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Microplastics, microfibers and associated microbiota biofilm analysis in seawater, a case study from the Vesuvian Coast, southern Italy

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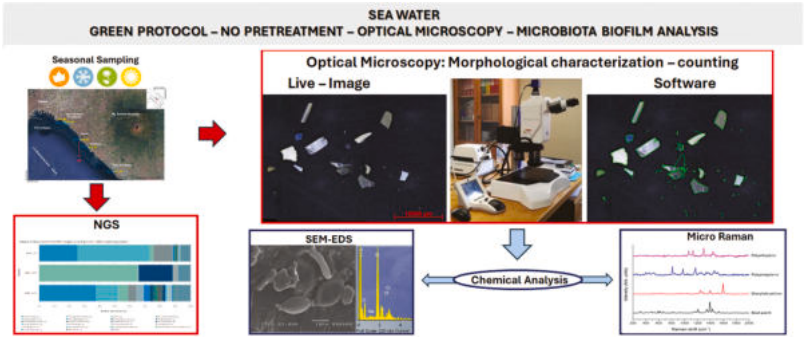
HIGHLIGHTS

- Microplastics and microfibers morphological characterization in seawater.
- Experimental protocol with optical microscopy without sample pretreatments.
- Optical microscopy on live image, photograph analysis and remote counting.
- Marine environment of Vesuvian Coast.
- Analysis of the microbiota of the MP-attached biofilm.

GRAPHICAL ABSTRACT



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ABSTRACT

The growing concerns regarding pollution from microplastics (MPs) and microfibers (MFs) have driven the scientific community to develop new solutions for monitoring ecosystems. However, many of the proposed technologies still include protocols for treating environmental samples that may alter plastic materials, leading to inaccurate results both in observation and in counting. For this reason, we are refining a protocol, based on optical microscopy without the use of pretreatments, applicable to different environmental matrices, which allows not only counting but also a complete morphological characterization of MPs and MFs. Previously, the protocol has successfully been tested on marine sediments from the Vesuvian area of the Gulf of Naples (Italy) with good results. In the present study, we tested the protocol on MPs and MFs in seawater samples collected

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from the same geographical area to provide a comprehensive overview of their distribution in the marine environments. The protocol enabled not only the morphological characterization of MPs and MFs but also the collection of information on the colonies of microorganisms present on the microparticles. Next Generation Sequencing (NGS) metagenomic technologies enabled us to characterize the microbiota composition of the sampled MPs, the so-called Plastisphere. The analytical approach allowed the characterization of several potentially pathogenic bacteria, which represent a potential threat to the environment and human health. In fact, they may exploit their ability to form biofilms on plastics to proliferate in marine ecosystems.

1. Introduction

1.1. Microplastics in marine environments

Plastic is a growing concern as it contaminates aquatic ecosystems through human activities, such as improper waste management and accidental loss, triggering several negative effects on biota. The mismanagement of plastic litter causes the contamination of freshwater ecosystems globally [38,85,86]. Environmental pollution caused by plastic debris is estimated at millions of tons, part of which may be generally discharged into the marine environment and fragmented into micro- and nano-plastics [72]. Plastics in the marine environment have numerous harmful effects, attaching to organisms, impairing their mobility, causing death, and negatively impacting benthic organisms by accumulating in sediments [3,81]. In the marine environment, microplastics (MPs) and microfibers (MFs) are ingested by marine organisms, leading to a range of adverse effects on their vital functions, including growth, reproduction, diet, gastrointestinal health, and immune systems [10,3,90]. Furthermore, MPs and the additives they contain are transferred through the food chain, moving from lower to higher trophic levels in the marine ecosystem, and eventually reaching humans [3] as in the study area [65,68]. MPs are categorized into primary MPs, which are raw materials used in products like toiletries, shampoos, and cosmetics, and secondary MPs, formed through the physical, chemical, and biological degradation of larger plastics in the environment [40,62,7,6]. Indeed, plastics then undergo processes of “fragmentation or deconstruction” into smaller pieces like microplastics, which range in size from 1 μm to 5 mm, or microfibers, defined as thin, elongated MPs with a high length-to-width ratio between 3:1 and 5:1 [3,60]. It has been reported in many studies that MPs and MFs such as polyethylene (PE), polypropylene (PP), polyamide (PA), polystyrene (PS), polyurethane (PU), polyvinyl chloride (PVC), polyethylene terephthalate (PET), polycarbonate (PC), polylactic acid (PLA), acrylonitrile-butadiene-styrene copolymer (ABS), polymethylmethacrylate (PMMA), polyacrylonitrile (PAN), polyvinyl acetate (PVAC), polyvinyl alcohol (PVA) and nitrile butadiene rubber (NBR) are found in marine environments [3,55].

1.2. Negative effects of MPS and MFs on marine organisms

Microplastics (MPs) can serve as unique substrates for microorganisms: biofilm formation on MPs increases their capacity to adsorb contaminants, facilitating the accumulation of pollutants. Consequently, MPs can act as vectors for contaminants and associated microorganisms into aquatic environments [46,58,96]. Chronic exposure may alter marine organism feeding, energy assimilation, growth, and reproductive output.

Plastic fragments are often colonized by bacterial species that may be considered autochthonous of aquatic environments, contributing in various cases to the biodegradation of specific materials. Overall, it is presumable that most species constituting the biofilm adhering to MPs are generic opportunistic colonizers. These microorganisms might be attracted not so much to the polymer surface but to the biofilm under formation, which would favor and increase their access to nutrients. Several studies have reported that members of the *Hyphomonadaceae* family commonly thrive on MPs [15,70], probably because of their capability of firmly adhering to the smooth surface of the polymers. The microbial communities of the marine *Plastisphere* generally differ from those constituting the marine environment itself, thus implying that the plastic residues act as a new ecological niche in the open marine environments [15,70]. On the other hand, plastic has a longer half-life compared to most naturally fluctuating marine substrates. Its hydrophobic surface can promote microbial colonization and biofilm formation, making it significantly different from the native substrates of surface marine layers.

The bacterial communities recognized as colonizers of MPs so far are: Flavobacteriaceae, γ-Proteobacteria, *Hydrogenophaga* sp., *Blastomonas* sp., *Pseudomonas* sp., *Marinomonas* sp.; *Erythrobacter* sp., *Sphingomonadaceae* [98], also *Vibrio spp. as Oceanoserpentilla sp.* and *Phormidium sp.*, [47], *Hyphomonas sp., Chloroflexi sp., Hyphomonas sp. and Haliscomenobacter sp*. [104]. Consortia of strains of *Pseudomonas sp*. and *Lysinibacillus sp*. [72] and the presence of *Neptunomonas naphthovorans* [49] have been also described in the literature.

MPs cause multiple adverse effects on phytoplankton, which are the primary producers of the marine ecosystem. It is known that the negative effects of MPs on phytoplankton include inhibition of cell growth, decrease in photosynthesis ability, decrease in chlorophyll content, formation of hetero-aggregates, inhibition of biomass productivity, restriction of interaction with the environment as a result of adsorption to the surface, and changes in gene expression [11,3]. Zooplankton are small aquatic animals that are the primary consumers of the marine environment and they feed by consuming phytoplankton. It has been reported that the growth and survival rates significantly decreased for some zooplankton species exposed to MPs. Studies revealed that MPs reduced the growth rate and body length of the microorganisms, causing severe disruption to their gut microbiota [11,3].

1.3. Characterization of MPs and MFs in marine environments

For many years, the scientific community has been dedicated to studying MPs and MFs, developing protocols that provide valuable information about their presence in the environment [9,36,37,60,71,75,93]. Currently, there is no standardized method for the analysis of MPs and MFs in the marine environment. Generally, the methodologies employed vary depending on the researchers’ objectives and can be used either independently or in combination. The most common methods include: microscopy, stereomicroscopy, fluorescence microscopy, and fluorescence stereomicroscopy, stereomicroscopy combined with ATR-FTIR, stereomicroscopy combined with μ-FTIR, fluorescence stereomicroscopy combined with μ-FTIR, stereomicroscopy combined with μ-Raman, GC-MS-based techniques [21,66]. In some cases, scanning electron microscope (SEM), FE-SEM, polarized light microscopy, electron energy loss spectroscopy (EELS), thermoanalytical analysis, thermogravimetry, atomic force microscopy (AFM) combined with IR, AFM combined with Raman microscopy, near-infrared (NIR) spectroscopy, and Vis-NIR spectroscopy are also used [12,21]. The lack of a standard MP analysis method makes direct comparisons of results in the literature very hard. Besides using different analytical methodologies for the characterization of MPs, the detection of MPs/MFs particles usually involves the following steps: 1) extraction from the matrix; 2) isolation and quantification; 3) characterization and identification [105]. For the study of MPs in water, the most common methods include adsorption, coagulation, membrane filtration, oxidation, and microbial degradation

[30]. Additionally, since no standardized procedure for MP analysis exists, the samples often undergo processing steps that can alter or erase critical information about transport, accumulation, and interactions with the surrounding environment [3,83]. It would therefore be important to study microplastics in their natural state, as they are found in the environment [13]. Liu et al. [58] study the phenomena related to the production of MP-derived dissolved organic matter (MP-DOM), including the factors that influence its quantity and quality (e.g., plastic types, aquatic environments, and external weathering conditions), as well as its environmental behaviors (biological degradation, adsorption on mineral surfaces, and interaction with pollutants). For instance, performing the chemical digestion process during the analysis of MPs can lead to an underestimation of the presence of MFs, a misinterpretation of the degree of alteration of MPs, and, above all, a lack of correlation with the microorganisms that often colonize them [3,21]. Recent studies, for example, continue to use digestion processes to destroy the organic matter using H_2O_2 [100,21,22], despite many studies have shown how this technique and these reagents cause damage to the surface of MPs [25,39,88]. Another consequence of the lack of a standard sampling method is that researchers report different morphological characteristics that are difficult to compare, making it impossible to directly compare the results [3]. Worldwide acceptance of a standard MP analysis method enables direct comparison of research studies with each other. Additionally, a standard MP analysis method helps to better understand the abundance and sources of MPs and environmental factors affecting MP abundance in marine environment. Several protocols primarily focus on the abundance and polymer type of MPs [24,78,87,89]; however, in recent years, the scientific community has also started to characterize them morphologically focusing on shape, size, color, and degree of alteration [1,27,75].

1.4. Protocol for MPs and MFs characterization on marine environment

Recent studies present various protocols developed to meet the scientific community's demands for the morphological and chemical characterization of microparticles using non-destructive and easy-to-apply methodologies. Li et al. [56] developed a portable, cost-effective polarization holographic imaging system combined with deep learning for efficient, high-throughput detection and dynamic analysis of MPs in water. The system captures holographic and polarization data simultaneously, enabling multimodal analysis to classify MP materials based on birefringence and automate real-time counting and morphological measurements.

Zhu et al. [107] presented an innovative technique, namely the Smart Polarization and Spectroscopic Holography (SPLASH), for automatic analysis of molecular structure and composition. By integrating multi-dimensional features, SPLASH achieves accurate and efficient identification of MPs using machine learning algorithms like ensemble subspace discriminant classifiers, k-nearest neighbors (k-NN), and support vector machines (SVM). The technique analyzes MPs based on anisotropy, interference fringes, refractive index, and morphology.

Xue et al. [100] investigate microplastics in near-shore seawater and propose a rapid, accurate detection method using a custom-built confocal Raman spectroscopy system. By optimizing the pretreatment of seawater samples, the method effectively removes organic matter interference and, through fluorescent labeling, overcomes issues such as lengthy detection times and high false-positive rates seen in traditional techniques. Differential Raman spectroscopy further reduces fluorescence interference, enhancing the signal-to-noise ratio. The method successfully detects microplastics like PE, PP, and PS within the 60–500 μm size range.

Di et al. [31] performed a quantitative analysis of 500 nm PS microplastics in seawater using the surface-enhanced Raman scattering (SERS) internal standard method. This method offers a lower detection limit and a wider quantification range while preserving the integrity of the analyte. It is simple to operate, it requires no addition of internal standard substances, and it is more environmentally friendly. This approach broadens the possibilities for the quantitative detection of MPs in seawater. However, this methodology does not allow for the morphological characterization of MPs and MFs and is unlikely to be applicable for studying MPs in other environmental matrices.

Meng et al. [63] presented a study on the identification of marine MPs using laser-induced fluorescence (LIF) combined with principal component analysis (PCA). The method involves measuring the fluorescence spectra of MPs samples in water, irradiated with a 405-nm laser, followed by PCA-based analysis. Nine types of MPs (ABS, PA, PE, PLA, PET, PS, PTFE, PVC, and PMMA) were successfully identified. Mixed MPs samples were positioned between pure MPs samples on the PCA score plot, with their position determined by the component ratio relative to the pure samples. While this analytical method provides information on the polymeric nature of the microparticles, it does not offer insights into their morphology. Additionally, this technique cannot be applied to other environmental matrices and has not been tested on MFs.

As commonly highlighted, it is increasingly urgent to develop a protocol applicable to the characterization of MPs and MFs in various environmental matrices. Such a protocol should enable the study of microparticles in their natural state, avoiding contamination from pretreatments, and should be standardized, with a particular focus on morphological characterization, as requested and emphasized by scientific community.

This work aims to demonstrate that it is possible to use a non-invasive method to study of MPs and MFs in marine environments across different matrices. The first application of our protocol focused on the characterization of MPs and MFs in beach sediments along the Vesuvius coast, specifically in the coastal sector between S. Giovanni a Teduccio and Torre del Greco, as presented in [83]. In the present study, the same protocol was applied to fragments of MPs and MFs found in seawater in the same coastal sector across different seasons. The method previously tested in [83], primarily using optical microscopy (without matrix extraction or digestion processes), allowed to identify the main sources of MPs and MFs pollution by studying the morphology of the microparticles and their correlations with the matrix they are contained in. In this study, the protocol, also applied using software for the automatic counting and characterization of microparticles, allowed us to highlight correlations with the environmental matrix, such as marine microorganisms, and provided a more comprehensive dataset on the pollution of the Vesuvius coast. The microbial consortia that form the biofilm adhering to the surface of the MPs were also characterized. This multidisciplinary study on MPs and MFs in seawater involves: 1) sampling seawater along the coast based on seasonal and current criteria; 2) investigating morphological characteristics using the [83] protocol and Zeiss-specific software for automatic microparticle counting; 3) investigating microorganisms colonizing microplastics (MPs) and microfibers (MFs) using SEM and qualitative chemical analysis (EDS); 4) applying Micro-Raman spectroscopy for chemical investigations; 5) analysing the microbiota of the MPs biofilm. For the first time, this study provides data regarding a single analytical protocol for the study of both MPs and MFs in marine environments and the presence of MPs and MFs in seawater along the coast of the Campania region, particularly in the Vesuvius area.

2. Materials and methods

2.1. Sampling procedure

The study was carried out on sea surface samples (LM1, LM2, LM3, LM4, LM5, LM6) along marine transept between Portici and Ercolano (Fig. 1 and Table 1). Samples have been collected in different seasons to verify correlations between MPs and MFs and meteorological conditions (water temperature, surface circulation, wind, wave motion, etc., Table 1). The sampling was conducted from autumn 2021 to spring 2022. The MPs and MFs in the first 50 cm below the seawater surface

Fig. 1. The Vesuvian Coast in the Bay of Naples. LM, Line Manta transept of the seawater samples. The samples along the beaches (S, shoreline; BE, berm) of San Giovanni a Teduccio (SG), Portici (PO), Ercolano (ER), and Torre del Greco (TG) are analyzed and described in Rossi et al. [83]. The geographic coordinate system is WGS84 (Google Earth Pro, 2024 mod.). For more details, refer to Table 1.

Table 1
The codes for the six marine surface samples collected along the Portici-Ercolano transect of the Vesuvian Coast and metereological conditions of sampling.

	LM1			LM2			LM3			LM4*		LM5		
Date	14/11/2021 Autumn			19/12/2021 Winter			21/10/2021 Autumn			28/5/2022 Spring		24/7/2022 Summer		
Transept	**1**	**2**	**3**	**1**	**2**	**3**	**1**	**2**	**3**	**1**	**2**	**1**	**2**	**3**
Time	09:30	10:30	11:18	10:37	11:19	12:00	10:30	11:00	11:30	16:24	17:04	10:41	12:10	12:41
Weather	Cloudy			Good			Cloudy			Stormy		Good		
Wind (m/s)	0.1 S/EST	1.0 S/EST	105 E/W	0.1 N	0.5 N/W	1.0 N/W	2.7 N	2.7 N	2.7 N	1,7 S/W	2,4 S/W	2 N/E	2 N/E	2 N/E
External T(°C)	19.1	20.1	19	15.8	16	18	17	17	17	27	27	34	34	34
M. current	W/E	W/E	E/W	N/O	N/O	N/O	N/O	N/O	N/O	S	S	N/W	N/W	N/W
W.H.	20 cm			Smooth			Long Wave			Choppy		slight sea		
Water T(°C)	18	17	17	14	14	15	15	16	16	25	25	30	30	30
pH	6.9	8.48	13.13	8	8	8	8.5	8.5	8.5	6.8	6.8	8.23	8	8
D.S. (ppm)	3015	1015	2015	3515	4754	3519	3800	3800	3800	7400	2966	4000	4000	4000
T (m)	2	2	2	8	8	8	3	3	3	5.5	5.5	3.5	4	3.5
$F._{TOT}$ (m^3)	220			208			251			114		219,3		
$P.W._{TOT}$ (g)	22			0.54			-			1.03		1.35		

M.=Marine, W.H.=Wave height, D.S.=Dissolved salt, T = turbidity, F=flowmeter, P.W.= Plankton weight. *The sampling of LM4 was interrupted due to adverse weather conditions. The codes for the six marine surface samples collected along the Portici-Ercolano transect of the Vesuvian Coast, along with the latitude and longitude coordinates of the transect's start and end points for the collection of MPs using the Manta trawl net, are as follows: 40°47'06.23'' N, 14°20'06.68'' and 40°48'04.60'' N, 14°20'07.90'' respectively. The geographic coordinate system is WGS84.

were collected. After a preliminary analysis of the number of MPs and MFs present in the water, the sample from the autumnal season (LM6) was selected to investigate gene sequence analysis to identify the microorganisms that colonize and form the biofilm on the surface of the MPs/MFs. Sample LM6 was collected in autumn, on October 22/2021, under the same weather conditions and at the same time as sample LM3. Then, sampling was repeat in autumn, in November, to verify whether the concentrations of MPs remained constant in sample LM6 and across different seasons.

2.2. Study area

The Vesuvius coast is part of the Gulf of Naples, a large bay in the Tyrrhenian Margin along the southeastern continental shelf of the Tyrrhenian Sea. This coastal marine ecosystem along volcanic rocks (De [80]) features a Mediterranean-type climate with a subtropical regime [28,54,91], weak tides (<30 cm of range), and a marked salinity contrast due to the combination of salty Mediterranean Sea waters (~38 ‰) and freshwater inputs by watercourses [53]. The communication between the internal and external waters of the Tyrrhenian Sea takes place through two main mouths, the Bocca Grande and the Bocca Piccola. From an oceanographic point of view, the Gulf of Naples is a coastland with unique morphological characteristics and a densely urbanized littoral [43]. The most recent studies have developed models that have deepened aspects relating to surface water circulation [43] and related transport processes [82]. The Gulf of Naples is characterized by a wide geographic fetch (over 700 km in length toward SW) with seasonal sea waves [84] and the circulation of two main water masses typical of the central-southern Tyrrhenian Sea ([50], and references therein): the Atlantic Water (AW) and the Levantine Intermediate Water (LIW). The result of the winter mixing is the Tyrrhenian Intermediate Water (TIW), and a seasonal destratification affected by turbulence [53]. Due to the summer heating, TIW is found at about 75 m of depth and forms the Tyrrhenian Superficial Water (TSW). Finally, another mass of surface water like the TSW forms due to the introduction into the gulf of fresh water from the Sarno and Volturno rivers, and the urban and industrial discharges are less salty and hotter than the TSW [19]. In summer, a marked stratification of the water column is observed, with consequent formation of the thermocline and a homogeneous surface layer of 20–30 m; in winter, there is strong mixing along the water column, with constant temperature and salinity values. The main drivers of this circulation can be identified as remote, i.e. linked to the general circulation of the Mediterranean, and local, *i.e.*, bathymetry and small-scale atmospheric circulation [43]. Among the remote forcing, the wind turns out to be the most significant [29,64] followed by the circulation of the Tyrrhenian Sea [43,79]. During the winter season, the surface currents driven by the wind orient in a south-westerly direction, moving away from the coast and forming a convergence area in front of the city of Torre del Greco, near the two zones adjacent to the city of Naples and Torre Annunziata [23]. In summer, surface circulation is controlled by the breeze regime, which is less intense during the other periods of the year [23,92]. In these conditions, the renewal times of the water masses are very long and the exchanges between the open sea and the coastal area are reduced [16,23,53,84].

2.3. Sea Sampling

In the sampling phase, the protocol published by Viršek et al. [94] and reviewed by [74] was followed, highlighting the pollution with MPs in the first 50 cm of the sea surface. Six samples in different seasons (LM1 - LM6) were collected along the LM transept, S-N oriented from offshore Ercolano to inshore Portici, 1.8 km in length, up to 400 m off and suborthogonal to the coastline (Fig. 1, Table 1). A 3m-long Manta trawl net, 30x57x243 cm and 330 μm mesh size, was used, and at the cod end a sieve of 330 μm, 11 cm in diameter, was placed. Manta net is deployed out of the wake zone, at 3–4 m of distance from the boat to prevent collecting water agitated by turbulence astern, sailing at 2–4 knots preferably in favor of the current, with calm seas and no wind or rain (approximately 1500/1900 m^3 of water has been filtered). Next, all the initial environmental parameters are collected (GPS geographic coordinates, initial time, meteomarine conditions, air and sea surface temperature, sea current, pH, dissolved salts, water turbidity, and flow meter revolutions). Then, the navigation route began in a straight direction with an average speed of approximately 2 knots for 30 minutes, and, in total, 6 nautical miles were covered in 2021–2022 samplings. At the end of the transect, the parameters are collected once again. The Manta net is rinsed thoroughly from the outside of the net with seawater, in the direction from its mouth to the cod end to concentrate all particles adhered to the net into the cod end. Then, the cod end is removed, and the content is sieved through a 330 μm mesh sieve. All the material was collected on the sieve and, using a funnel, stored in a glass jar closed with a metal cap. The material collected into the cod end was composed of a mix of plastic particles (in the range > 330 μm < 5 mm) and biological debris including microorganisms. Before performing the analyses, plastic items were separated from organic matter (mainly algae) with the density separation method, using a saturated NaCl solution (1.2 g cm^{-3}) [10,21,60,100] (Figure S1). The physical-chemical data were collected using a Secchi disk, a TDS, a pH meter, an anemometer, and a flowmeter. One sampling per season was collected, except for LM3 sampled in autumn and used as control.

2.4. Analysis of the microbiota of the MP-attached biofilm

To characterize the microbial consortia composing the biofilm adhering to the surface of MPs, LM6 sample was divided into three aliquots of 2 g each, prepared in triplicate. In order to assess the biofilm formation at different temperature conditions, hypothesizing seasonal changes in the plastisphere composition, samples were incubated at three different temperatures: i) at 22 °C for 24 hours (Code: M05); ii) at 37 °C for 24 hours (Code: M07); iii) at 4 °C for 24 hours (Code: M09).

For carrying out the analyses, 0.3 g of each sample was weighed in triplicate, and processed by a modified CTAB protocol [32] for the extraction of the total DNA, in triplicate; extracted DNA samples were amplified with PCR, utilizing V3 and V4 primers complementary to the V3-V4 hypervariable region of 16S rRNA bacterial gene [14]. Amplified bacterial DNA was sequenced with NGS analysis employing the MiSeq Illumina platform, with 2 × 300 bp paired-end, 600 cycles, according to manufacturer's instructions (Illumina MiSeq, USA; [34]). Diversities in the microbiota were evaluated utilizing weighted UniFrac distance and ANOVA (with Bray Curtis distance, Mothur), by anosim [20].

2.5. Anthropogenic MPs/MFs: counting and optical microscopy characterizing

LM1, LM2, LM3, LM4, and LM5 samples were sieved through a 330 μm size sieve and possible further MPs were separated manually. Then, all the natural and artificial litter objects were removed and air-dried in a sterile environment under a fume hood.

The samples were placed in their entirety in the Petri dish (20x magnification), photographed, and MPs and MFs were counted both from the images and during live observations, with each count repeated three times. This approach was performed to verify the correspondence between the on-site counting (directly under the microscope; Live) and the remote counting (from image; optical microscopy: OM). The characterization of MPs and MFs was carried out according to [83]. Each sample was counted and characterized by two different operators for 6 times each, 3 times for Live and 3 times for OM. The parameters related to the dimensions for both MPs and MFs, length and thickness, for MPs refer respectively to the longest side (length) and the shortest side (thickness). In this work, we experimented with the automatic counting of MPs from images on three samples (LM1, LM2, LM3), using the Zeiss Automeasure program (Fig. 2) and following the protocols described by Goytech et al. [42] and [71]. The measurement was performed three times on each image (Automeasure: AM). In the first step, as in [83], was images of all the microparticles contained in the Petri dish were acquired using the panorama module. Subsequently, the Automeasure module was used to count the particles, determine their size (μm^2), and analyze their shape. The data obtained were then processed by automatically selecting identified regions with a surface area and perimeter greater than 30,000 μm^2 and 1200 μm, respectively. For MFs, a surface area of 9000 μm^2 and a perimeter of 1300 μm were considered, choices dictated by the evidence of numerous artifacts resulting from image

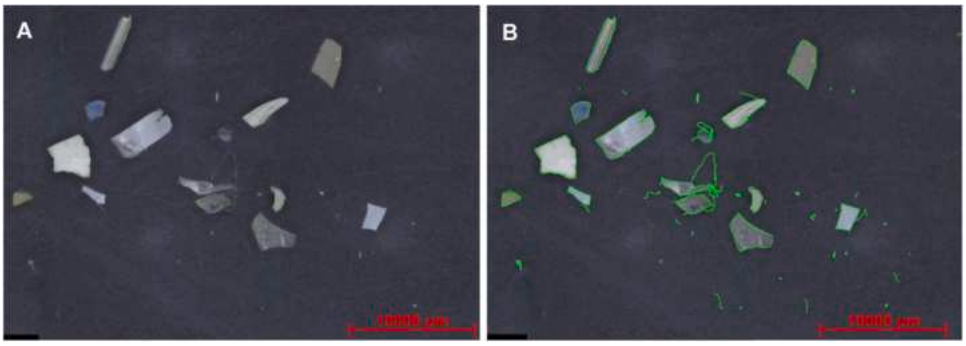

Fig. 2. Image from the stereomicroscope of sample LM1. In A, the microplastic fragments; in B, in green, the MPs e MFs identified by the Zeiss Automeasure program.

processing. We then analyzed the particle area-to-perimeter ratio (A/P), which never exceeded a value of 13. Regarding the shape of MPs, we based our classification on the system previously proposed for sediment analysis [83]: rounded (R.), sub-rounded (S.R.), sub-angular (S.A.), and angular (A.). Additionally, by considering area, perimeter, and area-to-perimeter ratio (A/P), we were able to establish correlations. Specifically, for rounded MPs, we considered an $A/P > 400$; for sub-rounded MPs, an A/P between 400 and 300; for sub-angular MPs, an A/P between 300 and 170; and for MPs with an $A/P \leq 170$, they were classified as angular. To consider the rounded and sub-rounded morphology, it is also possible to take into account the area and diameter of the MPs. The Automeasure program works on the grayscale images of the particles. By selecting the different colors of MPs and MFs, it is possible to identify the areas of the microparticles and obtain information about their diameter and area size. We could have distinguished the particles by color, but we chose following [42] and [71] to highlight differences and issues. Unfortunately, the use of Automeasure did not allow to define the shape, degree of alteration, opacity, and/or translucency of the MPs and MFs among the five samples, without assistance of an expert operator. As for the accuracy, we performed the counts of MFs and MPs using 3 different approaches (i.e., Live, OM and AM), and they gave consistent, and not significantly different, results. Furthermore, to verify the reliability of the method, the samples were counted and characterized by two different operators 6 times each, the samples LM1, LM3 and LM3 were counted 9 times each. The microscope used for counting and morphological characterization of MPs is the AXIO ZOOM V16 by Zeiss powered by the Museum Center of Natural and Physical Sciences. The microscope characteristics and analytical conditions are the same as previously described for sediment samples [83]. The software used are Axiovision SE64 Ref 4.9.1 with Z-Stack, Extended Focus, Panorama modules, and Automeasure.

2.5.1. Data analyses

All data were processed using Microsoft Excel®, and IBM SPSS Statistics for Windows (IBM, Version 27.0). The differences among the average number of MFs and MPs collected in the different season and applying the 3 (for MPs) or 2 (for MFs) observation methods were performed by one-way ANOVA followed, in case of rejection of the null hypothesis, by Tukey's post-hoc test ($p < 0.05$). The normality and homogeneity of the variances were assessed by Shapiro-Wilk's test and Levene's test, respectively.

2.6. MPS and MFS Detailed morphological and qualitative chemical analysis (SEM-EDS)

SEM analyses coupled to energy dispersive X-Ray spectroscopy (EDS) were carried out on samples to obtain information about the chemical nature of the MPs and MFs [26,35,41,48,61,77,97,108,106], to detect the presence of pollutants on the surface of the MPs and to confirm the morphological data collected in OM (shape, Surface Roughness and Sediment Grains). Detailed morphological and semi-quantitative chemical analyses of MPs and MFs samples were performed using JEOL-JSM 5310 SEM-EDS at CISAG, University of Naples Federico II. The setup is equipped with an Oxford Instruments Microanalysis unit: INCA X-act detector and operating at 15 kV primary beam voltage, 50–100 mA filament current, variable spot size, 20 mm WD, and 40 s net acquisition real-time. INCA X-act detector uses Energy software with an XPP matrix correction scheme and Pulse Pile-up correction. The data were processed with INCA software version 4.08 [73]. Semi-quantitative chemical and morphological analyses were performed, after coating the sample with gold and palladium.

2.7. Raman microspectroscopy

Randomly selected samples from 4 different sites (LM2, LM5, LM1, and LM4) were analyzed by Raman spectroscopy to identify the chemical nature of MPs [2]. The particles manually separated were attached with a thin layer of gum arabic on a sterile slide [83]. An aluminium foil was sometimes placed underneath the analysed samples to minimize fluorescence [51]. To prevent alterations and interferences, the samples were not treated with any substance and the spectra were recorded on the particle surfaces that were not embedded in gum arabic. The measurements were carried out in triplicate with a confocal Raman microscope (Jasco, NRS-3100). An integrated Olympus microscope was used to focus on the isolated samples the 514 nm line of an air-cooled Ar^{+} laser (Melles Griot, Rochester, NY, USA, 35 LAP431 220) with a laser spot diameter of approximately 1–5 μm. Utilising a 20X–100X objective, the sample was exposed to a final 2 mW power. The excitation laser line was rejected by means of a holographic edge filter. A diffraction lattice with 1200 grooves/mm and a 0.1 mm× 6 mm slit was used to collect Raman backscattering, producing an average spectral resolution of up to 7 cm^{-1}. A time of about 10–60 s was required to collect a complete dataset from a Peltier-cooled 1024 × 128-pixel CCD photon detector (Andor DU401BVI). Wavelength calibration was performed by using cyclohexane as a standard. Raman spectra were registered in the range of 200–2000 cm^{-1} (low frequency) and 2200–3600 cm^{-1} (high frequency). Inspection via optical microscopy allowed us to identify micrometric areas for Raman investigation. Raman assignment was based on the acquisition of in-house collected reference spectra, together with specific literature [18,2,69].

3. Results and discussion

3.1. Microbial communities composing MPs biofilm

The taxonomic composition at the Family level of the microbial communities of the MPs treated with different incubation conditions (M05, M07, and M09) (Fig. 3) included 24 main Families, with reported Relative Abundance values above 1 %: Alteromonadaceae, Pseudoalteromonadaceae, Flavobacteriaceae, Vibrionaceae, Saccharospirillaceae,

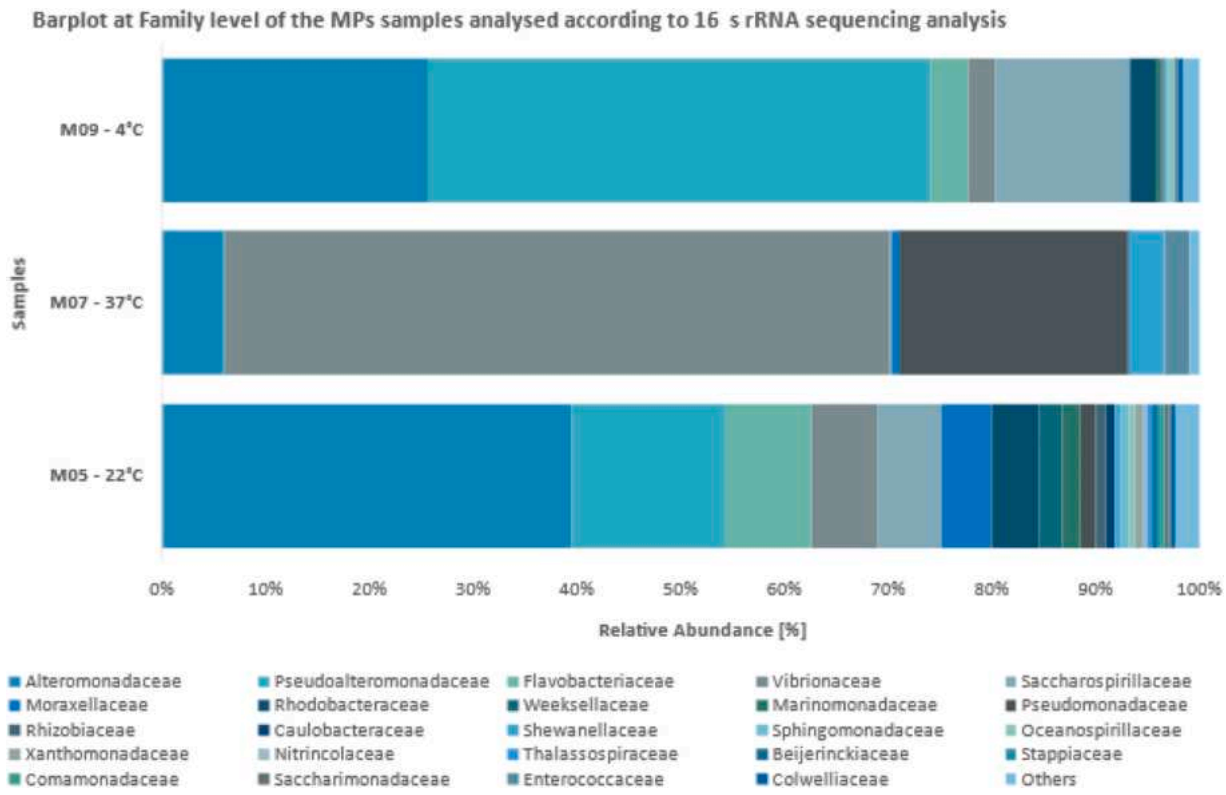

Fig. 3. Barplot at Family level, showing the most abundant Families detected in the MPs samples incubated at three different temperatures (M05: 22 °C; M07: 37 °C; M09: 4 °C).

Moraxellaceae, Rhodobacteraceae, Pseudomonadaceae, Shewanellaceae, Colwelliaceae and Marinomonadaceae.

The most represented genera are showed in Fig. 4 and resulted in *Alteromonas* spp., *Pseudoalteromonas* spp., *Vibrio* spp., *Oceaniserpentilla* spp., *Acinetobacter* spp., *Mesoflavibacter* spp., *Chryseobacterium* spp., *Marinomonas* spp., *Rhodobacteraceae* spp., *YooniaLoktanella* spp., *Flavobacterium* spp. and *Pseudomonas* spp. The hypothesis linked to the choice of the experimental conditions (i.e., the three different incubation temperatures) was confirmed by the differential abundance of taxa, whose percentages at Genus level is highlighted in Table S1. Table S1

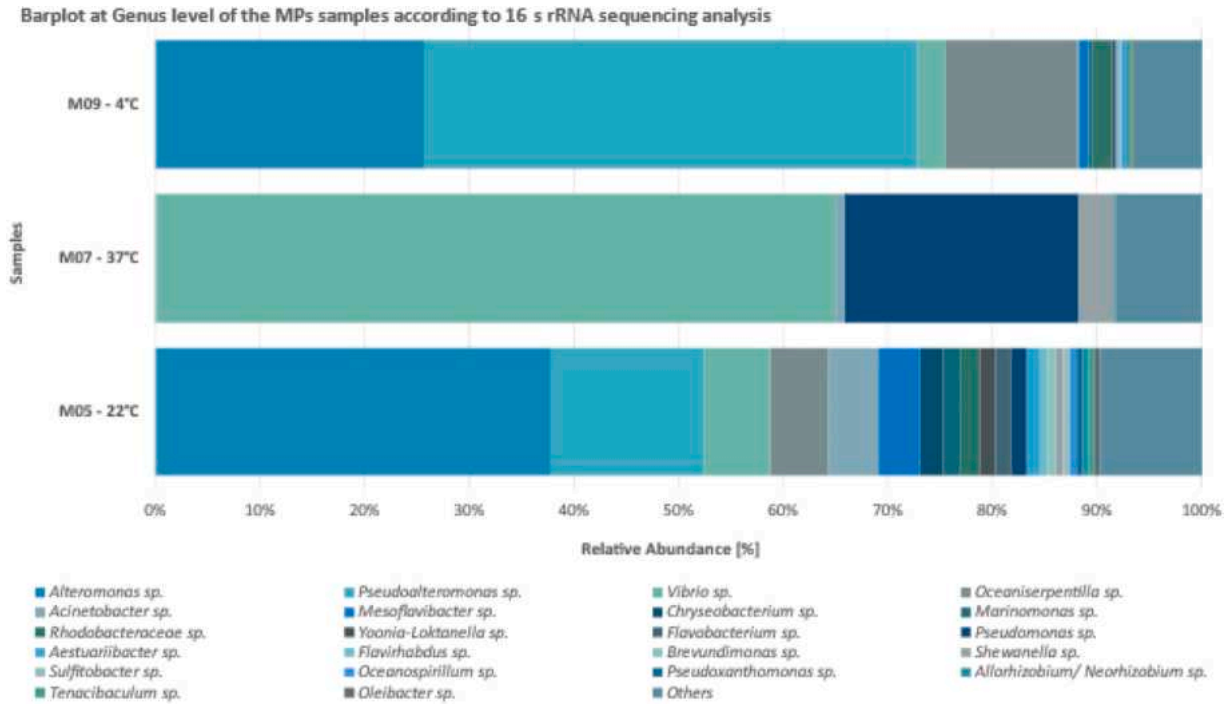

Fig. 4. Barplot at Genus level, showing the most abundant Families detected in the MPs samples incubated at three different temperatures (M05: 22 °C; M07: 37 °C; M09: 4 °C).

summarizes the percentages of Relative Abundance for each microbial genus detected in the samples, while Table S2 lists the most abundant species detected in the samples. The analysis of the three resulting microbiotas, indeed, shows a difference in the percentage composition of some genera in function of the different temperatures, allowing to presume a variation of the plastisphere based on the seasonality. In particular, *Vibrio* sp. increased at 37 °C (M07), with a relative abundance of 63.06 %, compared to the samples incubated at 22 °C (M05) and 4 °C (M09), whose relative abundance is of 6.28 % and 2.56 %, respectively. Also, *Pseudomonas* sp. was detected at a 21.64 % frequency for M07, compared to the 1.42 % (M05) and 0.18 (M09). In general, the genera detected with high frequency in sample M07 (37 °C) are less variable than those detected in samples M05 and M09. Furthermore, widely recognized pathogenic genera such as *Enteroccoccus* sp., *Shewanella* sp. and *Rheinheimera* sp. result predominant, together with the above-mentioned *Vibrio* sp. and *Pseudomonas* sp. The higher abundance of pathogens in the sample incubated at 37 °C correlate with the optimal growth temperature for pathogenic bacteria [44]. Several studies underlined the strict connection between the increasing temperatures in coastal and marine waters, also linked to global warming and heavy rain, the reduction of the salinity of coastal waters and the creation of favourable conditions for the proliferation of pathogenic species such as *Vibrio* sp. [33,8]. [76] reported the isolation of various *Pseudomonas* strains from the marine environment, highlighting the psychrotolerant and mesophilic-psychrotolerant characteristics of the isolates (bacteria able to grow at 0 °C with their optimum in the15–25 °C and 25–40 °C ranges) [76]. Thus, water temperature might influence biofilm formation on plastics, with an increase from winter to summer when thermal conditions and nutrient levels are more favorable. Compositional differences have also been found to vary in function to the temperature depending on the type of plastic, particularly for PE and PS. Furthermore, temperature also affects the adhesion of *Pseudomonas aeruginosa* to PS surfaces under certain conditions, as shown by [103]. Increased enzyme activity and microbial metabolism, able to promote community development, may also contribute to the augmented biofilm formation at high temperatures, also playing a role in the activity of adhesion organelles such as flagella [67].

In general, bacterial species reported with the highest Relative Abundance percentages resulted *Vibrio fluvialis*, *Vibrio furnissii*, *Vibrio alfacsensis*, *Oceaniserpentilla haliotis*, *Pseudomonas alcaligenes*, *Psychrosphaera saromensis*, *Neptunomonas Naphthovorans*, *Oceanospirillum sanctuarii* and *Shewanella putrefaciens*. The differential qualitative and quantitative bacterial compositions at the species level, considering the three different temperatures of incubation (M05: 22 °C; M06: 37 °C; M09: 4 °C) are described in Table S2. The interest of the present study was mainly aimed at understanding the interplay between the marine microorganisms and the plastic materials in the plastisphere habitat. In the last decade, the majority of research focused attention on the microbial compositions of biofilms on different types of plastics. The outcomes interestingly described a high similarity between the bacterial communities associated with PE, PS, and natural particles, while fewer studies demonstrated how some bacteria seem to specifically colonize PE (Flavobacteriaceae; γ-Proteobacteria), PS (*Hydrogenophaga* sp., *Blastomonas* sp., *Pseudomonas* sp., *Marinomonas* sp.), or further unknown polymer types (*Erythrobacter* sp., Sphingomonadaceae). Analyzing the most abundant microorganisms detected in the present research, it is worth noting that several scientific articles evaluated the role of microplastics as abiotic vectors for species of the genus *Vibrio*: some research additionally suggested a positive correlation between salinity and *Vibrio* species concentration on plastic materials. [98] showed how the colonization of plastics with different strains of *Vibrio* spp. can be highly dynamic, especially in low-nutrient and high-salinity systems, concluding that future studies focusing on the role of *Vibrio* spp. on MPs should consider the quality and physico-chemical parameters of water and temporal dynamics [52,98]. Species classified in the genus *Oceanoserpentilla* were also demonstrated active in the degradation of hydrocarbons [47]. *Oceanoserpentilla* sp. were additionally isolated from models of co-presence between members of the Plastisphere, together with *Phormidium* sp., *Hyphomonas* sp., *Chloroflexi* sp., *Hyphomonas* sp. and *Haliscomenobacter* sp. [104].

A study published in 2021 described the adhesion properties and structure of microbial biofilm on HDPE (High-Density Polyethylene) in deep water. The research reported that a consortium of strains of *Pseudomonas* sp. and *Lysinibacillus* sp. showed increased extracellular matrix production, capable of promoting adhesion to HDPE in less than 24 hours [72].

The formation of microbial biofilms on HDPE surfaces also led to changes in the chemical structure of the polymer, thus suggesting the potential involvement of these strains in the biodegradation of plastic pollutants [72]. *Neptunomonas naphthovorans* (belonging to the subgroup of γ-Proteobacteria, closely related to the genus *Oceanospirillum*) has been demonstrated capable of exploiting naphthalene as the unique carbon and energy source, beyond being able to degrade polycyclic aromatic hydrocarbons (PAHs) in artificial seawater (such as 2-methylnaphthalene, 1-methylnaphthalene, 2,6-dimethyl naphthalene, phenanthrene) [49]. Future perspectives will focus on the evaluation and identification of bacterial species involved in the degradation of micro- and nano-plastics generally detected in the marine environment.

3.2. Anthropogenic MPs/MFs: counting and optical microscopy characterizing

3.2.1. Protocol application

The data collected through optical microscopy indicate that among the MPs present, fragments constitute 81 %, while the remaining 19 % are MFs. The highest number of both MPs and MFs was found in the winter sample, followed by spring, summer, and finally autumn. However, the values for the last three seasons are very similar, as shown in Table 2a.

The method proposed here does not involve any pretreatments but rather a simple gravity separation from organic matter using a supersaturated NaCl solution. Therefore, the collected material is analyzed in its entirety, without losing information such as the presence of bacteria, phytoplankton, zooplankton, and other organic elements, besides calculating and recognizing the degree of alteration of MPs [83]. The count performed on the data collected from images (OM), compared to the live count (live) and Automeasure (AM), indicates no statistical differences between the results provided by the three approaches.

The calculated errors and statistical analyses confirm the validity of the analytical methodology tested. Nonetheless, Table 2a reports the collected descriptions with standard deviations and relative errors for MPs, showing good consistency between values. The total count (nine times) of MFs for each sediment sample, performed using each methodology (three times), also shows standard deviations and relative errors in good correlation. Table 2b presents the averages, standard deviations, and relative errors for all types of measurements (live, OM and AM), broken down by sample and for all the described parameters. Quantitative analyses of parameters such as color, brightness, dimensions, shape, degree of alteration, and the method of acquisition (live, microphotographs, or Automeasure) are comparable, as evidenced by Tables 2a 2b.

As for the observation methods, no statistically significant differences were found among methods (lowercase letters in Fig. 5). However, based on our observations, AM method seems to underestimate the number of MPs and MFs since the software cannot discriminate among those cases in which smaller particles overlap with larger ones. To statistically confirm this evidence, further data should be collected and compared. Since no significant difference was found among the observation methods, for the seasonal comparison, we combined the data from the three methods (capital letters in Fig. 5A, B). For both categories of MPs and MFs, their release into the sea is influenced by seasonality. Specifically, the winter season followed by spring are the most impacted,

Table 2a
Results of counting and morphological analyses on MPs in seawater.

	Number	C. %	W. %	O. %	T. %	L. μm	Th. μm	A. %	S.A. %	S.R. %	Round %	R. %	P.I.	I.%
LM1 - Autumn														
Mean/live	41	62	23	53	47	2108	1139	69	11	9	10	52	20	29
σ	1	1	1	2	2	2115	788	1	1	1	1	2	1	2
E.R.%	2	2	2	3	3	88	67	1	9	10	5	3	3	5
Mean/OM	42	61	24	51	49	2066	1151	70	12	8	10	52	20	28
σ	1	1	1	2	2	2155	736	1	1	0	1	2	1	1
E.R.%	2	1	3	3	3	93	58	1	4	3	10	4	5	4
Mean$_{tot}$	42	61	23	52	48	2087	1145	70	12	9	10	52	20	28
σ	1	1	1	2	2	1910	682	1	1	1	1	2	1	1
E.R.%	4	2	4	6	6	92	66	2	9	11	10	4	5	5
Mean/AM	38	-	-	-	-	5054141*		76	12	5	7	-	-	-
σ	5	-	-	-	-	561224*		2	3	2	2	-	-	-
E.R.%	13	-	-	-	-	11		3	27	28	22	-	-	-
LM2 - Winter														
Mean/live	106	37	49	76	24	2783	1291	52	22	9	17	50	30	20
σ	2	3	3	1	1	1403	44	2	1	1	2	2	1	1
E.R.%	2	7	5	1	2	44	2	4	2	5	9	4	3	5
Mean/OM	105	37	47	76	24	2969	2006	51	22	10	17	50	29	21
σ	1	1	1	1	1	1374	201	1	1	1	1	2	1	1
E.R.%	1	1	1	1	4	43	15	2	2	5	6	3	2	5
Mean$_{tot}$	105	37	48	76	24	2876	1648	51	22	10	17	50	29	21
σ	2	2	2	1	1	1246	413	2	1	1	1	2	1	1
E.R.%	2	7	5	1	4	46	29	4	5	5	9	4	5	7
Mean/AM	100	-	-	-	-	2720807*		55	20	10	15	-	-	-
σ	6	-	-	-	-	836711*		5	2	2	2	-	-	-
E.R.%	5	-	-	-	-	30		8	9	14	13	-	-	-
LM3 - Autumn														
Mean/live	46	64	10	90	10	2417	994	71	12	7	10	66	20	14
σ	1	1	1	1	1	2756	65	1	1	0	0	1	1	0
E.R.%	1	1	5	1	5	100	7	1	5	1	2	1	3	1
Mean/OM	46	64	9	90	10	2257	1285	71	12	8	10	66	20	14
σ	1	0	0	1	1	2897	665	1	0	0	0	1	1	0
E.R.%	1	0	2	1	6	114	49	1	2	3	3	1	5	2
Mean$_{tot}$	46	64	9	90	10	2337	1139	71	12	7	10	66	20	14
σ	1	0	1	1	1	2531	451	1	0	0	0	1	1	0
E.R.%	1	1	8	1	6	110	56	1	5	7	4	1	5	2
Mean/AM	52	-	-	-	-	3405847*		68	12	8	12	-	-	-
σ	7	-	-	-	-	638663*		4	3	2	2	-	-	-
E.R.%	13	-	-	-	-	18		5	24	20	16	-	-	-
LM4 - Spring														
Mean/live	56	15	28	96	4	2629	1196	37	22	29	13	31	48	21
σ	1	1	1	1	1	1766	753	1	1	1	0	1	1	0
E.R.%	1	3	4	1	14	67	62	3	2	2	2	3	2	0
LM5 - Summer														
Mean/live	54	38	24	80	20	4370	1711	37	18	25	20	71	19	10
σ	1	1	1	1	1	2992	1363	0	2	1	1	1	1	1
E.R.%	1	3	2	1	3	65	71	0	7	4	3	1	4	7

Legend: OM.=microphotographs, C.=colorless, W.= withe, O.=opaque, T. =translucent, L=length, Th= thickness, A.= angular, S.A.= sub angular, S.R.=sub round, R.=regular, P.I= partially irregular, I.=Irregular, *μm^2.

then summer and autumn.

In the analysis conducted using AM, despite the involvement of an experienced operator, small differences in the counting and measurement of MPs and MFs are noticeable. These discrepancies in the count (LM1 =-4; LM2 =-6; LM3 =+6 for MPs; LM1 =-1, LM2 =-3, LM3 =0 for MFs) and in the particle sizes are due to the presence of overlapping microplastics, tangled microfibers, or microfibers attached to microplastics, which prevent an accurate count. It is well known that the ionic strength and pH of water influence the stabilization, agglomeration, and aggregation of suspended MPs [55].

Regarding the automatic counting and description method (i.e., AM), we highlighted the following: 1) color recognition of the MPs allows for more accurate counting and measurement of MPs particles, especially when they overlap; 2) the program must be configured to prevent the generation of artifacts; and 3) a program for surface analysis would be useful, as it would provide information on the degree of alteration of the MPs and MFs. On the other hand, we were able to count MPs with satisfactory results, determine the particle size, and recognize the morphology. In our case, the operator, performing an accurate count and a correct description, had to manually intervene in the separation of overlapping MPs, in the correct recognition of some perimeters, particularly when there is an overlap of MPs and MFs, and in the identification of artifacts. As shown in Tables 2a 2b, any information can be obtained using AM regarding the degree of alteration. Further developments are possible with the continuous improvement of technologies. For example, the protocol by [71], based on optical microscopy and image analysis using a self-written Python script, presents the same issues encountered with the AM method, related to the overlap of microparticles, the failure to recognize color and sheen, and, in particular, the absence of surface analysis to determine the degree of alteration. The same can be said for the protocol mentioned in Goytech et al., 2023, which, among other things, uses virgin microplastics not derived from marine ecosystems.

Instead, [95] highlighted one of the main challenges in applying artificial intelligence (AI) to the recognition of marine MPs. While AI can already be used for counting and measuring, the main issue remains its ability to describe the complex characteristics of MPs found in seawater, such as biological encrustations, overlapping particles, optical features (e.g., lustre and color), and varying degrees of alteration. To overcome some of these limitations, an AI system should be trained on a dataset

Table 2b
Results of counting and morphological analyses on MFs in seawater.

	Number	C. %	W. %	O. %	T. %	L. μm	Th. μm	Flat %	Round %	R. %	P.I.%	I.%
LM1 - Autumn												
Mean/live	11	36	56	25	75	3150	17	36	64	64	36	-
σ	0	0	0	1	1	1097	3	1	1	1	1	-
E.R.%	-	-	-	2	1	35	14	2	1	1	2	-
Mean/OM	11	36	56	27	74	3717	18	36	64	64	36	-
σ	0	1	1	1	1	685	1	1	1	1	1	-
E.R.%	-	1	1	2	1	18	8	1	1	1	1	-
Mean$_{tot}$	11	36	56	26	74	3434	18	36	64	64	36	-
σ	0	0	0	1	1	875	2	1	1	1	1	-
E.R.%	-	1	1	4	1	32	14	2	1	1	2	-
Mean/AM	10	-	-	-	-	4449*		-	-	-	-	-
σ	1	-	-	-	-	587*		-	-	-	-	-
E.R.%	10	-	-	-	-	12		-	-	-	-	-
LM2 - Winter												
Mean/live	26	8	81	14	86	2590	20	4	96	92	4	-
σ	1	0	0	1	1	1450	1	1	1	1	0	-
E.R.%	2	-	-	3	1	56	3	12	1	1	-	-
Mean/OM	26	8	80	14	86	2557	19	4	96	92	4	-
σ	1	0	1	1	1	1000	1	0	0	0	0	-
E.R.%	2	-	1	3	1	39	5	2	-	-	2	-
Mean$_{tot}$	26	8	81	14	86	2573	19	4	96	92	4	-
σ	1	0	1	1	1	1114	1	0	0	0	0	-
E.R.%	2	-	1	3	1	56	5	14	1	1	2	-
Mean/AM	23	-	-	-	-	9270*		-	-	-	-	-
σ	3	-	-	-	-	1139*		-	-	-	-	-
E.R.%	13	-	-	-	-	12		-	-	-	-	-
LM3 - Autumn												
Mean/live	9	22	56	20	80	2213	18	44	56	89	11	-
σ	0	0	0	0	0	1274	3	0	0	0	0	-
E.R.%	-	-	-	-	-	57	14	1	-	-	-	-
Mean/OM	9	22	56	20	80	2342	19	44	56	89	11	-
σ	0	0	0	0	0	1517	1	0	0	0	0	-
E.R.%	-	-	-	-	-	64	5	-	-	-	-	-
Mean$_{tot}$	9	22	56	20	80	2277	18	44	56	89	11	-
σ	0	0	0	0	0	1255	2	0	0	0	0	-
E.R.%	-	-	-	-	-	66	14	1	-	-	-	-
Mean/AM	9	-	-	-	-	6193*		-	-	-	-	-
σ	2	-	-	-	-	1060*		-	-	-	-	-
E.R.%	16	-	-	-	-	17		-	-	-	-	-
LM4 - Spring												
Mean/live	13	7	85	15	85	1960	21	46	54	85	15	-
σ	0	0	0	0	0	950	2	0	0	0	0	-
E.R.%	-	-	-	-	-	48	7	-	-	-	-	-
LM5 - Summer												
Mean/live	10	30	50	25	75	1570	17	30	70	90	10	-
σ	0	0	0	0	0	681	2	0	0	0	0	-
E.R.%	-	-	-	-	-	41	12	-	-	-	-	-

Legend: OM.=microphotographs, C.=colorless, W.= withe, O.=opaque, T. =translucent, L=length, Th= thickness, R.=regular, P.I= partially irregular, I.=Irregular, *μm^2.

that includes not only 'clean' MPs images but also examples of MPs in natural conditions, with biological encrustations, oxidation, or overlapping particles. Training with deep learning techniques on multispectral or three-dimensional images could enhance the software's ability to recognize and characterize these complexities. [57] utilized a laser infrared imaging spectrometer (LDIR) to quantify MPs in oysters and their farming seawater at 18 sites along Chinese coastlines. The technique they apply allowed for counting, measuring, and recognizing the chemical composition of MPs, but the quality of the imaging data does not permit highlighting the optical characteristics and the degree of alteration. Furthermore, the sample undergoes digestion processes that, as already shown by several studies, can physically and chemically alter the MPs [55,60]. Li et al. [56] developed a portable, cost-effective polarization holographic imaging system combined with deep learning for efficient, high-throughput detection and dynamic analysis of MPs in water. Although their study also focuses on the use of optical microscopy, it is not conducted on samples collected from marine environments and does not perform a comprehensive morphological characterization (e.g., color, degree of alteration, or connection to the environmental matrix of origin). For example, in real seawater samples, multiple scattering and absorption occur, typically mixed with numerous impurities, which affect imaging quality. Despite, the technique they propose could prove extremely useful, even though it is not applicable to marine sediment samples. Zhu et al. [107] present SPLASH technique with integrating multi-dimensional features that allows the identification of MPs. While promising for assessing MP pollution and monitoring water quality, the study lacks detailed morphological characterization and quantification of MPs and, in particular, of MFs. SPLASH could be explored for characterizing microparticles and MFs in marine sediments. Xue et al. [100] investigate MPs in near-shore seawater and propose a rapid, accurate detection method using a custom-built confocal Raman spectroscopy system. While this approach provides chemical and morphological insights into MPs and MFs in seawater, it relies on density separation, digestion, and filtration processes, which could limit its applicability to other environmental matrices, such as marine sediments.

Unlike the aforementioned methods, the methodological approach we propose allows for the collection of comparable information on the morphological characteristics of both MPs and MFs in sediments and in water, without any pretreatment of the samples required. This allows for

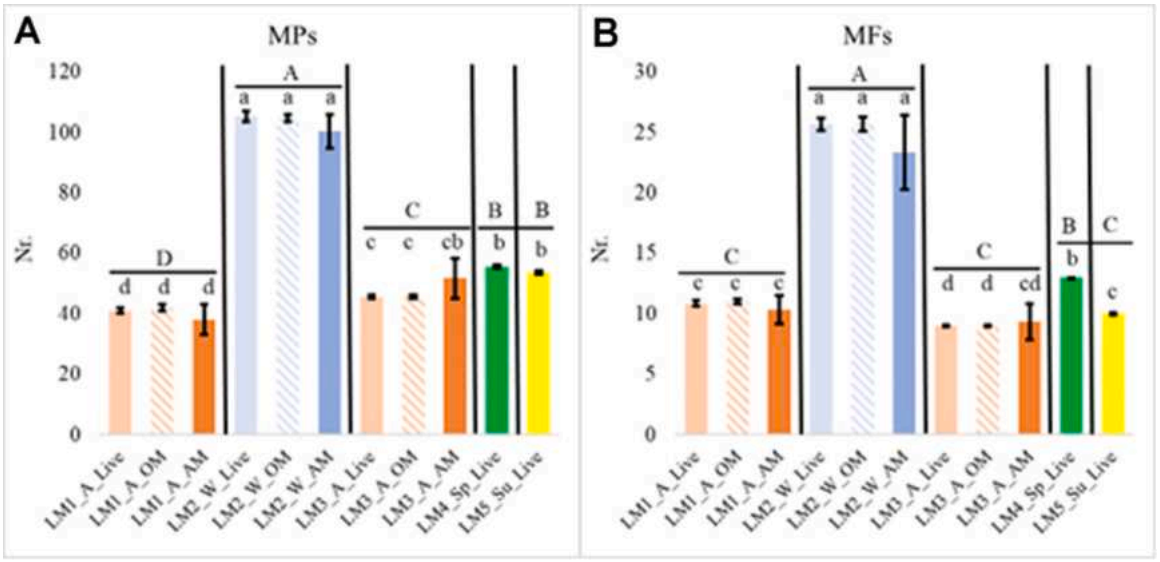

Fig. 5. Bar charts of MPs (a) and MFs (b) number to compare the effect of the observation method (e.g., OM, Live and AM) and seasonality (A: Autumn, W: Winter, Sp: Spring, Su: Summer) on microplastic content in the samples. Bars are the average values, and the error bars are the standard deviations. Different letters indicate significant differences according to Tukey's test, $p < 0.05$; lower case for observation methods, upper case for seasonality.

the collection of not only quantitative but also qualitative data, providing a snapshot of the interaction of these microparticles with the surrounding environment. However, the disadvantages relate to the lack of information on their polymeric nature, which can subsequently be obtained using various spectroscopic methodologies, e.g. Raman Microscopy.

To verify the accuracy of their obtained data, each sample was counted three times by two different operators, using live observation (Live), pre-acquired images with an operator (OM), and the automeasure software (AM). In total, each sample was counted 18 times. This required a considerable amount of time (1 week per operator with access to a single microscope), but once the the procedure has been optimized, the estimated time for the analysis of one sample is: 1 hour (Live), 2 hours (OM), 2 hours (AM). The time differences are due to the additional time required for the image data acquisition in the cases of OM and AM methods. However, the advantage of working with the acquired image is that the image data can be preserved and subsequently compared for further studies and potential redefinitions.

Comparing the study of MPs and MFs found on beaches [83] with that of the same sampling area in seawater, we can observe that the analytical approach has remained consistent. The same information on the microparticles was collected, such as color, shape, opacity and translucency, size, degree of alteration, and interaction with the matrix (if present). This approach allows for obtaining comparable data on MPs and MFs, enabling a deeper understanding of transport and accumulation dynamics. Below we report on the collected data and their interpretation.

3.2.2. *Morphological characterization*

The sizes of the microplastic fragments are very variable as shown by the standard deviation in Table 2a. Regarding the shape they angular in 53 % of cases, sub angular in 17 %, sub-rounded in 16 %, and rounded in 14 %. The most prevalent color is colorless (43 %), along with white (27 %), and blue (15 %), colors green, yellow, black, and red representing the 15 %. The majority of MPs are opaque (79 %), while the remaining are translucent (21 %). As for the degree of alteration, MPs appear to be slightly more altered (regular 54 %). Fragments of larger MPs are often associated with smaller fragments, especially with tangles of MFs of various sizes (Fig. 6A and B).

Regarding the characterization performed through optical microscopy on MFs it has been highlighted that, for the parameters related to optical features, the prevalent colors are white (66 %) and colorless (21 %), while for lustre the prevalent ones are translucent (80 %) and opaque (20 %). In terms of dimensions, the average length is 2363 μm,

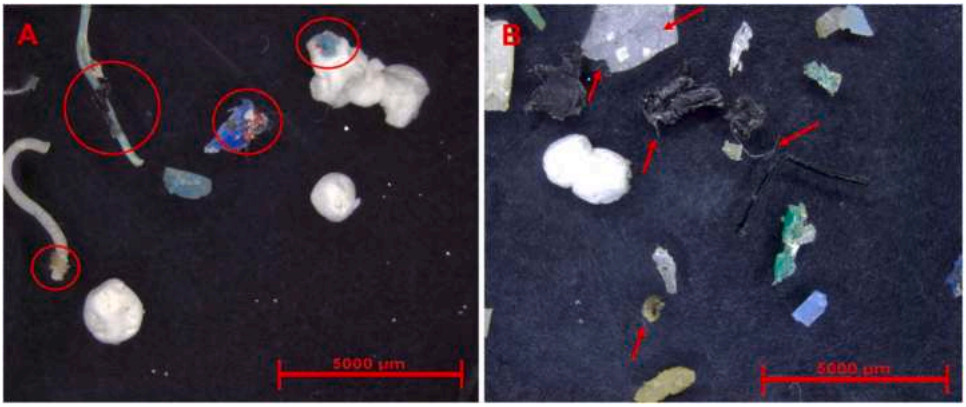

Fig. 6. In the stereomicroscope images A and B, fragments (LM4 and LM3 samples) with different colors, shapes, and luster can be observed. In image A, there are particles composed of multiple overlapping fragments, mainly recognizable by different colors (circled). In image B, the presence of microfibers closely associated with the MPs fragments is indicated by arrows.

while average thickness is 19 μm. For shape, we recognize round and flat MFs average percentage of round MFs of 68 % and flat ones of 32 %. In many cases, MFs are rolled up, forming tangles often associated with microplastic fragments (Fig. 6B). From the analysis of the degree of alteration, MFs appear regular in 84 % of cases and partially irregular in the remaining 16 % (Table 2b. Regarding quantitative data, it is evident that the water contains a higher number of MPs compared to MFs. If we compare the data presented by [83] on MPs and MFs found in the sediments of the beaches adjacent to the marine transect under study, it becomes evident that MP fragments tend to remain suspended in the water column rather than settling on the beaches. This is likely due to their flattened shape, as, while MFs, especially the flat ones, anchor themselves to the sediments, MPs remain in the water or are more easily reintroduced into the water column. If we consider the data in Table 2b and compare it with the data presented in [83], we can deduce that MFs, particularly those with a flat morphology, accumulate along the coastlines, while MFs with a cylindrical (round) morphology hardly bind to the sediments and tend to re-enter the water column. The majority of MFs found in water have a cylindrical shape. In a recent study by [17] performed in the same geographical area on airborne anthropogenic microfibers deposited on native moss *Hypnum cupressiforme*, the authors found the opposite trend, with MFs constituting 99 % and MPs 1 % of all fragments observed, thus suggesting very different diffusion and distribution dynamics in air and seawater. We could also hypothesize that the microfibers found on beaches reflect an accumulation coming not only from seawater but also from land areas.

The analysis of MPs and MFs in water reveals a greater color variability, which could be related to their low degree of alteration, as indicated by the parameters considered (size, shape, and degree of alteration). Indeed, both MPs and MFs show larger sizes compared to those found in the sediments, predominantly angular shapes for MPs, and a low degree of alteration. The sheen is predominantly matte due to the organic encrustations present on the surface.

The higher concentration of MPs and MFs observed during the winter period could be linked to lower temperatures, which tend to bring particles to the surface, and increased water mixing driven by meteorological and littoral dynamics. Conversely, the lower presence of MPs and MFs in the autumn season, contrary to expectations, is likely due to calmer weather conditions compared to the other sampling periods. As noted by Cincinelli et al. [24], the heterogeneity in MP distribution and concentration levels can be influenced by various factors, including seawater circulation (currents, wave action, and wind-driven turbulence), biofouling, salinity, temperature, land-based litter sources (e.g., river inputs), coastal activities, and hydrodynamic processes such as upwelling, downwelling, gyres, or fronts. In the Mediterranean Basin, particularly in the Gulf of Naples, coastal dynamics during the autumn-winter season are characterized by low temperatures and winds and marine currents predominantly from the NW and NE, which activate upwelling [16,23,43,82,92]. Conversely, during the spring-summer period, higher temperatures and SE and SW winds and currents trigger downwelling [53,84]. Considering the presence of a seasonal thermocline at approximately 20 m of depth, it can be hypothesized that during the winter period, the activation of upwelling facilitates the transport of MPs and MFs in the more surficial areas.

The study of tides and winds highlights that MPs and MFs primarily originate from the Port of Naples and the surrounding industrial areas. The supply from the channelized watercourses [4] flowing into the study area is not negligible. In this Mediterranean climate coastland, the prevailing winds blow from southwest and southeast, especially during the autumn-winter period. Considering the long geographical fetch (over 700 km) and the NW-SE coastal orientation, these winds generate waves over 2 m high moving from offshore to inshore the floating microplastic debris towards the wave-prone littoral. In winter, the less frequent northeast wind and the related waves in the sheltered coastal area move from the shoreline offshore the microplastic fragments. Instead, in the spring-summer period, wind calms, calm, and rough sea alternate, triggering a complex water mass circulation involving upwelling and downwelling phenomena. As a result of these interactions, the main littoral drift occurs from northwest to southwest, *i.e.*, from the Port of Naples toward the Vesuvian Coast, due to sea waves, longshore and rip currents remobilizing the debris. The marine currents act in shallow waters and a few hundred meters from the coast, from the shoreline down to 4–6 m, and within 8 m of depth, representing the depth of closure [45,5]. These processes cause microplastic debris to remain briefly offshore and then transported southeast and landward. Here, marine clockwise and counterclockwise secondary cell circulations close to the coast and winds disperse the MPs and MFs along the emerged beach where they accumulate in the sediments for more or less time [83].

3.3. Detailed morphological and qualitative chemical analysis

Chemical EDS analyses and morphological data correlate with the findings of surface encrustations of various types on the MPs and MFs. Specifically, bacterial colonies, mostly cocci and rod-shaped microorganisms, were observed on the surface of both MPs and MFs (Fig. 7A). On the surface of the MPs alone, different types of pinnate Diatoms (Figs. 7C and 7D), twisted MFs (Fig. 7E), and microcrustacean fragments (Fig. 7F) were found. EDS analyses confirm the morphological data: the presence of S, Ca, Mg, K, and O detected in bacterial colonies (Fig. 7A and B). The occurrence of Diatoms pennate was confirmed by the presence of SiO_2, as they are organisms with a siliceous shell (Fig. 7C and D); while microcrustacean shell fragments were confirmed by the presence of $CaCO_3$, typical of the exoskeletons of these organisms (Fig. 7F). Based on the chemical composition and comparing the microbiota composition data (Table S1), the presence of species belonging to the *Sulfitobacter* genus might be confirmed [101]. Indeed, in addition to oxidizing sulfite and thiosulfate, *Sulfitobacter* sp. strains also have the capability to degrade diatom-derived dimethylsulfoniopropionate (DMSP), an organic sulfur-containing compound present globally in very large amounts (109 tons or more per year) [102]. As a result, *Sulfitobacter* sp. strains are considered significant contributors to the organic sulfur cycle in marine environments [99]. SEM analyses indicate a generally smooth surface of the MPs and MFs, but they are nonetheless colonized by microorganisms, especially phytoplankton (Diatoms) and zooplankton. These microorganisms, along with MFs and inorganic deposits such as sulfates and salts (NaCl), form true encrustations that make the MPs appear opaque (Fig. 7F). Moreover, qualitative chemical analyses of microplastic fragments have revealed the presence of polymers composed either of carbon (C) or, in some cases, of carbon and chlorine (C and Cl). We were able to attribute a polymeric classification by observing the shapes of the MPs and their optical characteristics. For example, the round, whitish, opaque MPs composed only of C are likely polystyrene, while the round or sub-round, translucent MPs composed of C and Cl could be PVC. To gain further insight, chemical analyses of the polymers that constitute the MPs were conducted using Raman microscopy. Traces of Al and potassium (K) and calcium (Ca) sulfate incrustations have been found on the surface of the MPs, likely due to pollution from fertilization.

3.4. Raman analysis

Samples collected at LM1, LM5, LM2, and LM4 sites were analyzed with Raman microspectroscopy to identify their chemical nature. Since at the laser line used for the measurement (514 nm), 10 out of 24 samples are fluorescent, Raman features of these samples and, consequently, their chemical nature cannot be identified. For other 2 samples, collected at LM2 and LM5 sites, Raman spectra (Table 3 and Figure S2) show signals of diarylide yellow (pigment yellow 83) or a complex mixture consistent with boat paint. Raman spectra of the remaining 12 samples allowed to determine the chemical nature of the examined MPs (Table 3, Figure S2, and Figure S3). All the samples show features that

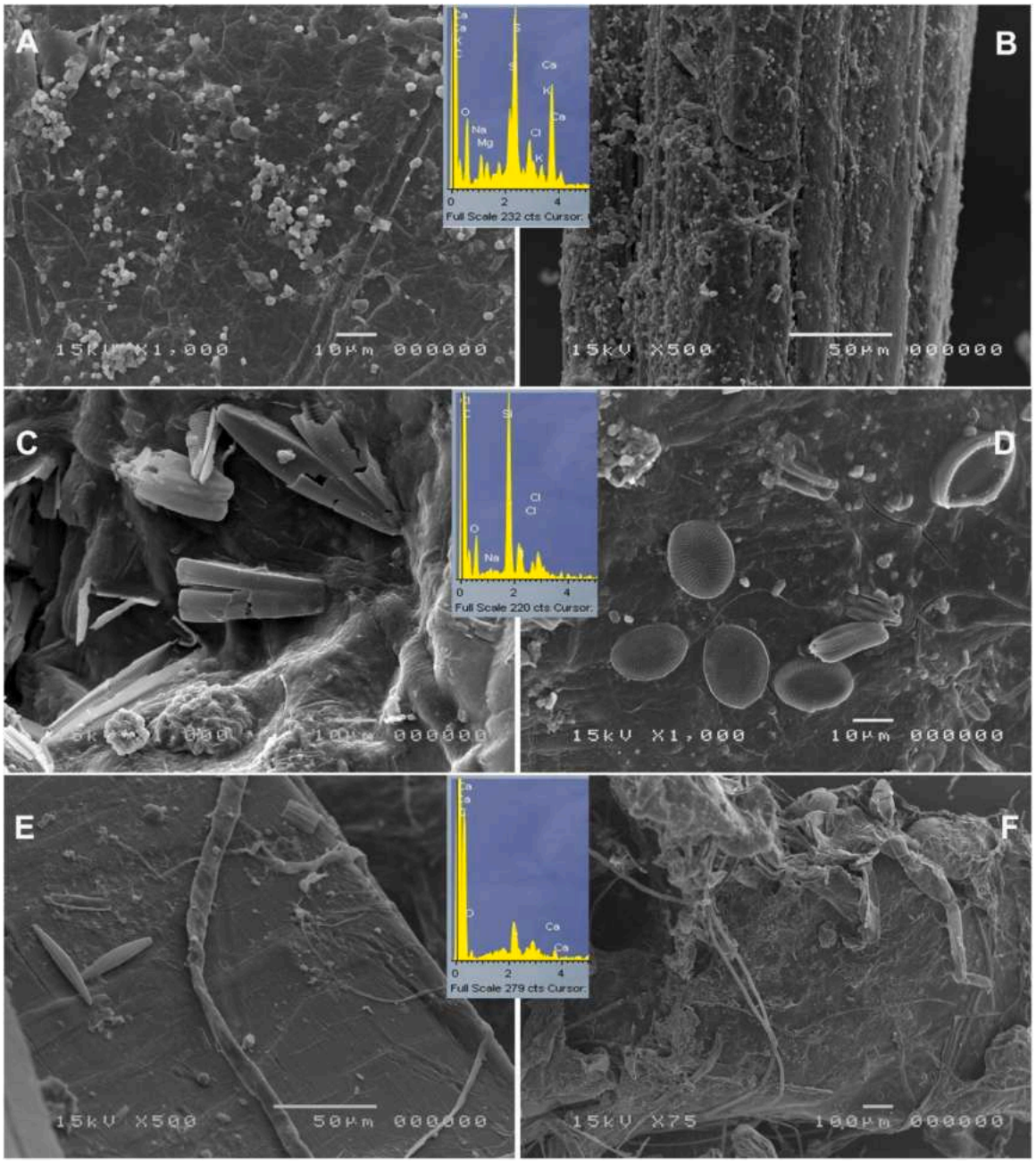

Fig. 7. SEM images and EDS spectra. Images of bacterial colonies on MPs (A) and MFs (B); the spectrum is representative of the chemical composition of the bacterial colony present on the microparticles. Panels C and D show the presence of various types of pinnate Diatoms encrusted on the surface and the representative spectrum of their chemical composition. In panels E and F, it is possible to observe pinnate Diatoms, MFs, and fragments of microcrustaceans on the surface of the MPs. The spectrum indicates the chemical composition of the microcrustacean fragments.

Table 3
Samples identified by the Raman analyses.

Site ID	Sample ID	Raman assignment
LM2	1	Polypropylene (PP)
	2	Diarylide yellow (pigment yellow 83)
	3, 4	Polyethylene (PE)
LM5	5	Boat paint
	6, 7, 8	Polyethylene (PE)
LM1	9, 10	Polyethylene (PE)
LM4	11, 12, 13, 14	Polyethylene (PE)

were clearly compatible with PE, except for one collected at LM2 site that showed unambiguous features corresponding to PP. A quantitative distribution of the identified samples is reported in Fig. 8.

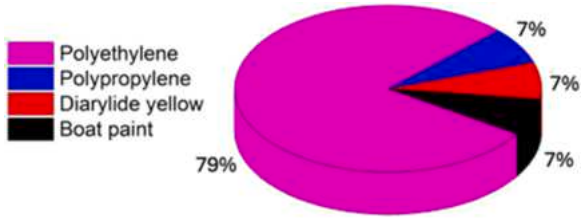

Fig. 8. Quantitative distribution of the samples identified by Raman analyses.

Summarizing, Raman analyses highlighted that 50 % of the samples were unambiguously identified as synthetic materials. In detail, PP particles are found only in the LM2 site, while PE particles are found in all the sampling sites. The polymers recognized in this study are those most commonly found in seawater [55]. In two cases (8 %), the identification of a synthetic pigment (diarylide yellow) and a solvent/pigment/additive/resin mixture (boat paint) suggest the anthropogenic nature of the examined particles. The remaining 42 % of the analyzed samples are fluorescent, and it was not possible to identify the nature of these particles, due to the presence of specific types of fluorescent dyes and/or organic substances on their surface as shown by SEM-EDS analysis. However, these samples could still correspond to additional polymer species. Several studies highlight the valuable contribution of Raman microscopy in determining the polymer composition of MPs. For instance, [59] use the micro-Raman technique with excellent results, but their protocol involves digestion and separation processes. Additionally, the laser they use differs from the one applied in this study.

4. Conclusion

This work highlights how it is possible to analyze MPs and MFs present in water without pretreatments, based on observation under optical microscopy. The observation can be conducted both live and by acquiring image data. Moreover, it highlights that automatic counting by AM software must be carefully supervised by an experienced operator when analyzing MPs and MFs in environmental matrices such as water and sediments, due to the complexity of the material and to prevent observational artifacts. The advantages of the method concern the possibility of collecting comparable information on MPs and MFs in different environmental matrices, without any sample treatment. In this way, the study of microparticles provides a realistic snapshot of their presence and their interactions with the environment. The disadvantages relate to the lack of information on their polymeric nature, which can subsequently be obtained using various spectroscopic methodologies.

This method allows for the recognition of bacterial colonies, phytoplankton, and zooplankton, highlighting the presence of specific ecosystems on the surface of these microparticles. In addition, this method permits to understand the accumulation processes of MPs and MFs, immediately highlighting anomalies corresponding to seasonal marine conditions such as water temperature, wind, and wave motion. Furthermore, it can provide crucial insights into pollutant sources, thus enabling timely intervention to address sensitive environmental issues.

Moreover, the exploitation of NGS techniques in environmental monitoring, specifically in the assessment of microplastic pollution in diverse habitats, complementing traditional methodologies (i.e., microscopy), consents to understand the capability of specific microorganisms to adhere to the plastic materials. NGS constitutes an asset, whose results might improve the microscopy analysis, allowing for confirmation of the classification of the viable microorganisms on the plastic materials in several habitats. Furthermore, the above-mentioned protocols might aid the identification of potential pathogenic strains composing plastic biofilms, posing threats to human health.

Environmental implication

Microplastics in the marine environment cause multiple negative effects, attaching to organisms, reducing mobility, and compromising their vital functions. Furthermore, they can serve as vectors for bacterial colonies threatening human health. Given this impact on the ecosystem, they must be studied and monitored carefully. Current protocols for studying MPs and MFs involve a series of treatments that can alter or erase crucial information regarding their transport, accumulation, and interactions with the surrounding environment. Our protocol based on optical microscopy is eco-friendly, fast, and requires no pretreatment. This allows for the identification of the dynamics of microplastic accumulation, their interactions with microorganisms present in the water, including bacteria, as well as the pollution sources on the beaches of the Vesuvian coast, heavily impacted by human activity.

CRediT authorship contribution statement

Carraturo Federica: Writing – original draft, Formal analysis, Data curation, Investigation, Methodology, Writing – review & editing. **Salamone Michela:** Investigation, Formal analysis, Data curation. **spagnuolo valeria:** Visualization, Writing – review & editing. **Ambrosi Filippo:** Software. **Giordano Simonetta:** Writing – original draft, Supervision, Conceptualization, Writing – review & editing. **Capozzi Fiore:** Writing – original draft, Validation, Data curation, Formal analysis, Writing – review & editing. **Vedi Vincenzo:** Investigation, Data curation, Formal analysis, Methodology. **Vergara Alessandro:** Writing – original draft, Supervision, Formal analysis, Data curation, Funding acquisition, Validation, Writing – review & editing. **Guida Marco:** Writing – original draft, Methodology, Investigation, Conceptualization, Funding acquisition, Supervision. **Troisi Romualdo:** Writing – original draft, Formal analysis, Data curation, Writing – review & editing. **Donadio Carlo:** Writing – original draft, Supervision, Resources, Data curation, Formal analysis, Investigation, Writing – review & editing. **Rossi Manuela:** Writing – original draft, Validation, Software, Methodology, Investigation, Formal analysis, Data curation, Conceptualization, Project administration, Supervision, Writing – review & editing. **Scognamiglio Viviana:** Writing – original draft, Visualization, Writing – review & editing. **Alberico Miriam:** Investigation, Formal analysis.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.jhazmat.2025.137468.

Data availability

Data will be made available on request.

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