers, including both pristine and post-consumer fossil fuel based, as well as biopolymers, were used in this study to understand whether polymer type is one of the factors influencing trace metal accumulation onto the plastisphere. Plastic is a known substrate for the growth of micro- but also macroorganisms, including bryozoans and barnacles (Li et al., 2016), and is described as a potential vector for invasive species and/or potential pathogenic bacteria and viruses, hosting antibiotic resistance genes (ARGs) and metal resistance genes (MRGs) (Pedrotti *et al.,* 2022; Li et *al.*, 2022; Sababadichetty et al., 2024). The accumulation of both

organic and inorganic pollutants is expected to increase the resistance genes within the plastisphere (Li et al., 2022); this, in turn, increases the exogenous pressures of metals, triggering a cause-effect pattern which is the rationale behind this study. Separate papers assessing persistent organic pollutants (Concha-Graña et al, 2022) and the genetic diversity (Pedrotti et al., 2022; Lacerda et al., 2023) of the plastisphere from the same incubation experiment were already published, and will be published in parallel in this special issue.

In scientific literature, *in situ* immersion tests assessing pristine and recycled plastics and relating these to trace metals bioaccumulation are scarce, if nonexistent. Therefore, this study provides a valuable dataset and provides insights into a problem that deserves more attention. Trace metal bioaccumulation is incredibly complex, and as seen in this work, where geographical location, seasonality and polymer type were assessed, it depends on many variables. Regarding polymer type, PET, PET-Post, PS and PLA showed the highest bioaccumulation of metals by the plastisphere. This widens the toxicity linked to PET, PS and PLA, as they were already demonstrated to affect the gut microbiota of marine fish, even in their pristine form (Zhang et al, 2023). Understanding the physico-chemical properties of these polymers (hydrophobicity, surface charge, crystallinity, tensile structure, surface-area ratios, etc.) under natural environmental conditions will allow a better understanding of how weathering, degradation and fragmentation and/or inclusion of chemical additives contribute to the formation of biofilm layers. This will also allow us to understand how biofilm genetic diversity is linked to the adsorption and/or absorption of persistent organic and inorganic pollutants. The complexity of this assessment requires a transdisciplinary approach where microbiologists, chemists and material scientists collaborate to mitigate potential risks, as highlighted by Pedrotti et al. (2022), particularly with potentially pathogenic bacteria and viruses hitchhiking in plastics and representing vectors for invasive species and potential spread of diseases.

This work explores both pristine and recycled plastics. A recent study on the impact of recycling on PLA physical properties concluded that a succession of recycling operations would not affect the polymer properties (Davies et al., 2023). Here, from a microbiological and a chemical point of view, recycling had an effect, even though the trends were not possible to generalise. Yet, it has to be underlined that PET-Post (and PP-Post in Villefranche) is on average promoting higher metal bioaccumulation than the pristine counterpart.

PolyLacticAcid (PLA), a popular commercial biopolymer used in this study, has become a popular alternative to fossil fuel based polymers, as its synthesis uses bioproducts that can be easily degraded in recycling plants (Park et al., 2020; Sun et al., 2022). PLA is a brittle and stiff polymer that requires chemical additives such as plasticizers to be added during production to enhance flexibility and toughness (Park et al., 2020). This polymer has similar fragmentation rates to other fossil-fuel based polymers (Sun et al., 2022), making it ideal for comparative studies Overall, PLA promoted higher metals bioaccumulation at all immersion sites of this study, which could potentially be linked to its structural composition. In Toulon, PLA always led to the higher bioaccumulation for all evaluated trace metals (Figure S2). In all immersion sites and seasons cumulated, PLA presented significantly higher Sn bioaccumulation (Figure S2). It can therefore be concluded that PLA, at least in our tested *in situ* conditions, represents a threat as important as fossil-fuel based polymers. These results also underline that the term “biodegradable” should be supported by the conditions used for the test (Paul-Pont et al., 2023).

This study assessed environmental risk (using RQ values), to understand ecotoxicological impacts. Arsenic (As) was shown to be a metal with very high risk across all sampled sites. This could be explained by the chemistry of arsenic species, as it was mainly found in its oxic phase. As a matter of fact, in oxic conditions like the ones encountered at all immersion sites, arsenic is mainly present as arseniate, an oxyanion whose size, with charge and structure are really close to that of phosphate (Willsky and Malamy, 1980). These arsenic species could therefore use phosphate pumps that are present within the bacteria and the biofilm (Pothier et al., 2018). This could explain the high bioaccumulation at all immersion sites. Cadmium (Cd), molybdenum (Mb) and titanium (Ti), on the other hand, have low risk across all sites.

Apart from Villefranche (which can be considered our reference site), all immersion sites showed potential ecological risk. This is particularly relevant because colonized microplastics are more likely to be ingested by marine organisms (Yu et al., 2023), and this would result in bioaccumulation of metals across marine food webs, potentially representing a human and environmental threat. However, to our knowledge, there are no studies that reflect this causal relationship. Nonetheless, given the fishing (e.g. Naples) and aquaculture (e.g Toulon, and Galway) activities occurring at some of the sites, precaution should be taken.

In the literature, there appears to be a positive relationship between the plastisphere and metals bioaccumulation, as some studies report that trace metals bioaccumulation increase with growth of the plastisphere (Richard et al., 2019; Forero-Lopez et al., 2022). Once again, our results show that this is a complex matter, as metal bioaccumulation was not systematically higher in areas with higher biofilm growth. Galway is a typical counter-example of this statement, as for some metals, the highest bioaccumulation occurred in winter, when the temperature was the lowest, and biofilm growth was relatively slower. Additionally, all polymer types (without biofilm) tend to adsorb similar concentrations of metals, as shown for San Diego Bay (Rochmann et al., 2014). Instead, our results seem to show otherwise, even though the sites investigated are all substantially different in terms of environmental characteristics. We could hypothesise that metal bioaccumulation would be higher in areas with extensive anthropogenic activities, however, the opposite was recorded here, where in Villefranche Bay the bioaccumulation of Ti and V were both high.

The results presented here demonstrate that the nature, rather than solely the biomass, of the plastisphere play an important role in accumulating trace metals from the surrounding environment.

5. Conclusion

The results presented in this study clearly established a discernible correlation between seasonal variations and the bioaccumulation of trace metals in the plastisphere. The seasons conducive to higher biofilm proliferation consistently exhibited elevated levels of metals bioaccumulation, each site exhibiting unique characteristics. Notably, Galway displayed a sustained peak in bioaccumulation during the winter season. The site with the lower anthropogenic activities, namely Villefranche, demonstrated the lowest metals bioaccumulation. Conversely, sites characterized by extensive anthropogenic influences consistently exhibited elevated metals bioaccumulation in the plastisphere. Villefranche therefore emerged as a reference area in the Mediterranean Sea for evaluating trace metals bioaccumulation in the plastisphere. The polymers promoting biofilm development with higher metals bioaccumulation were PET, PET-Post, PP, PS and PLA. Polylactic Acid (PLA), a biopolymer usually used as a replacement of fossil fuel based polymers, was shown here to promote similar or higher bioaccumulation of trace metals by the plastisphere. RQ values highlight that

trace metals bioaccumulation can pose ecological risk, however, the complexity of this process requires further investigation. The data provided here provides new insights on how bioaccumulation relates to seasonality and polymer type. Further research into the impacts or interactions between the plastisphere and organic and inorganic pollutants is required to understand and potentially mitigate their effects to marine and human health.

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Figure Captions

Figure 1 - European *in-situ* cage deployment sites (map produced using ArcGIS Pro, Source: ESRI)

Figure 2 - Experimental design. a) *In-situ* immersion deployment, b) Digestion of plastisphere and ICP-MS processing (image produced in Adobe Photoshop. Source: Biorender and Canva).

Figure 3 - Bioaccumulation (µg/L) of trace metals in the plastisphere. When required, Naples results are shown in separate plots due to changes in bioaccumulation scale.

Figure 4 -Principal Component Analysis of the metals bioaccumulation as a function of season and polymer, in each immersion site.

Figure 5: Risk quotients (RQ) for metal bioaccumulation at all immersion sites. The red dotted lines represent RQ value of 1, above which the environment is considered to be at high risk (Nika et al., 2020).

Table Captions

Table S1: List with predicted no-effect concentration (PNEC) and other reference values for trace metals in this study.

Table S2: RQ values for each trace metal, according to site, season and polymer type.

Figure S1: Seawater temperature (°C) in each immersion site. The season is evidenced by a symbol.

Figure S2: Cumulative metal bioaccumulation at all sites and seasons, per polymer.

Supplementary Material

Table S1: List with predicted no-effect concentration (PNEC) and other reference values for trace metals in this study.

Trace metal	CAS Number	NORMAN Ecotoxicology Database
Arsenic (As)	7440-38-2	PNEC: 0.5 µg/L (freshwater) PNEC: 0.05 µg/L (marine water)
Cadmium (Cd)	7440-43-9	PNEC: 0.08 µg/L (freshwater) PNEC: 0.2 µg/L (marine water)
Cobalt (Co)	7440-48-4	PNEC: 2.36 µg/L (marine water)
Chromium (Cr)	7440-47-3	PNEC: 3.4 µg/L (freshwater) PNEC: 0.34 µg/L (marine water)
Cupper (Cu)	7440-50-8	PNEC:1.0 µg/L (freshwater) PNEC: 5.2 µg/L (marine water)
Manganese (Mn)	7439-96-5	PNEC: 123 µg/L (freshwater) PNEC: 3.4 µg/L (marine water)
Molybdenum (Mo)	7439-98-7	PNEC: 136 µg/L (freshwater) PNEC: 1910 µg/L (marine water)
Nickel (Ni)	7440-02-0	PNEC: 4.0 µg/L (freshwater) PNEC: 8.6 µg/L (marine water)
Lead (Pb)	7439-92-1	PNEC: 1.2 µg/L (freshwater) PNEC: 1.3 µg/L (marine water)
Tin (Sn)	7440-31-5	PNEC: 10 µg/L (marine water)
Titanium (Ti)	7440-32-6	PNEC: 600 µg/L (marine water)
Vanadium (V)	1314-62-1	PNEC: 2.5 µg/L (marine water)

Table S2 in a separate document

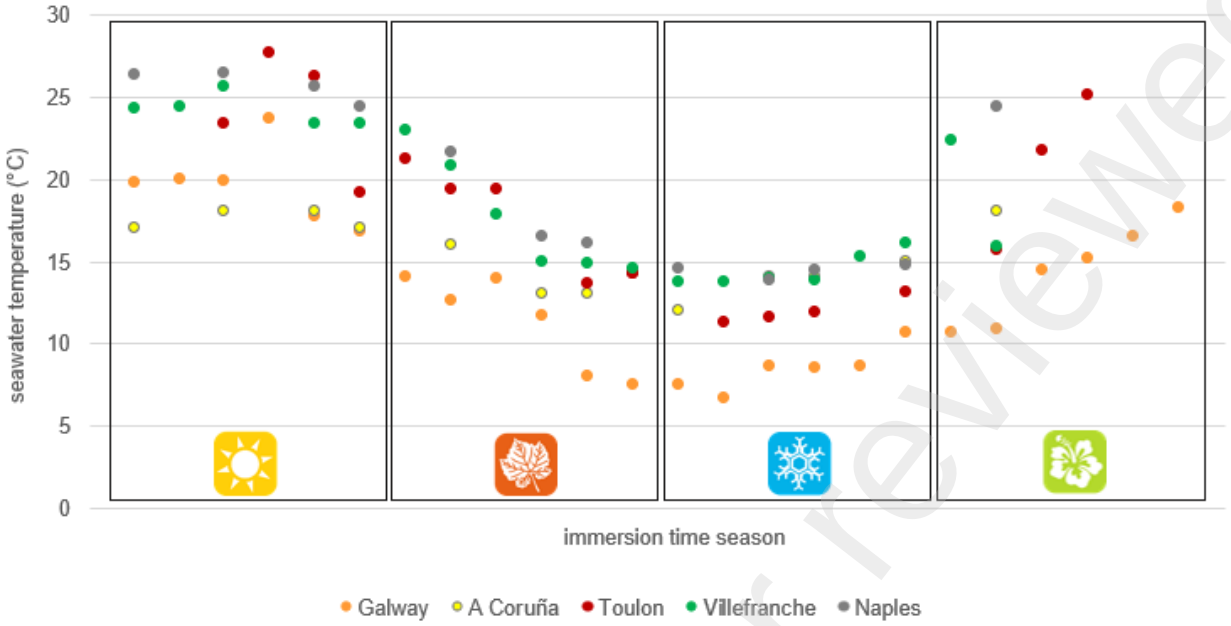
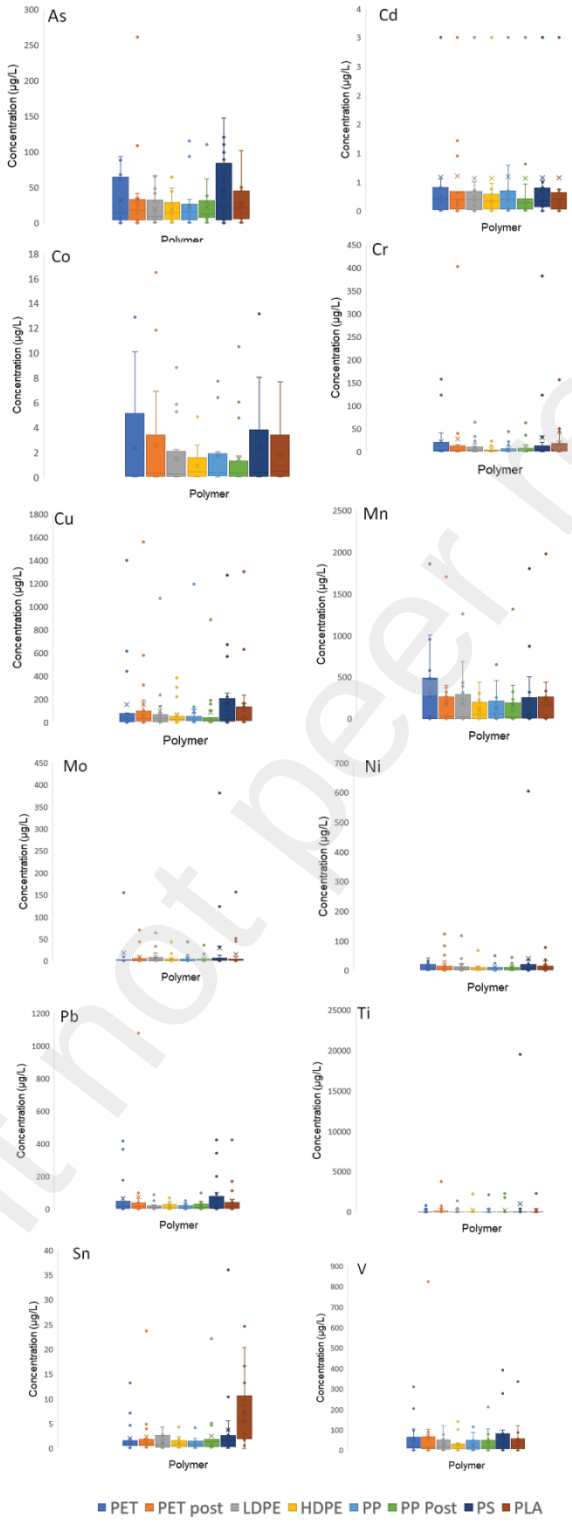


Figure S1: Seawater temperature (°C) in each immersion site. The season is evidenced by a symbol.



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929 Figure S2: Cumulative metal bioaccumulation at all sites and seasons, per polymer.