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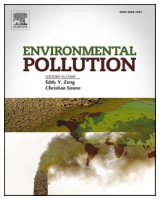

Epipelagic community as prominent biosensor for sub-micron and nanoparticles uptake: Insights from field and laboratory experiments☆

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ABSTRACT

Nowadays, ENMs/NPLs particles have not yet been extensively measured in the environment, but there is increased concern that this size fraction may be more widely distributed and hazardous than larger-sized particles. This study aimed to examine the bioaccumulation potential of engineered nanomaterials and nanoplastics (ENMs/NPLs) across marine food webs, focusing on plankton communities and commercial fish species (*Engraulis encrasicolus* and *Scomber colias*) from the Gulf of Naples. Laboratory experiments on plankton assemblages exposed to fluorescent polystyrene nanoplastics (PS-NPs, 100 nm) for 24h at concentrations ranging from 0.01 to 10 mg/L confirmed nanoplastic uptake in phytoplankton and zooplankton, indicating a dose-dependent internalization in plankton communities. Notably, in natural samples no particles were detected in fish muscle or liver tissues, suggesting limited translocation. Unexpectedly, titanium oxide particles (<1 μm) were found in natural phytoplankton, highlighting the potential presence of other nanoparticles in marine systems. These findings suggest that, despite detection challenges, plankton communities are major biosensors of ENMs/NPs contamination and highlight the need for ongoing environmental monitoring to assess ecological impacts and potential risks of nanoparticle bioaccumulation in marine ecosystems.

1. Introduction

Anthropogenic pressure threatens marine communities in coastal areas and the deep sea, leading to environmental deterioration and reduction in ecosystem services (Bulleri et al., 2020). Marine ecosystems, historically seen as major sinks for anthropogenic contaminants, are continuously receiving significant amounts of engineered nanomaterials (ENMs) and nanoparticles (NPs) (Corsi et al., 2014; Selck et al., 2016). These emerging contaminants can be deliberately produced with specific properties at the nanoscale or can arise incidentally as a side product from physical reactions or human activity (El-Kalliny et al., 2023). Since life first appeared on the Earth, natural nanomaterials deriving from dust storms, vulcanos or the weathering of rocks and living things coevolved (Hochella et al., 2019). By the early 21st century, owing to their unique size-dependent physicochemical properties, anthropogenic nano-sized particles have been widely used in a large number of medical, industrial, electronic, consumer and personal care applications (Keller et al., 2024). Most current NPs and ENMs can be include carbon-based material (i.e., fullerenes-C60, carbon nanotubes, carbon nanofibers) and metal/metal oxide such as Au or Ag NPs, TiO_2 and ZnO NPs, all ENMs/NPs extensively used in biomedical and healthcare products as well as for wastewater treatment (Kiser et al., 2009; Jeevanandam et al., 2018; Zahmatkesh et al., 2023).

In recent years, interest in a specific type of nanomaterial, nanoplastics (NPLs), has grown significantly (Piccardo et al., 2020). These particles represent the smaller nanoscale fraction of these colloids and

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are most likely to be incidentally produced from the fragmentation of larger plastic debris as also demonstrated under laboratory conditions (Lambert and Wagner, 2016). The transformation of microplastics (MPs) into NPLs is a process that remains largely overlooked. However, it has been clearly demonstrated, for instance, that the breakdown of MPs into NPLs occurs continuously during wastewater treatment (Monira et al., 2024). While the mechanistic processes underlying this transformation are poorly understood, even less is known about the contribution of biological interactions to the fragmentation of MPs into NPLs. Once in the marine environment, the surface of microplastics is rapidly colonized by microorganisms, forming a unique ecological niche known as the Plastisphere (Zettler et al., 2013). Certain members of the Plastisphere include plastic-degrading organisms, and microbial hydrolysis of plastic polymers or extreme oxidation through microbial biotransformation can lead to the generation of nanoplastics (Amaral-Zettler et al., 2020). Moreover, it has been shown that Antarctic krill (*Euphausia superba*) ingested microplastics of approximately 31.5 μm and fragmented them into pieces less than 1 μm in diameter (Dawson et al., 2018). Similarly, both marine (*Brachionus plicatilis*) and freshwater rotifers (*Brachionus calyciflorus*) have been observed to rapidly generate over 3.48×10^5 and 3.66×10^5 submicron particles per rotifer per day, respectively, from photo-aged microplastics through grinding mechanisms (Zhao et al., 2024). However, there is currently no universally accepted definition of NPLs: they can be described as plastic particles smaller than 1 μm according to the metric scale or as having at least one dimension between 1 and 100 nm according to the International Organization for Standardization (ISO) and the European Union (EU) (ASTM, 2020; ISO, 2008; Gigault et al., 2021). Beyond any definition, there is a real technological challenge to overcome the difficulties of sampling and analysing NPLs in the environment. As particle size decreases, detecting plastics in the environment becomes increasingly challenging, if not virtually impossible. As a matter of fact, no established analytical methods exist for the detection of NPLs in the aquatic environment causing a paucity of information to implement an environmental risk assessment (ERA) approach (Cunningham et al., 2023). Environmental concentrations of NPLs are expected to rise over time due to their increased use in various products, the global increase of plastic items and the significant release from the fragmentation and degradation of larger plastics (Bergmann et al., 2015). According to the recent estimates, NPLs concentrations are expected to become more than 10^{14} times higher than the current concentrations of microplastic particles in the ocean (Besseling et al., 2019). The first report of the chemical signatures of NPLs contamination in ocean surface samples has been provided by (Ter Halle et al., 2017) in the North Atlantic Subtropical gyre in which through pyrolysis GC-MS highlighted the presence of polyvinyl chloride, polyethylene terephthalate, polystyrene and polyethylene (1–999 nm). More recently (Moon et al., 2024), retrieved nylon nanofibers (~20 nm, diameter) and a ball-stick–shaped PET nanoplastics along Long Beach, CA. Moreover, in this study PET nanoplastics were also found in water samples from the offshore location in the Gulf of Mexico, which are collected from 311-m deep under the water surface (Moon et al., 2024). Looking at Mediterranean Sea, NPLs contamination in Mediterranean marine ecosystems is still unknown, although a first study from (Schirinzi et al., 2019) reported traces of nano-sized polystyrene (PS), in the range of 1.08–136.7 ng L^{-1} in estuarine and surface waters of the West Mediterranean Sea. NPLs end up in marine ecosystems arising from landfill operations, wastewater treatment plants, improper disposal of untreated sewage as well as marine-based sources such as direct discharging of litters from ships/boats, fishing nets, aquaculture practices (Kumar et al., 2021). Once in marine environments, a manifold transformation occurs, often characterized by dynamic and ephemeral phases, determining their environmental fate, behaviour as well as their bioavailability driving the interaction with marine organisms through different exposure routes. The stability of colloids is sensitive to the interplay between the properties of the dispersed colloid-phase and the abiotic and biotic factors of the surrounding environment, physically often described in the framework of the classical DLVO theory (Derjaguin and Landau, 1993). In the marine environment, aggregation is a key issue in understanding the fate of NPLs (Wang et al., 2021). The paramount interfacial interactions, absorbome-like, concern diverse coronal biological coatings (i.e. eco-corona and protein-corona), natural organic matter (NOM) and even some dissolved organic matter (DOM) as well as microbial colonization (Galloway et al., 2017; Corsi et al., 2020; Zhang et al., 2022; Shi et al., 2023).

From an ecotoxicological point of view, NPLs, and more generally ENMs, thanks to the increased surface/volume ratio and consequently presenting a higher number of atoms at the surface compared to the core, show greater chemical, physical and biological reactivity, thus imparting unique properties on a material, which does not happen at other sizes (Ferreira et al., 2019; Corsi et al., 2020). Nanoplastics are anticipated to pose a greater hazard than microplastics due to their increased potential for cellular interaction. This enhanced interaction includes the likelihood of internalization via mechanisms such as endocytosis, phagocytosis, micropinocytosis, or even diffusion through the lipid bilayer (Firdessa et al., 2014; Rossi et al., 2014; Sendra et al., 2020). These processes allow NPLs/ENMs to penetrate cells more easily and potentially cause more significant biological effects up to a direct interaction with the nucleus (Augustine et al., 2020). Laboratory-controlled studies have shown that NPLs/ENMs can adversely affect the growth, feeding behaviour, mortality, and immune response of marine organisms across different trophic levels, including microalgae, echinoderms, ascidians, bivalves, and fish (Della Torre et al., 2015; Balbi et al., 2017; Bergami et al., 2017; Bellingeri et al., 2020; Eliso et al., 2020; Murano et al., 2021). In detail, it has been seen that at the basis of the toxicity mechanism of these colloids occurs the overproduction of reactive oxygen species which initialize phenomena of neurotoxicity, embryotoxicity, genotoxicity (reviewed in Hu and Palić, 2020; Corsi et al., 2021; Wang et al., 2021; Geremia et al., 2023). Because of their small size, the surface of NPLs/ENMs is an ideal spot for interaction with a variety of hazardous contaminants, such as heavy metals and persistent organic pollutants, which may enhance their bioavailability to living organisms and raise their potential biological and toxicological effects (Mattsson et al., 2018).

The ambitious goal of this study was to assess the potential for internalization of submicrons and nanoplastics (<1 μm) in the pelagic environment in this uncertain context, without a proper ecological risk assessment and without a recognition of the best indicators underlying risk assessment features. We specifically examined phytoplankton and zooplankton assemblages, as well as commercial fish species including anchovies (*Engraulis encrasicolus*), and mackerel (*Scomber colias*) communities, in attempt to replicate the classical marine food-web in the Gulf of Naples (Mediterranean) on a small scale. Before analyzing natural samples from the Gulf of Naples, we conducted controlled laboratory exposures where phytoplankton and zooplankton natural assemblages were treated with polystyrene nanoplastics (PS-NPs) as proxy for environmental particles. These preliminary experiments helped to establish a baseline for understanding particle internalization in plankton communities.

2. Methods

2.1. Experimental design and in vivo exposure

Phytoplankton and zooplankton sampling were carried out in the Gulf of Naples through the LTER-MC program (https://deims.org/0b87459a-da3c-45af-a3e1-cb1508519411) (Tesson et al., 2014; Di Capua et al., 2023) between September and October 2023. Specimens of *E. encrasicolus* and *S. colias* were fished in the same area in the same days. Once in the laboratory, the phytoplankton and zooplankton samples were immediately fixed in 4% PFA, counted and stored at +4 °C while the anchovy and mackerel specimens were stored individually at

−20 °C. In order to obtain information on the potential accumulation pattern, a positive control case study was performed in which specimens of phytoplankton and zooplankton were exposed to fluorescent polystyrene nanoplastics (PS-NPs, 100 nm, Polysciences Europe GmbH, Eppelheim, Germany) as a proxy for nanoplastics, at different concentrations 0.01, 0.2, 2, 5, 10 mg/L for 24h, under controlled laboratory conditions. In detail, 6 equal aliquots (100 mL) of the total phytoplankton/zooplankton/sample were inserted into six different glass beakers to which filtered sea water was added until reaching a final volume of 500 mL. Working solution aliquots were then added to obtain the final PS-NPs concentrations (0.01, 0.2, 2, 5, 10 mg/L). The *in vivo* exposures were performed in triplicate for both phytoplankton and zooplankton and took place in a chamber with controlled temperature and light conditions (18 °C) for 24 h. After 24 h of exposure, samples were washed 5 times with FSW in order to eliminate the not-internalized particles, then the organisms were fixed in 96% ethanol. All samples (natural and treated) were sent to the Bioscience Research Center (Orbetello, Italy) for submicron and nanoparticles and PS-NPs quantification. Here, different approaches, including optical microscopy, fluorimetry, Raman spectroscopy and SEM microscopy, were employed to detect the presence of PS-NPs in both phytoplankton and zooplankton specimens at all tested concentrations.

2.2. Polystyrene nanobeads

Fluorescent-labelled polystyrene nanobeads (100 nm, PS-NPs) (441 excitation/486 emission) were purchased from Polysciences (Warrington, PA, U.S.A.). According to the supplier, the particles were packaged as 2.5% aqueous suspension (4.55 x 10^{13} PS-NPs/mL) without biocides or stabilisers. In addition, these PS-NPs are internally dyed and not surface-labelled. This confers a greater resistance to photobleaching and to surface-leaching. PS-NPs working solutions (10 mg/mL and 1 mg/mL) were prepared in ultrapure water and then added to filtered natural sea water (NSW, 0.22 μm) to reach the final concentration of 10, 5, 2, 0.2, 0.01 mg/L. Stock solution were briefly sonicated (bath sonicator 40 kHz, 3 min), while working solutions were vortexed for 3 min prior to use. In order to investigate PS-NPs behaviour under the experimental conditions, we monitored the evolution of the stability of a solution of PS-NPs (250 μg/mL) under static conditions and after 3 min of stirring at controlled temperature for 24 h using Turbiscan (TURBISCAN LAB, Microtrac). The same solution was used to evaluate the aggregation state of the PS-NPs through a Mastersizer 3000 (Malvern Panalytical, Malvern, UK, WR14 1XZ) laser granulometer with manual entry of operating conditions and material properties. Here, we further confirmed the stability of PS-NPs YG fluorophore during the exposure condition (24 h in SW) using the protocol proposed in Murano et al. (2021) using Microcon® (10 kDa). Polystyrene was chosen as proxy for ENMs/NPs due to the fact that it's one of the most largely used non-biodegradable plastic worldwide and unlike other polymers, it shows a greater stability in sea water suspension with low styrene release (Moon et al., 2024; Monira et al., 2024; Keller et al., 2024). Additionally, particles in PS disperse in suspension or float because their density (1.04–1.06 g/cm^3) is comparable to that of natural water (Li et al., 2021).

2.3. Sample treatment

2.3.1. Zooplankton and phytoplankton communities

Both natural samples of plankton assemblages (phytoplankton and zooplankton) and PS-NPs exposed ones, after a thorough analysis of the digestion methods, were processed with a solution of H_2O_2 30% (1:5 v/v) followed by a sonication step for 60 min at maximum frequency (40 KHz) at 60 °C (Murano et al., 2022). After the digestion of the organic matter, the natural samples were filtered on Anodisc® filters (0.22 μm mesh) using a vacuum pump. In the case of PS-NPs exposed samples the digestion occurred directly on a glass slide. Before the digestion, at least 10 specimens from the treated samples (both phyto- and zooplankton) were used for microscopy analyses. In addition, a portion (1 mL) of the digested product was retained for direct fluorescence measurement (Ex 441/Em 485). All filters and slides obtained were dried in an oven at 39 ± 1 °C and stored in a glass Petri dish at room temperature (Todaro et al., 2023).

2.3.2. Fish specimens

50 specimens of *Engraulis encrasicolus* and 6 specimens of *Scomber colias* were dissected in order to obtain, of each individual, the muscle and the liver. Each individual was first weighed, measured and during dissection their sex and stage of maturity were determined according to (Ferreri et al., 2009). Then the males were divided from the females and pools of liver and muscle were created from these according to the stage of maturity (I, II, III, IV, V, VI) (see Supporting Material). In the case of fish specimens, the use of potassium hydroxide (KOH) was adopted. Briefly, the pools of livers and muscles were digested with KOH + NaOH solution (1:3 v/w) incubated at room temperature overnight on a plate stirrer. After the digestion of the organic matter, the samples were filtered in this case on glass fiber filters (0.22 μm mesh) using a vacuum pump. The resulting solution of filtration was used for the detection of nanoplastic by RAMAN spectroscopy.

2.4. Microscopy and spectroscopic analyses

The effective internalization of PS-NPs in the phytoplankton and zooplankton and samples was assessed using optical microscopy with a Nikon microscope at 100x magnification and fluorometric analysis with a Tecan spectrophotofluorometer (Infinite M1000 Pro). For this last analysis, the solution resulting from the digestion was analysed, of which 200 μL in triplicate were placed in a black plate and the fluorescence intensity expressed in arbitrary units was analysed by subtracting the background values of digestion solution (H_2O_2 solution 1:5). All filters and slides obtained from the digestion phase (natural and treated organisms) were analysed under RAMAN microscope spectroscopy (μRAMAN, DXR2, s/n AIY1716158, Thermo Scientific®), equipped with Olympus confocal microscopy for the determination of chemical nature of targeted particles (laser set emission source at 532 nm). Thermo libraries for microplastic detection by RAMAN spectroscopy integrated with BsRC ones were used. Phenom Pro G6 SEM microscope (Thermo) 160-350.000x equipped with EDS (Energy Dispersive X-ray Spectroscopy) microsonde for quantitative elemental analyses.

2.5. Quality control

The most critical phase for possible contamination concerns all the steps starting from the dissection to visual identification. Therefore, during all phases of this study the operators worked under controlled conditions to reduce the interference from laboratory air contamination and/or other inputs (Todaro et al., 2023). For this purpose, samples were treated in a controlled area (glove box; Iteco engineering, mod. SGS20-13599, serial number 103,421) to ensure the absence, down to 200 nm of size, of contamination from air and other laboratory sources. All materials, equipment, bench surfaces, and gloves were carefully cleaned before and after the analysis of each sample.

3. Results and discussion

In the Anthropocene era, the environment is increasingly subjected to an ever-growing production and accumulation of emerging contaminants, such as microscale and nanoscale particles, including microplastics, NPLs, and ENMs. Despite the significant interest that these contaminants have garnered in recent years, there remains a critical need to understand how they behave in an ecosystem context. In this study, we addressed the imperative of understanding the bioaccumulation behaviours of submicron and nanoparticles through the pelagic food-web to identify potential biosensors for this pollution-type.

Therefore, this study was conceived with the intent to determine the biomagnification of nanoplastics (<1 μm) along a two-tiered food-web, focusing on the planktonic assemblages (phyto- and zooplankton), and extending to commercial fish species, as well as anchovies (*Engraulis encrasicolus*) and mackerel (*Scomber colias*). While phytoplankton and zooplankton were considered as an assemblage in their entirety, the focus for evaluating the potential translocation patterns in fish was the muscle, the edible part, and the liver. Before studying the content of natural assemblages, a laboratory controlled study was performed to evaluate the bioaccumulation pattern of fluorescent PS-NPs (100 nm) used as a proxy for nanoplastics in the same phytoplankton and zooplankton specimens exposed to concentration starting to environmentally relevant one 0.01 mg/L up to 10 mg/L for 24 h.

3.1. PS-NPs exposure

3.1.1. Polystyrene nanoparticles behaviour

Polystyrene (PS) is one of the most abundant non-biodegradable plastics among marine debris (Plastic Europe, 2006). PS is one of the most investigated plastic particle types, largely adopted as a proxy for nanoplastics toxicity (Catarino et al., 2019). The time-solved dispersion stability of the PS-NPs suspension in SW was measured for 24 h at 10-min intervals using a Turbiscan both in completely static conditions (Fig. 1A) and after 3 min vortex stirring (Fig. 1B). PS-NPs suspension remained stably suspended in the liquid between the first 20 mm of height with a little change in transmission and back scattering. Instead, in the case of post-stirring suspension there was a PS-NPs distribution across the entire height with time-dependent variations in transmission and back scattering (Fig. 1A and B). Moreover, the results revealed the immediate tendency towards a constant increase in the TSI (Turbiscan Stability Index) already in the first hour while the PS-NPs suspension after stirring input did not present particular variations in TSI for up to 2 h followed by an increase (Supporting). The dimensional characteristics of the obtained PS-NPs suspension in SW determined by laser-granulometry show high aggregation occurring (Fig. 2). Indeed, the volume distribution (%) revealed that after 24 h approximately 90% of the particles are in a size between 0.01 and 4 μm with a peak at approximately 0.7 μm (Fig. 2). These data are coherent with the optical microscopy observation reported below in Tables 1–2. PS-NPs are susceptible to homo-agglomeration (i.e., between NPs themselves) and hetero-agglomeration (i.e., between non-homologous particles) when suspended in SW or any alkaline and high ionic strength milieus (Therezien et al., 2014; Praetorius et al., 2020). Table 1 summarizes the chemical-physical characteristics (pH, salinity, O_2) of the sea water of

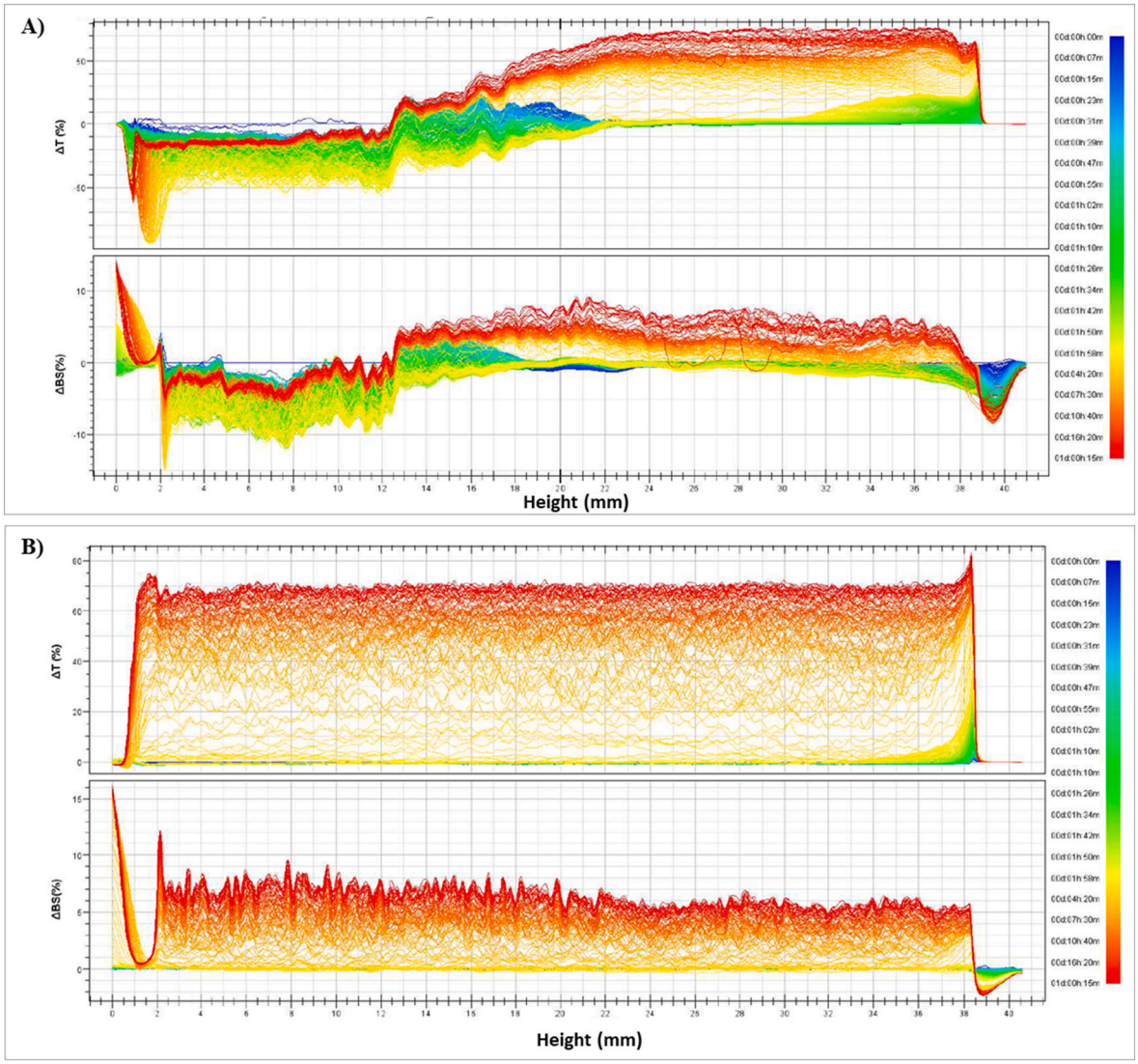

Fig. 1. Profile of PS-NPs Dispersion Stability in static condition (A) and after 3 min stirring (B) for 24h in sea water. Data presents the mean backscatter and transmission in percent of PS-NPs in SW. Analysis was done with Turbiscan Lab (Formulaction, France) and analysed with TurbiSoft LAB software.

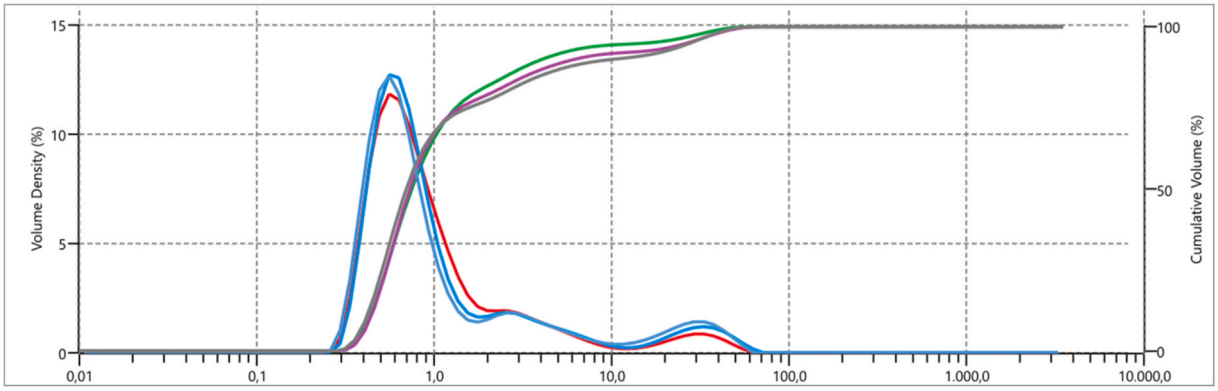

Fig. 2. Laser-Granulometer analysis of size distribution of PS-NPs suspension in SW (250 μg/mL) in SW after 24h (log scale).

Table 1
Chemical-physical characteristics of the sea water used during the experiment.

	pH	Salinity			O_2
		g/L	PSU	mS/cm	mg/L
SW MC site	8.13	26.4	34.88	52.9	5.14

Table 2
Total number of PS-NPs internalized by the zooplankton community. Results are referred to 10 random specimens for each concentration tested.

Zooplankton			
[PS-NPs]	Area (μm^2)	N° PS-NPs	PS-NPs (μm)
10 mg/L	2520	74	0.54 ± 0.1
5 mg/L	2500	48	0.57 ± 0.1
2 mg/L	2540	23	0.43 ± 0.1
0.2 mg/L	2490	13	0.46 ± 0.05
0.01 mg/L	2500	1	0.51 ± 0

the Gulf of Naples (LTER- MC site, 40°48.500′N, 14°15.010′E) used during the *in vivo* PS-NPs exposure of zooplankton and phytoplankton. For instance, under laboratory conditions bare PS-NPs along with an increase in NaCl concentration, decreased net repulsive energy barrier between NP particles according to DLVO theory, causing PS-NPs aggregation up to 4 μm at 35 ‰ (Wu et al., 2019). Stability of plain PS-NPs in NSW has been described in other studies in which is reported in the time-dependent agglomeration that occurred later at 24 and 48 h (Brandts et al., 2018; Tallec et al., 2019; Mishra et al., 2019).

3.1.2. PS-NPs toxicokinetics

Integrated spectroscopy and microscopy approaches allowed the detection of a concentration-dependent uptake of PS-NPs in zooplankton and phytoplankton communities after 24 h of exposure to 10, 5, 2, 0.2, 0.01 mg/L of PS-NPs. In detail, in the case of zooplankton for each surface area analysed (2500 μm^2) the maximum internalization rate was observed at a PS-NPs concentration of 10 mg/L was of 74 ± 21.74 (PS-NPs) going down to 1 ± 0.3 at PS-NPs concentration of 0.01 mg/L (Fig. 3, Table 2). For phytoplankton, the maximum internalization rate was 66 ± 33 (PS-NPs) at 10 mg/L of PS-NPs going down to 0.7 ± 0.8 at 0.01 mg/L of PS-NPs (Table 3). Even through advanced microscopy techniques, the probable presence of PS-NPs aggregates in zooplankton and phytoplankton samples treated at the highest concentration was highlighted (Fig. 4). In this way, by exploiting the fluorescent properties of nanoplastics, we described a nearly similar pattern of accumulation between natural communities of phytoplankton and zooplankton from the Gulf of Naples, dependent on concentration. It was interesting to note that up to a concentration of 5 mg/L, the internalization was almost similar between zooplankton and phytoplankton, but at the maximum

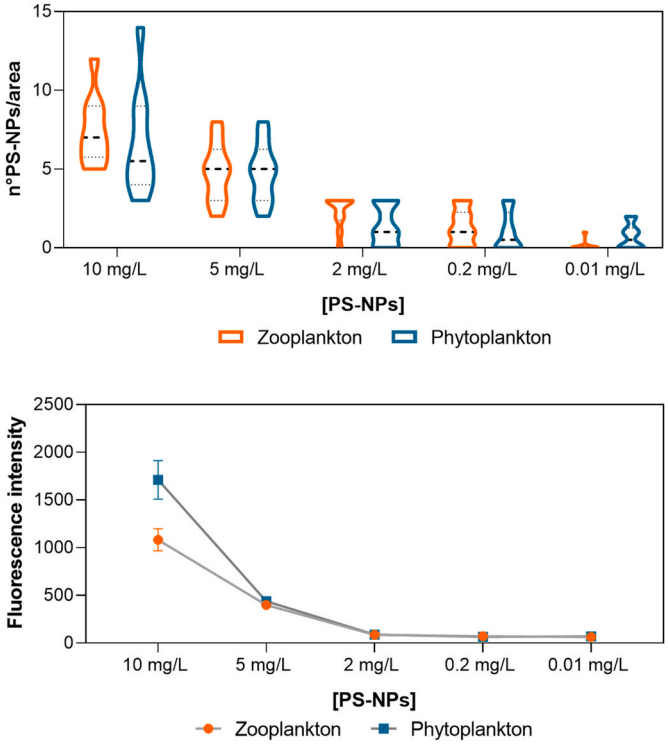

Fig. 3. Quantification of PS-NPs uptake by zooplankton and phytoplankton communities after digestion process. (A) Results represent the number of particles within an optical surface area of 250 μm. (B) Fluorescent intensity of digested samples of zooplankton and phytoplankton.

Table 3
Total number of PS-NPs internalized by phytoplankton community. Results are referred to 10 random specimens for each concentration tested.

Phytoplankton			
[PS-NPs]	Area (μm^2)	N° PS-NPs	PS-NPs (μm)
10 mg/L	2500	66	0.60 ± 0.2
5 mg/L	2500	48	0.57 ± 0.14
2 mg/L	2550	14	0.46 ± 0.14
0.2 mg/L	2420	10	0.46 ± 0.13
0.01 mg/L	2520	7	0.73 ± 0.14

tested concentration of 10 mg/L, the phytoplankton exhibited a greater accumulation capacity. To the best of our knowledge, this is the first report in which it is described how the interaction occurs by mimicking the environmental conditions between nanoplastics and a community of phytoplankton or zooplankton, naturally composed of such diversified

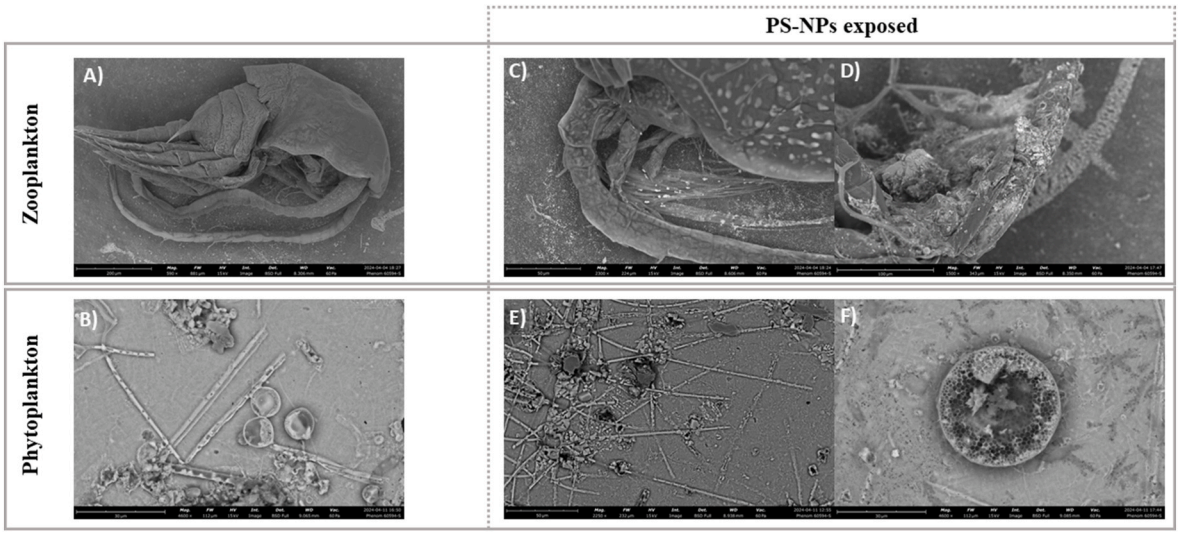

Fig. 4. SEM examples of untreated zooplankton A) and phytoplankton (B) and respective PS-NPs treatment [10 mg/L] (C–F).

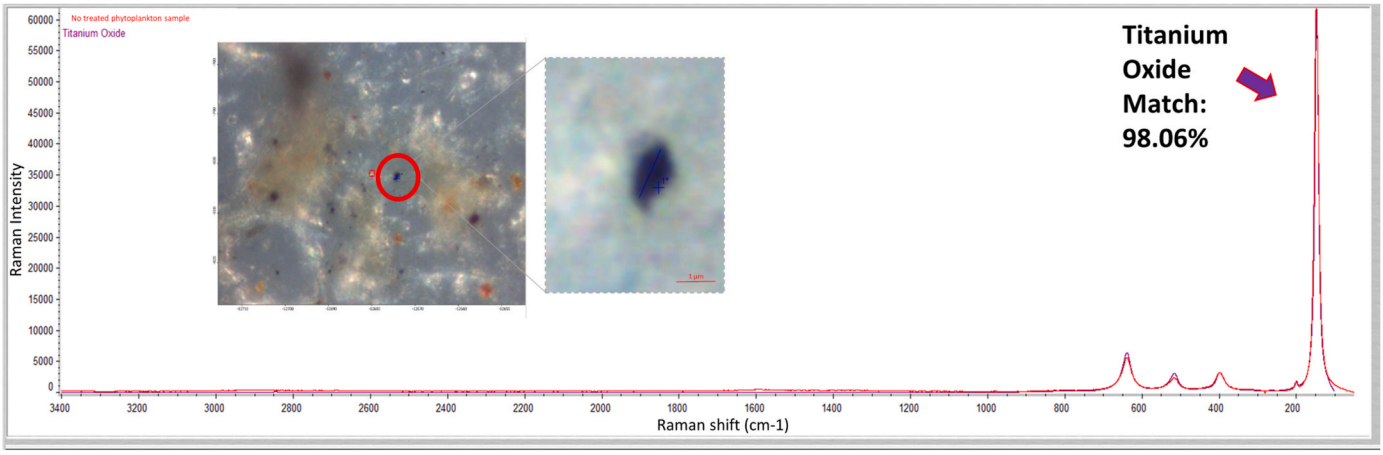

Fig. 5. Raman spectra of Titanium oxide sub-micron particle (in red) recorded in the Phytoplankton community from the Gulf of Naples overlaid with the original Titanium oxide spectrum in purple. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

taxa and genera, and not focusing attention on particular selected taxa. Working with natural, multi-species plankton communities allows us to capture the complexity of microbial behaviors in natural water systems, which can differ significantly from the behaviors observed in studies conducted under simplified laboratory conditions with single-species cultures (Yallop et al., 2022). The use of fluorescence to facilitate the identification of particles is a widely used technique, this also happens with regards to tests with microplastics, until at least seventy-two percent of nanoplastic bioaccumulation studies used fluorescently labelled particles. Over time, other detection strategies such as the formulation of 14C-labelled PS or metal coupled to overcome false positives/negatives obtained from the loss of the fluorophore (Mitrano et al., 2019; Al-Sid-Cheikh et al., 2020; Redondo-Hasselerharm et al., 2021). As specified, the fluorophore of labelled PS-NPs used in this study was embedded within the polymer during synthesis eluding any leaching of the dye during the exposure. However, as underlined by the most recent literature, the stability of PS-NP fluorophore should also be proven prior to use to avoid artefacts when studying NPLs uptake and translocation by fluorescence imaging (Catarino et al., 2019; Schür et al., 2019). Here, we further confirmed the stability of YellowGreen fluorophore under the experimental conditions (24 h in NSW) excluding the dispersion/loss (see Fig. S3).

3.2. Untreated natural samples

The complexity level is evidently higher in samples not treated with labelled particles. In terms of abundance the phytoplankton assemblage reached 2.5 x 10^4 cell/L, dominated by small undetermined flagellates, including naked dinoflagellates (58%), followed by coccolithophorids (21%), diatoms (15%) and by other dinoflagellates (6%). *Coccolithophorids* group is dominated by small undetermined taxa, *Syracosphaera pulchra* and *Coronosphaera* sp.; diatoms group is dominated by *Pseudo-nitzschia delicatissima* complex, Achnanthes sp., *Paralia sulcata, Cerataulina pelagica, Thalassionema nitzschioides;* dinoflagellates group, excluded naked ones, is characterized by *Torodinium robustum*, *Heterocapsa/Azadinium* sp., *Gymnodinium* spp. > 20 μm and *Gyrodinium* sp. Our search for sub-micron particles and nanoparticles did not yield the expected results. Specifically, no sub-micron particles were detected in the muscles and liver of anchovies and mackerel. Similarly, zooplankton samples showed no presence of plastic or other materials including the neritic species *Temora stylifera* and *Clausocalanus furcatus*. However, in the September phytoplankton sample, we observed five titanium oxide particles, with sizes ranging from 700 nm to 1 μm (Fig. 5). In light of the fact that the collection of the phytoplankton sample involved the filtration of 24.3 m^3 of SW through the net, and considering the concentration of the sample, it is possible to estimate that 5 nano-TiO

particles were found in 6 x 10^9 cells. In detail, Raman spectroscopy identified particles of Titanium Oxide/Titanium Dioxide with a match of 98.06-98.05% using different libraries. Understanding the bio-accumulation is essential to determining a substance's potential eco-toxicity (Petersen et al., 2023). The discovery that the phytoplankton community from the LTER-MC location contains 700 nm–1 μm TiO_2 particles is a significant finding of this study. Due to its high photo-catalytic potential, this material is extensively utilized in self-cleaning paints, self-sterilising surfaces, cosmetics and personal care products, water treatments, and solar cells (Vance et al., 2015). As a result, it has become one of the most prevalent and widely employed metal oxide nanomaterials globally. Undoubtedly, the widespread use of titanium dioxide particles, especially nano-TiO_2 (<100 nm) has raised substantial concerns about their possible impacts on both the aquatic environment and human health (Klaine et al., 2008). Furthermore, it is important to emphasise that TiO_2 naturally occurs in sediments and the water column, generally in three crystalline phases: anatase, rutile, and brookite (Du et al., 2019). Due to TiO_2 ENMs' low concentrations compared to naturally occurring nanoparticles and their similar characteristics, it is currently difficult to distinguish between anthropogenic and natural particles (Gondikas et al., 2018). Recent model predictions estimate that titanium dioxide nanoparticle concentrations in European waters are around 16.8 μg L^{-1}. However, during the summer period, these concentrations can exceed 900 μg L^{-1} in surface waters near popular beaches (Labille et al., 2020). At the higher organisational level, nano-TiO_2 toxicity is manifested as the negative effects on fitness-related organismal traits including behavioural, feeding, reproduction and immunity in aquatic organisms through the main mechanisms of TiO_2 toxicity based on genotoxicity, damage to membranes, inflammation and oxidative stress processes (Corsi et al., 2014). Delving further, in the case of phytoplankton the toxicity mechanisms for TiO_2, in NPs and bulk form, are related to the interaction between nanoparticles and cell surface (Sendra et al., 2017). On this ground, Manzo et al. (2015) demonstrated that TiO_2 (20 nm nominal size-aggregates grew up to 4 ± 5 μm) seemed to exert its toxic action in the first hours of exposure, mostly via cell entrapment and agglomeration on marine microalgae *Dunaliella tertiolecta* (Manzo et al., 2015). Accordingly, Wang and co-authors (2012) described nano-TiO_2 aggregates were found to entrap algae cells *Phaeodactylum tricornutum*, which is likely responsible for the observed toxic effects exerting its most severe inhibition effects on the first day of exposure, at a lower EC50 of 12.65 mg/L (Wang et al., 2016). Moreover, direct physical effects such as cell wall damage, affecting also chlorophyll *a* content, soluble sugar content, malondialdehyde (MDA) content, SOD and POD activities (Deng et al., 2017). At the basis of the interaction between nanosized particles and phytoplankton is the ability of the phytoplankton to release exopolymeric substances (EPS), or polysaccharide-rich anionic colloidal biopolymers, which perform a plethora of functional roles including the protection against various environmental threats (Chiu et al., 2017). Accordingly, EPS can significantly edit the colloidal behavior of NPs suspended in seawater deeply contouring their bioavailability and toxicity (Grassi et al., 2020). Simultaneously, it is noteworthy that no particles were detected in samples of zooplankton, or in the muscle and liver tissues of *E. encrasicolus* and *S. colias*. This result may be attributable to the methodologies employed, highlighting the lack of standardised and efficient techniques for such studies. In part, it could be attributed that the concentration of NPLS/ENMs is below the detection limit in line with others studies focused on the presence of fullerenes, carbon nanotubes or graphene-family nano (Petersen et al., 2016; Goodwin et al., 2018; Petersen et al., 2019). However, the ability to identify TiO_2 submicron particles in phytoplankton samples substantiates the robustness of the findings and excludes the possibility of operation-contamination. The absence of particles in zooplankton and fish tissues/organs may be due to the rapid elimination of such particles and the lack of translocation phenomena. Being top predators, fish are expected to be exposed to NPLs and ENMs, directly through water column ingestion, gill tissue or skin or indirectly through trophic transfer from preys (Brandts et al., 2021). Literature available highlighted the presence of micro-nano-plastic (MNPLs) polymers (polysiloxane, polyethylene, poly-dimethylsiloxane) in muscles of *Oreochromis niloticus* collected in a commercial European recirculated aquaculture system farm (Blonç et al., 2023). Interestingly, Grasso and co-authors (2020) provide a chemical characterization and quantification of TiO_2-NPs in processed canned fish products belonging to different trophic position of the pelagic compartment demonstrating that canned tuna had levels of TiO_2-NPs significantly higher (0.117 mg/kg) than canned mackerel (0.02 mg/kg) and anchovy (0.06 mg/kg) (Grasso et al., 2020). In our study, we specifically focused on analyzing the edible muscle tissues and liver of *Engraulis encrasicolus* and *Scomber colias*, omitting the digestive system to evaluate not only potential bioaccumulation but also particle translocation. This approach allows us to better assess the risk of NPLs/ENMs presence in edible parts and in liver that undergo biotransformation, which is crucial for food safety assessments. The absence of detectable particles in these tissues could be attributed to several factors: it may reflect current detection limits, the lack of translocation from the digestive system to other tissues, or even the general absence of NPLs/ENMs contamination within the samples. These considerations highlight the challenges in establishing reliable detection methods for ENMs/NPLs and underscore the exploratory nature of our study in assessing nanoplastic behavior within a real-world marine food web context.

3.3. Ecological implications

Phytoplankton communities are an extremely important component of the functioning of ecosystems and climate regulation. Let's just sum up by saying that primary phytoplanktonic production accounts for at least 50% of the oxygen we breathe (Falkowski, 2012) and that phytoplankton profoundly affect the biogeochemical cycle of many elements, such as carbon, nitrogen and phosphorus (Falkowski et al., 2004). In marine ecosystems, are the dominant primary producers providing the base of oceanic food-webs supporting production of higher trophic levels (Tweddle et al., 2018). Furthermore, according to the Millenium Ecosystem Assessment (2005), phytoplankton provides at least thirty different ecosystem services, including those that are essential to the maintenance of ecosystems, water purification, provisioning, climate regulation, and cultural functions (Reid et al., 2005). In the era of global change, human-induced environmental factors are rapidly evolving and interact with phytoplankton in complex ways. These interactions can lead to a range of outcomes on the growth and productivity of phytoplankton, including stimulation, neutrality, or negative impacts (Häder and Gao, 2017). For instance, according to recent model projection, phytoplankton biomass is projected to decrease over much of the tropical and subtropical ocean due to lower nutrient supply rate (Henson et al., 2021). The loss of biodiversity appears to affect ecosystems as much as climate change, pollution and other major forms of environmental stress (Hooper et al., 2012). As abundant small (0.2–200 μm) single or colonial organisms with high surface-to-volume ratios suspended in water, phytoplankton have high probability of encountering suspended particles, including pollutants of emerging concern, especially in coastal zones where the input of these contaminants could reach the highest concentrations (Miller et al., 2012). In this regard, interactions between phytoplankton assemblages and ENMs/NPLs may be crucial to the health of marine ecosystems, with possible drawbacks for primary producers that could affect trophic chains and ecosystem services. The results of this study fit into this context, demonstrating the effective interactions between sub-micron particles and natural phytoplankton communities. Although perhaps out of topic, this work serves as a valuable example demonstrating that the ecotoxicological approach is an innovative tool in the context of long-term time series. On one hand, the data obtained can be used to create functional exposure models, while on the other, ecotoxicology could form the basis for

shifting the paradigm from controlling classical or canonical parameters of the communities under study towards the frontier of emerging contaminants and, importantly, towards conceptualizing the socioeconomic dimension.

4. Conclusions

A primary challenge in understanding the bioaccumulation behaviours of ENMs/NPLs is that the analytical methods for quantifying their concentration in different tissues are still being developed, as no potential biosensors for their monitoring have yet been identified. This study represents a first attempt to evaluate the accumulation potential in the first links of the natural pelagic trophic chain. While no particles were found in zooplankton specimens and in the liver and edible part of fish with high commercial impact, in phytoplankton natural communities were found particles <1 μm of titanium oxide. If on the one hand this observation defines phytoplankton as a prominent biosensor, on the other the elective interaction with NPs/ENMs may also affect water quality and ecosystem services, underscoring the need for stringent environmental monitoring and regulation of nanoparticle pollutants. Our research provides a critical foundation for understanding the ecological consequences of ENMs/NPLs in marine environments. With a view to improving ENMs/NPLs detection techniques, future studies should focus on long-term monitoring of nanosized particle concentrations, their interactions with other environmental pollutants, and the mechanisms driving their ecological effects. These finding serves as an exploratory pilot study aimed at enhancing our understanding of contamination phenomena and will serve as a support to focus attention on fundamental aspects such as the effects of this contamination on processes such as photosynthesis and primary production and to build new studies on the translocation and effects of nanoparticles. Additionally, future research should use the developed analytical workflow to focus on assessing and quantifying ENMs in other food-webs or organisms with commercial strength. As technologies for detecting nanoparticles and nanomaterials in marine environments continue to improve, addressing the dynamics of particle transformation in marine environment and their migration through more complex food web systems will become a categorical imperative for advancing our understanding of their ecological impacts.

CRediT authorship contribution statement

Carola Murano: Writing – original draft, Visualization, Validation, Software, Methodology, Investigation, Formal analysis, Data curation. **Tecla Bentivoglio:** Writing – review & editing, Software, Methodology, Formal analysis. **Serena Anselmi:** Writing – review & editing, Visualization, Validation, Software. **Leonilde Roselli:** Writing – review & editing, Validation, Software, Methodology, Investigation, Formal analysis. **Iole Di Capua:** Writing – review & editing, Validation, Software, Methodology, Investigation, Formal analysis. **Monia Renzi:** Writing – review & editing, Validation, Supervision, Resources. **Antonio Terlizzi:** Writing – review & editing, Supervision, Resources, Project administration, Funding acquisition, Conceptualization.

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. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.envpol.2024.125566.

Data availability

Data will be made available on request.

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