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The aim of this study was to characterize and quantify microplastics (MPs) at the chlorophyll maximum layer (CML), around 30 to 60 m depth, during a cruise dedicated to the study of contaminants in plankton, the MERITE-HIPPOCAMPE project, along a north-south transect in the western Mediterranean Sea (Tedetti et al., 2023). Plankton were collected by horizontal net tows in this layer using a multinet Hydrobios Midi equipped with 60 µm mesh-size nets. The collected plankton were fractionated through a sieve column for various later contaminant measurements and for zooplankton analysis (Fierro-González et al., 2023). For all stations, samples were also fully examined for microplastics (MPs) for fractions greater than 300 µm. MPs were found at all stations in the CML layer (mean: 42.9 ± 45.4 MPs m^{-3}), of which 96 ± 4% were fibers. The ratios of mesozooplankton/MPs and detritus/MPs in this CML were respectively 223 ± 315 and 2544 ± 2268. These data are analyzed together with MPs concentrations from sea- surface sampled with a 300 µm net-size Manta net at the same stations. Overall, our observations highlight the very high density of fibers at the CML, mainly associated with aggregates, raising the hypothesis of their interactions with marine snow. Therefore, the importance of marine snow and vertical layering will have to be considered in future MP distribution modelling efforts.

Keywords: (maximum 5): Microplastics, Mediterranean Sea, chlorophyll maximum, zooplankton, detritus

Highlights

- High MPs densities observed at the chlorophyll maximum layer in Mediterranean Sea
- Fibers were the dominant MPs at the CML
- Fibers were mostly associated with detritus in aggregates
- At the CML, zooplankton/MPs ratio varied from 5 to 1000

1. Introduction

MPs in the oceans are assessed as a growing threat to marine food chains (Cole et al., 2011). The vast majority of observations of MPs in the ocean environment are made at the surface and on the seabed substrate, and much less in the water column (Cincinelli et al., 2019; Lefebrve et al., 2019; Chevalier et al., 2023), even though the redistribution of surface MPs in the first 10 meters of the water column by mixing due to wind stress has received more attention in order to correct assessments of surface MPs concentrations (Kukulka et al., 2012; Chevalier et al. 2023 and quoted references therein). Numerous studies have been carried out on the sedimentation of surface MPs, mostly using modelling or experimental approaches, even including the impact of potential degradation and biofouling processes (e.g., Mendrik et al., 2023). To date, very few studies have attempted to quantify variations in the distribution of MPs in the water column (Choy et al., 2019, in offshore areas; Chevalier et al., 2023 in inshore areas), and they have highlighted accumulation layers. The fate of MPs in the pelagic food chain is also of great interest, as the size spectrum of MPs ranges from hundreds of µm to a few mm, and shows a strong overlap with the size spectrum of planktivorous fish prey (Chen et al., 2022). However, potential risks for planktivorous fishes ingesting MPs are inferred from potential predation at the surface (e.g., Fabri-Ruiz et al., 2023), while major trophic interactions occur on zooplankton patches within the water column (Benoit-Bird, 2009; Möller et al., 2012). Studies combining MPs sampling within layers of the water column and observation in planktivorous fish guts are still very rare (Lefebrve et al., 2019). In the marine environment, the water column is also the site of an abundant load of particles, called 'marine snow', composed of aggregates of organic and inorganic matter, whose size spectrum ranges from 0.5 mm to tens of cm (Alldredge and Silver, 1988), strongly overlapping with the MPs size spectrum. This marine snow is also

characterized by layers of higher density within the water column (Möller et al., 2012). How marine snow interacts with MPs in unknown. Therefore, it is essential to know and quantify the MPs that can be found in key layers of the water column, together with the associated concentrations of zooplankton and detritus.

In order to better understand the role of plankton as a pump of contaminants, the MERITE-HIPPOCAMPE project (Tedetti et al., 2023) has developed a massive sampling protocol at the chlorophyll maximum layer (CML). Zooplankton sampling was carried out by horizontal tows of nets through filtration of large volumes. This approach provided a unique opportunity to examine the MPs content in samples intended for counting and characterizing the collected zooplankton (Fierro-González et al., 2023). The **aims of this study** were (1) to count, classify and measure MPs collected at the CML during the MERITE-HIPPOCAMPE cruise, (2) to identify differences in concentrations and size structures between the MPs collected at the CML and those collected at the sea surface, (3) to compare the concentrations of MPs to zooplankton and detritus densities in the same samples, (4) to identify potential differences between the coastal and offshore stations.

2. Material and methods

Study area and environmental data. The MERITE-HIPPOCAMPE cruise was carried out between 13 April and 14 May 2019, along a north-south transect in the western Mediterranean Sea, from the French coast (Toulon, Marseille) to the Tunisian coast (Gulf of Gabès) aboard the French Research Vessel *Antea* (see **Fig. 2** in Tedetti et al., 2023). Ten stations were sampled from the bays of Toulon and Marseille in the north (4 north coast stations: St 01: coastal station in the inner bay of Toulon; St 02: station offshore of the bay of Toulon; St 03: offshore of the bay of Marseille, above the head of the Planier Canyon on the

shelf break; and St 04 within the bay of Marseille) to the south of the Tunisian continental shelf (3 south coast stations: St15 in the Gulf of Hammamet, St17 and St19 in the Gulf of Gabes), with three deep oceanic stations along the transect, one in the Ligurian Sea (St09 offshore station to the north of the Balearic Thermal Front) and two stations west of the Sardinian coasts (St10 is situated southwards of the Balearic Front and north of Sardinia, and St11 southwest of Sardinia). For each station, the date of sampling, the geographical position, the station depth, and the depth of the different samples (CTD and net tows explained below) are presented in **Table 1** in Tedetti et al. (2023). At each station, an oceanographic carousel equipped with a CTD Seabird SBE 911+ to measure the hydrological variables (temperature, density and salinity) was deployed down to 250 m for open-sea stations or near the bottom for shallower stations. In addition, the CTD was coupled with several sensors including chlorophyll-a fluorescence (Chla; Aqua Tracka, Chelsea ctg), LISST and LOPC optical sensors.

***Zooplankton and MPs sampling and observations*.** Micro- and mesoplankton were sampled with a Multiple Plankton Sampler (Hydro-Bios Midi type, square aperture surface of 0.25 m^2, HYDRO-BIOS Apparatebau GmbH) towed horizontally at the depth of the CML at a constant speed of around 2 knots (see sampling depth for each station in **Table 1**), with five successive shut-off nets of 60 µm mesh-size, each of them filtering a water volume of around 50 to 80 m^3 (estimated by automatic detection with internal and external flowmeters, and according to plankton load before clogging). The analysis of zooplankton and MPs at the CML was done from aliquots of samples taken with these net tows, and then fractionated by sieving on board in a dedicated container under clean conditions (fractions 60-200; 200-500; 500-1000; 1000-2000 and > 2000 µm). All fractions were treated for zooplankton studies (see Fierro-González et al., 2023). Fractions above 500 µm and the fraction 300-500 µm, from the zooplankton sample 200-500 µm sieved on 300 µm mesh, were fully examined

under a binocular microscope for MPs. We separated the sample in aliquots of small quantities of plankton in Dolfus chambers, then separated all the aggregates as far as possible using forceps. The small number of stations (10) meant that all samples could be exhaustively and meticulously observed, even though this was time-consuming.

In addition, a Manta net (opening 60 cm x 20 cm, mesh size 300 µm) was towed at the surface for 20 min at 2 knots to sample an area of about 1.000 m^2, for counting and measuring of MPs following the IFREMER / MSFD - Marine Strategy Framework Directive - protocol (Hanke et al., 2013). The sample from station 3 was lost. For comparison with microplastic concentrations at the CML, surface MPs counts per m^{-2} were transformed into concentration per m^{-3} by multiplying by 0.1 based on sampling a layer 10 cm below the surface. The relative sampling efficiency of the two different sampling gears (Manta net with 300 µm mesh for surface layer sampling and 60µm mesh Multinet for horizontal CML sampling) for MPs categories and size classes is discussed in Supplementary Material 1. The use of the Hydrobios midi net and Manta net is nowadays the best combination of nets for sampling horizontally the surface layer and oceanic layers in the water mass with accurate depth position, and with fairly comparable filtered volumes (several tens of m^3).

Zooplankton surface counts were obtained from another Manta net with a 60 µm mesh-size net, towed at a constant speed of 2 knots for 10 min, and the sieved-fractions above 200 µm of this sample were used for determining MPs/zooplankton ratio at the surface. Zooplankton analysis for multinet Hydrobios and Manta nets are fully described in Fierro-González et al. (2023).

The quality of MPs observations was based on (members of the consortium's skills as plankton observers and as microplastics specialists. Compact microplastics (pellets, films, fragments) are easily

recognizable, with shapes and angles that are not found in plankton organisms. Fibers can be more confusing but experienced zooplankton observers can easily differentiate elongated body parts from MPs fibers (for instance segmentation and setae of copepod antennules, used in taxonomy). Because no chemical treatment was used in this study, the term 'fiber' is used here according to this broader definition and it is possible that not all fibers are made from plastic. A plate with examples of photos taken with a binocular magnifying glass to identify and measure microplastics in plankton samples collected at the CML is presented in Supplementary Material 2. In the laboratory, these various stages of treatments were carried out in a closed laboratory used exclusively for these observations and following the handling recommendations to avoid sample contamination (Brander et al., 2020).

Measuring MPs dimensions. MPs collected with the 300 µm mesh-size Manta net were sieved and sorted by size class, with four classes for the MPs (300 µm-1 mm, 1-2 mm, 2-5 mm, > 5 mm). For each size class, MPs were counted, and their typology determined (fragment, pellet, filament/fiber, foam (mainly polystyrene) and film) under a binocular microscope (Gérigny et al., 2022). Observations dedicated to MPs for the Hydrobios net were made with a Leica M 165C binocular microscope with camera. Length and width of each MPs item were measured. MPs were classified following the same typology as described above.

Statistical analysis. Results of MPs concentrations, frequencies in size fractions, zooplankton /MPs ratios, detritus/MPs ratios, in the surface water and at the CML are presented as mean values ± standard deviation and ranges in square brackets. Differences in mean values between regions for surface and CML layers were tested using ANOVA, after log X+1 data transformation to obtain normality and homoscedasticity using the Kolmogorov-Smirnov and Levene tests. This analysis was performed using Statistica v7 software. Differences in MPs size distribution between surface and CML were compared by calculating the Fisher-Pearson coefficient of skewness. For the spatial patterns of abundances of MPs and % of fibers in MPs,

zooplankton and detritus, and of the ratios zooplankton/MPs and detritus/MPs, a station matrix was created with square-root transformed data to estimate Bray–Curtis similarity distances. The similarity matrix was then ordinated using non-metric multidimensional scaling (NMDS). This analysis was performed using PRIMER v7 software.

3. Results

Figure 1 presents the distributions of MPs, zooplankton and detritus collected at the surface and at the CML, together with temperature, fluorescence and salinity profiles for the different stations. Concentrations of MPs, zooplankton and detritus are presented in Log10, with MPs x100 for easy comparison. In total, for the surface Manta samples, 1184 MPs items (for 9 stations) were counted and classed in 4 size fractions, and for those of the Hydrobios at the CML, 3165 MPs items were found and identified at all 10 stations, and for 3 of the stations size measurements were made for 1317 MPs and then distributed in the same 4 size fractions (**Figure 2**). At the CML, MPs abundance ranged from 10.1 to 117.9 MPs m^{-3} across the 10 stations, whereas in the top 10 cm of the sea surface the range was from 0.1 to 3.4 MPs m^{-3} (**Table 1**). MPs at the CML were on average 96 ± 4 % fibers, the rest being either fragments or films (**Figure 2 top**). In comparison, MPs found in the surface water at these stations were largely dominated by fragments (89 ± 7 %). At the 3 stations where comprehensive size measurements of all MPs were made for CML samples (mostly fibers), the size distributions presented a similar shape declining in proportion with increasing size classes (on average, 40 %, 35 %, 22 %, 3 % in classes 0.3-1, 1-2, 2-5, 5-200mm, respectively). In contrast, the size distributions of the MPs collected at the surface showed variable proportions in size classes (**Figure 2 bottom**). Fisher-Pearson coefficient of skewness was on

average 0.52 (± 0.12) for the distribution at the CML, and 0.29 (± 0.29) for the distributions at the surface.

The microplastics concentrations and the ratios zooplankton/MPs and zooplankton/detritus in the two sampled layers and visited regions showed some clear aspects (Table 1). In the surface water, MPs concentrations were found in the order of 10^{-1} to 10^{0}, but with no significant differences between regions. Zooplankton/MPs and detritus/MPs ratio were in the order of 10^{2} to 10^{5}. At the CML, MPs concentrations were found in the order of 10^{1} to 10^{2}, whereas zooplankton/MPs and detritus/MPs ratio were in the order of 10^{0} to 10^{3}. For MPs concentrations at the CML, significantly lower values were found at the 3 deep sea stations compared to coastal stations ($p=0.03$).

The Non-Metric Multi-Dimensional scaling (NMDS) (**Figure 3**) provides a very good visualization of the similarities and differences between surface and CML sampling for the different stations, in relation with detritus, zooplankton and MPs contents, fibers percentage, and detritus/MPs and zooplankton/MPs ratios. Samples from the CML and surface layers are clearly distinct for all stations, in relation to the MPs content / fibers percentage axis. Secondly, while the samples from the CML tend to be grouped by zones with the exception of St.15, the samples from the surface stations are very scattered, even within same zones: this can probably be explained by the effect of differences in the conditions of the surface patchiness on MPs under the effect of high-frequency forcing (winds, surface currents).

Samples from the CML are dependent on the water column dynamics. For these samples, there is (1) a strong clustering of offshore oceanic stations (St.9, St.10, St.11) with similar MPs loads, and slight differences in mesozooplankton concentrations (as shown in Fig.1 and Table 1), (2) a clustering of NC and SC coastal stations with higher MPs loads (St.1, St.2, St.3, St.4,

St.17, St.19), except for St.15 whose load is more comparable to that of the offshore oceanic stations. Among the coastal stations, stations 4, 17 and 19, all very shallow (< 60 m) and sampled close to the bottom, form a very similar group because of their load of detritus and zooplankton.

4. Discussion

During MERITE-HIPPOCAMPE, MPs were found at all stations both at the surface and at the CML. The observation of MPs at the surface is widely documented and is now carried out in accordance with standards (Hanke et al., 2013). In the Mediterranean, it is estimated that MPs are found over almost all the sea-surface (Collignon et al., 2012; Fabri-Ruiz et al., 2023). Our observed MPs surface concentrations did not show identifiable regional differences due to high variability between stations, related to the characteristics of local transport (Rwawi et al. 2023). The surface MPs quantified during our campaign with the 300 µm mesh-size Manta net (0.005 to 0.343 MPs m^{-2}) are within the classical range of observations in the Mediterranean as presented in the review by Cincinelli et al. (2019).

On the other hand, MPs observations in the water column are much less numerous, and also much less standardized in terms of protocols. They are either obtained by vertical net tows for zooplankton (e.g., Lefebvre et al., 2019), or by using bottles that can be closed at the desired depths but with a limited volume (e.g., Tamminga et al., 2018; Dai et al., 2018), or by sediment traps for long-term observation of sinking MPs (e.g., Galgani et al., 2022). Collection of MPs by pumping is restricted to sub-surface samples in the upper 15 m (see Table 2 in Montoto-Martinez et al., 2020). The use of a Hydrobios Midi Multinet for MPs has been done before, but for closing net vertical tows (Zhao et al., 2022, with 100 µm mesh-size

nets), but its use for perfectly horizontal tows with a layer thickness of 0.25 m within the densest in situ plankton layers, as in our study, is, to our knowledge, new. Our observations of MPs concentrations at the CML (with 60 µm mesh nets; fraction > 200 µm) varied between 10 and 120 MPs m^{-3} at the level of these dense planktonic layers. The rare publications providing concentrations in the water column give very variable values depending on the sampling device. With a 90 µm mesh-size closing net, Gorokhova (2015) found that intermediate layers (30-60 m) in the 100 m deep water column have more MPs per unit volume than upper (0-30 m) or deeper (60-100 m) layers, and reach 10^2-10^4 MPs m^{-3} in this intermediate layer. Similarly, Uurasjärvi et al. (2021) using both closing nets with a 100 µm mesh-size towed vertically in the intermediate layers and 30 L volume closing bottles found variations of 10^0-10^3 MPs m^{-3}. Choy et al (2019) present a distribution of MPs over the whole water column in the deep-sea area of Monterey Bay (from surface to 1000 m) sampling with a ROV (their Fig1). The distribution suggests a first local maximum of MPs below the surface at 25 m (which corresponds to the local Chl*a* maximum layer, see Monterey Bay Time Series Data, https://www.mbari.org/data/mbts-data/) followed by a decrease down to 75 m, and then a rapid increase below this depth down to the bottom of the mixed layer (around 200 m), to slowly decrease again downwards. They specify that most of these MPs are fibers (see their Figure S4 in their online supplementary and the associated legend text information - https://doi.org/10.1038/s41598-019-44117-2-), and they are in the same order of magnitude as those we observed.

Our MPs results are rare because they were obtained for large volumes in thin horizontal layers at the level of the CML using the Hydrobios net, and they show that the densities of MPs observed in this CML layer are higher than those at the surface, and that this pattern is more marked in coastal areas than in offshore areas. It is likely that in coastal areas,

resuspension of suprabenthic detritus including MPs can maintain a relatively high particle load in the intermediate layers (Chevalier et al., 2023), whereas at offshore ocean stations, the observed concentrations of MPs at the CML are not greatly affected by vertical resuspension, and certainly not from the benthic reservoir.

The very high proportion of fibers distributed in all size classes (based on their length) (Figure 2) is probably partly linked to the mesh size of our nets and our vertical sampling protocol in the densest planktonic layers, which inevitably leads to clogging. Larger mesh sizes and towed nets avoiding clogging are clearly not suitable for collecting fibers. Conversely, closing bottles are probably the best sampling tool for this. Dai et al. (2018), when studying the fine vertical distribution of MPs in the water column of Bohai Bay using 10 L Niskin bottles, detected fiber proportions of 75 % to 96.4 % of the total MPs, with accumulation in the intermediate layers. The distribution of fiber sizes in the few samples where these measurements have been made (Figure 2) deserves further investigation: the relative regularity of these fiber size distributions at the CML may be related to mechanical or biological phenomena of fiber fractionation.

Fibers, films and fragments from CML samples have often been observed under a binocular loupe to be trapped in an organic matrix (marine snow, aggregates). This may be due to aggregate-microplastic complexes formed *in situ* but may also be enhanced by the sample collection and sieving protocol. However, the fate and transport of MPs has previously been reported to be intrinsically linked to marine snow formation (Möller et al., 2012; Porter et al., 2018; Kvale et al., 2020), but the resulting aggregate-MPs complexes have instead been mostly considered as a factor increasing the vertical transport of MPs to the seabed and potentially impacting the benthos (Porter et al., 2018; Kvale et al., 2020). For MPs having a density close to the water density, such as polypropylene fibers, it is possible that they are

trapped and accumulated in thin layers of perennial marine snow (Zhao et al., 2022) promoting denser layers of fibers, which accumulate and float over a long period (Prairie et al., 2015). Our observations even suggest that the fibers become micro-skeletons of marine snow or aggregates. Nevertheless, it is also possible that the larger fibers structure larger aggregates of marine snow which ultimately trap other particles which increase sedimentation. Fiber size distributions at the CML may be a consequence of differences in the stability of aggregate-MP complexes at density interfaces.

The zooplankton/microplastic ratio is considered a valid index of the impact of MPs on planktonic biota (Gerigny et al., 2022), and can be used to characterize a potential impact of microplastics loading on the prey available to planktivorous fish (Fabri-Ruiz et al., 2023). However, it can be seen that these ratios obtained during the Hippocampe cruise are extremely variable from one station to another (Table 1), for both the surface and CML layers. An in-depth analysis of the relative vertical distributions of zooplankton and microplastics would require taking into account, at each station, the recent dynamic history of the water masses in the epipelagic layer and the dynamics of the water column (over a few days), which have a very strong influence on the establishment of physical gradients in the water column and therefore of chemical and biotic gradients (Rwawy et al., 2023; Chevalier et al., 2023). All the features are also highlighted by the NMDS analysis.

Our observations show that detritus and phytoplankton aggregates are always more abundant in the CML than in the surface layer, which is also the case for microplastics (Figure 1), mainly of the fiber type (Figure 2). It is well recognized that thin layers of phytoplankton and marine snow particles are associated with strong density gradients inducing reduced fall rates (Alldredge et al., 2002), so it is hardly surprising to find here higher proportion of buoyant microplastics.

Fierro-Gonzalez et al. (2023) analyze in detail the observed differences in stocks of zooplankton, detritus and microphytoplankton aggregates and in the relative distributions of zooplankton taxonomic groups at the level of the surface and CML layers (see their Fig. 4), and over the whole water column (0-200 m, or 0-bottom for stations with bathymetry < 200 m). In general, the surface layer is distinguished from the rest of the water column by relatively stronger representations of certain taxa (copepods, crustaceans or gelatinous plankton) with species that are often characteristic of the hyponeuston, and which can proliferate there (Champalbert, 1971).

At the CML layer, the general pattern over the Hippocampe cruise highlights specific features in the distribution of taxonomic groups compared with the average concentrations in the water column: greater proportions of copepods, nauplii, and other crustaceans, much smaller proportions of herbivorous gelatinous plankton (appendicularians, salps) and pteropods, and carnivorous plankton (chaetognaths, jellyfish). Recent studies using cameras allowing a fine vertical resolution, either towed on a platform (Möller et al., 2012; Greer et al., 2020) or placed on a glider (Whitemore and Ohman, 2021), show similar patterns of different relative distributions of taxonomic groups between dense layers of fine particles and the rest of the epipelagic water column.

Studies of the distribution and behaviour of mesozooplanktonic organisms show that certain groups tend to aggregate in these fine layers, while others seek to avoid them (Greer et al. 2013). Zooplankton taxa that are strict phytoplankton filter feeders, either with appendages or with filtering systems, tend to avoid layers with high densities of detrital particles that obstruct their filtering systems (appendicularians, salps, some of calanoid copepods) and will feed on phytoplankton and particles in more diluted layers. Inversely, many omnivorous filter- feeders and ambush feeders, either omnivorous-herbivorous (most calanoid

copepods), or omnivorous-carnivorous Cyclopoida and Poecilostomatoida (i.e. Oithonidae, Oncaeidae and Corycaeidae), or omnivorous-detrivorous (harpacticoids), tend to aggregate in dense layers (Möller et al., 2012; Koski et al., 2017) associated with bacterial and microbial development (Kiorboe, 2001). In addition, other planktonic crustaceans (larvae and juveniles of mysids, ostacods and amphipods) also consume particles and microzooplankton that are colonizing marine snow aggregates (see quoted papers in Möller et al., 2012).

These zooplankton taxonomic groups found in the CML are among the favorite prey of small pelagic fish (Chen et al, 2022), that exploit dense layers of particles and zooplankton (Benoit-Bird, 2009; Möller et al, 2012), and it is therefore important to assess the quantity and quality of MPs in these layers that can be swallowed accidentally by fish. In their joint study of MPs concentrations in the water column and the stomach contents of fish in the Gulf of Lion, Lefebvre et al. (2019) showed a relatively low occurrence of MPs in fish stomachs (around 10% of individuals), and fibers were the only type of MPs encountered in the digestive tracts. Their results are consistent with the relatively high ratio zooplankton/MPs ($> 10^4$ in our study) in the dense layers (CML for us) preyed on by planktivorous fish.

In our results, the lowest zooplankton/microplastics ratios were obtained for stations in outer continental shelf zones (St. 3 and St. 15) or in oceanic zones close to the slope (St. 2 and St. 11), while the highest ratios were obtained for either inshore littoral zones (St. 1, St. 4, St. 17, St. 19) or offshore oceanic zones (St. 9 and St. 10). Espinasse et al. (2014), in their study of particle and zooplankton distributions in the Gulf of Lion, showed that beyond the 100 m isobath from the continental shelf to the oceanic waters above the shelf break, the zooplankton is aggregated in very dense layers located between 20 and 40 m, whereas in the area close to the coast (below the 100m isobath), the water mass is strongly stirred with homogenized distributions of particles and zooplankton throughout the water column. This

suggests that further studies on the associated distributions of microplastics and zooplankton should take into account knowledge of the structuring of planktonic habitats, as done for example by Espinasse et al (2014), and characterize the MPs/zooplankton ratios in key layers of the water column, not only at the surface.

5. Concluding remarks

Two major interfaces promote the accumulation of MPs: the air-sea surface interface with the accumulation of MPs less dense than seawater, and the water-sediment interface where MPs denser than seawater accumulate. Our observations based on horizontal sampling with a Multinet Hydrobios midi in the densest planktonic layers of the water column, close to the chlorophyll maximum, highlight a high concentration of MPs mainly fibers (sometimes films and fragments) associated with high levels of detrital aggregates. Although our protocol may have amplified these MPs-aggregate complexes, our results enable us to propose that sub-surface dense layers of marine snow and plankton represent a third major marine interface for MPs types with a density close to that of water and floating in the water column and will have to be considered in future MPs distribution modeling efforts. Clearly, the use of Multinet nets allowing targeting of particular layers associated with dedicated bottle sampling represents a suitable methodological approach to better characterize the MPs load of these productive oceanic layers. This is all the more important as the major trophic interaction layers between zooplankton and their prey and between fish and their zooplankton prey are mainly these dense layers.

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Figure captions:

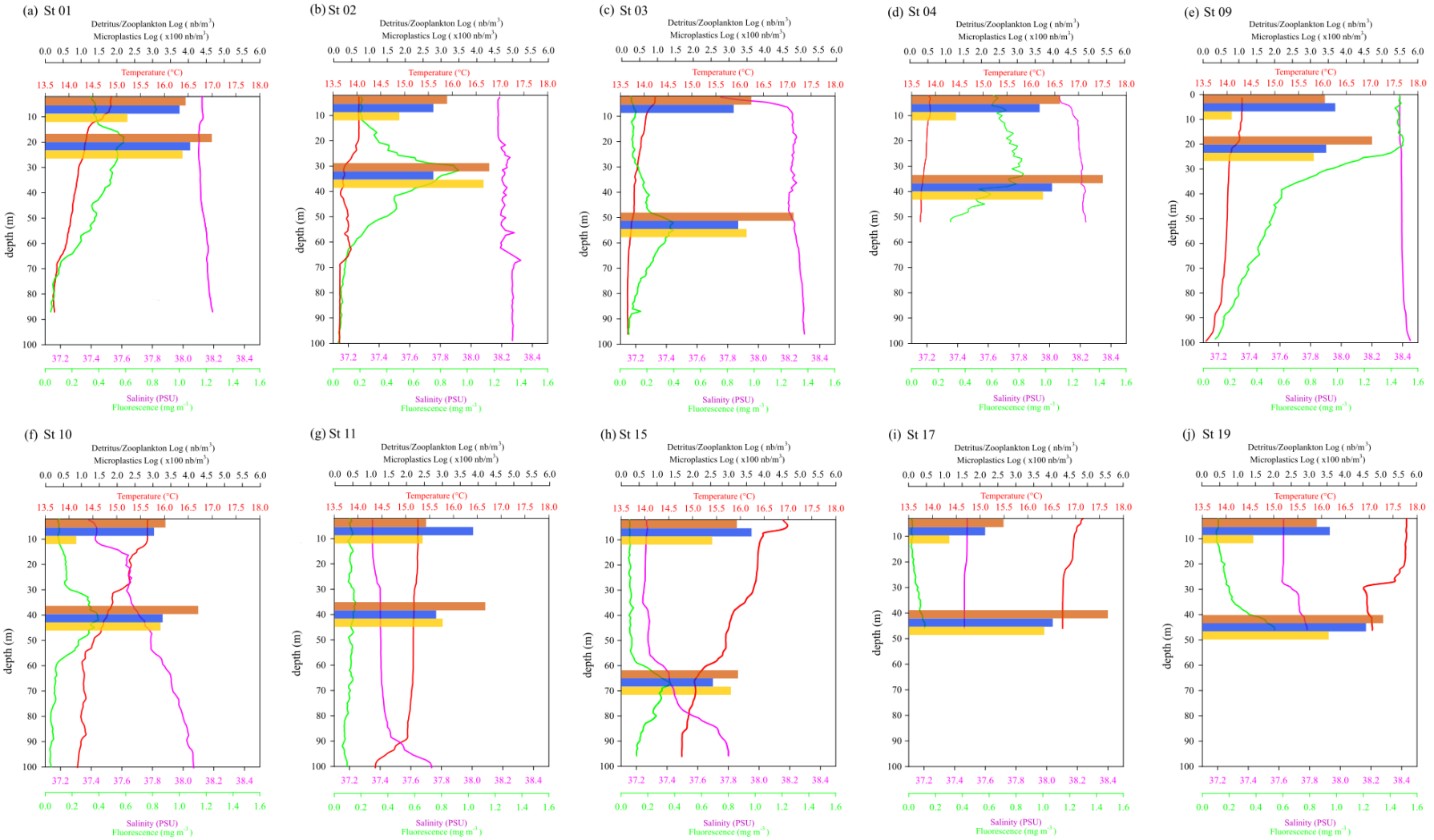
Figure 1: Distribution of detritus (brown bar), mesozooplankton (blue bar), and microplastics (MPs, orange bar) at the surface and at the chlorophyll maximum layer (CML) at the 10 stations of the MERITE-HIPPOCAMPE cruise (see presentation in the text, and detailed information in Tedetti et al., 2023 and Fierro et al., 2023). Values in Log10 for detritus and zooplankton, Log10 (x 100) for MPs. Red, pink and green lines are respectively the vertical profiles of temperature, salinity and fluorescence.

Figure 2: Distributions of the different types of microplastics (MPs) between fibers. fragments, films, polystyrenes and pellets. Top: Distributions of the different types of MPs between fibers, fragments, films, polystyrenes and pellets at the surface and the chlorophyll maximum layer (CML). Sample of St.3 at the surface is missing. Bottom: Distributions of MPs in size fractions at stations St.1, St.10 and St.19 at the surface and at the CML.

Figure 3: Non-Metric Multi-Dimensional scaling (NMDS) on abundances of MPs, % of fibers in MPs, zooplankton and detritus, and on ratios zooplankton / MPs and detritus / MPs. NC: northern coastal stations (St.3 is missing in surface waters); SC southern coastal stations; DS: Deep-oceanic stations; Open symbols: Surface samples; Full symbols CML samples.

Table 1: Values of environmental variables (temperature, salinity, density, chlorophyll-*a*), concentration of detritus, zooplankton and microplastics (MPs), and ratios detritus/ MPs and zooplankton/MPs at the CML and at the surface. Zooplankton, detritus and MPs collected with Hydrobios 60 µm and then sieved > 200 µm at the CML, and collected with Manta net tow and sieved > 300 µm at the surface.

Zones	North Coast Stations				Deep Oceanic stations			South Coast Stations					
Stations	St1	St2	St3	St4	St9	St10	St11	St15	St17	St19		Mean	SD
Isobath (m)	91	1770	100	58	2575	2791	1378	100	50	50			
Surface													
Temperature (°C)	14.9	14.0	14.2	13.9	14.3	15.5	15.3	17.3	17.2	17.8			
Salinity (‰)	38.1	37.9	37.8	38.1	38.4	37.5	37.4	37.3	37.5	37.6			
Density (x-1000 g l^{-1})	28.4	28.4	28.3	28.6	28.7	27.8	27.7	27.2	27.4	27.3			
Chlorophyll-*a* (mg Chla m^{-3})	0.34	0.24	0.08	0.54	1.39	0.10	0.15	0.05	0.02	0.11			
Manta net Fraction > 300 µm													
Zooplankton (ind m^{-3})	6216	563	1573	3948	4224	1037	7069	4208	133	3307		3228	2366
Detritus (# m^{-3})	8014	1893	4382	14742	2646	2543	378	2322	448	1725		3909	4393
Microplastics (MPs) (# m^{-3})	2.3	0.7		0.2	0.1	0.1	3.0	3.4	0.2	0.3		1.1	1.4
Ratio Zoo/MPs	2652	759		20718	82368	13666	2395	1242	856	11467		15125	26193
Ratio Det /MPs	3418	2554		77365	51595	33522	128	685	2894	5983		19794	28080
CML depth	20-30m	25-35m	50-60m	30-40m	15-25m	45-55m	40-50m	65-75m	35-45m	35-45m			
Temperature (°C)	14.3	13.8	13.7	13.7	14.2	14.6	15.2	15.1	16.7	17.0			
Salinity (‰)	38.1	38.2	38.2	38.2	38.4	37.8	37.4	37.4	37.5	37.8			
Density (x-1000 g l^{-1})	28.5	28.7	28.8	28.7	28.8	28.2	27.8	27.8	27.5	27.6			
Chlorophyll-*a* (mg Chla m^{-3})	0.55	0.65	0.36	0.76	1.37	0.26	0.16	0.35	0.10	0.41			
Hydrobios – Fraction > 200 µm													
Zooplankton (ind m^{-3})	11312	567	2017	8643	2956	2106	727	346	12403	37474		7855	11363
Detritus (# m^{-3})	40601	30532	68837	248097	41005	22355	16043	2508	342449	118535		93096	113514
Microplastics (MPs) (# m^{-3})	68.6	117.9	31.9	43.4	12.1	16.8	10.1	11.7	50.7	34.5		39.8	33.5
Ratio Zoo/MPs	165	5	63	199	244	125	72	30	245	1086		223	315
Ratio Det /MPs	592	259	2158	5717	3389	1331	1588	214	6754	3436		2544	2268



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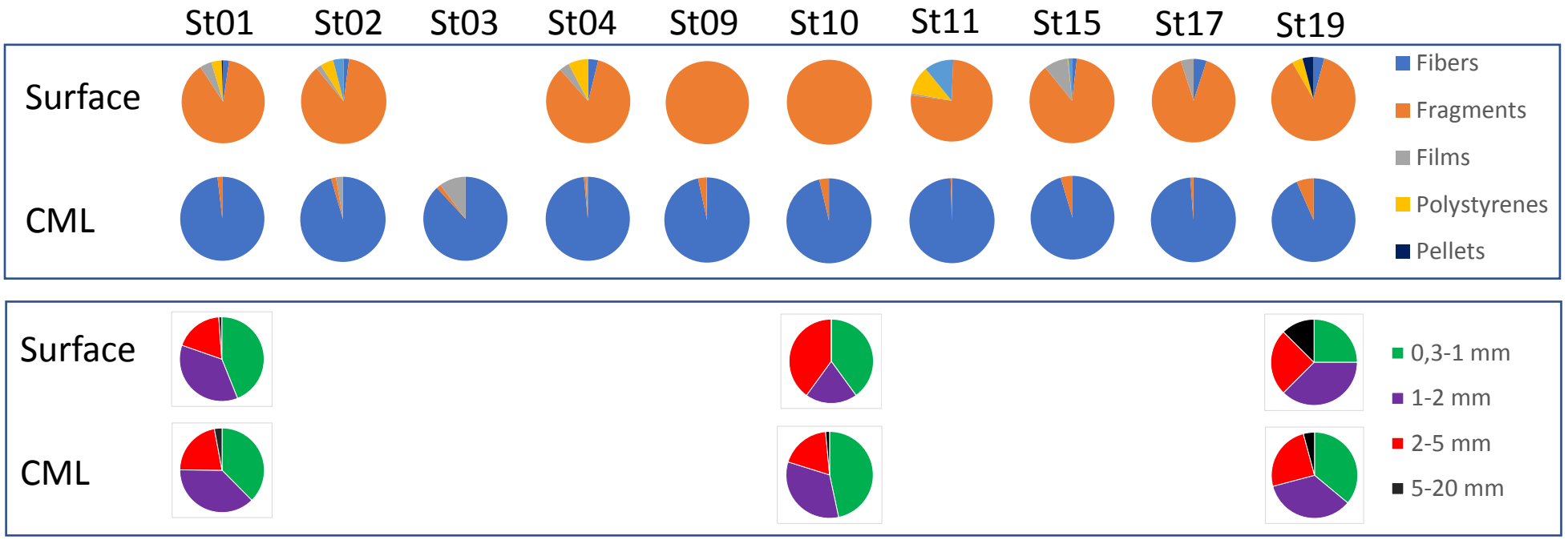

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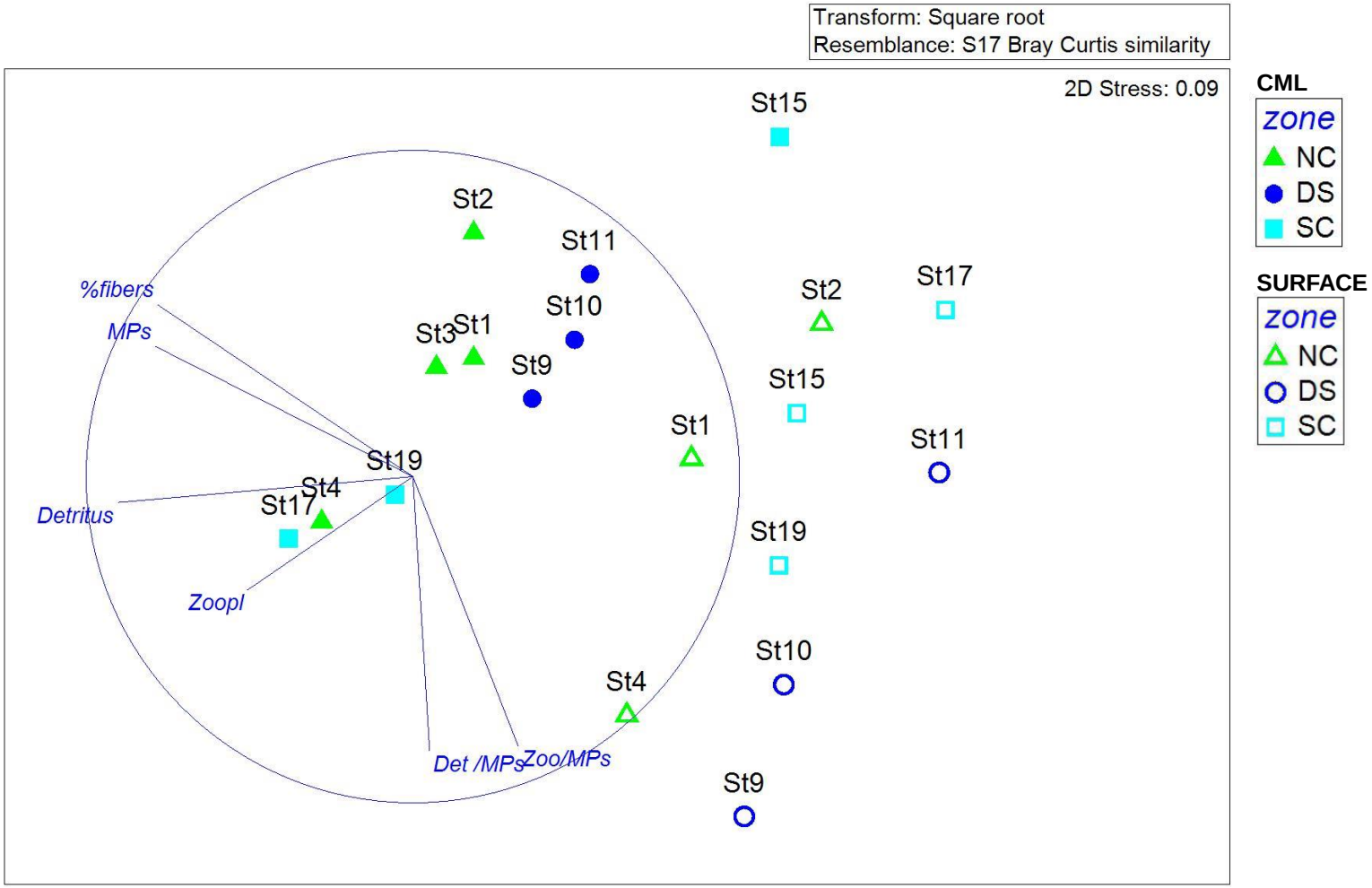

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Supplementary Material 1: **Comparison of sampling efficiency of the two different sampling gears (Manta net with 300 µm mesh for surface layer sampling and 60µm mesh Multinet for horizontal CML sampling) for MP categories and size classes.**

This additional material reproduces the detailed response to one of the reviewers on the comparability of the used two sampling gears and mesh sizes of the nets.

The Hippocampe cruise (and the scientific program of the same name) focused on organic and metallic contaminants in plankton collected at maximum chlorophyll.
In addition, Manta nets (300 µm mesh size) were programmed to quantify surface microplastics according to MSFD standards (Hanke et al., 2013). Initially, during our analysis of zooplankton communities from Hydrobios samples (60 µm) collected at maximum chlorophyll of the various stations (Fierro et al., 2023), a few microplastics items were observed in the aliquots studied for zooplankton treatment and identification with both Zooscan and binocular loupe. The idea then occurred to us to attempt to exhaustively quantify the microplastics after fractioning on 300 µm sieve of these samples obtained with the Hydrobios (60 µm mesh) at maximum Chlorophyll, in order to compare them with the microplastics data obtained using Manta net surface tows with a mesh size of 300 µm.

Obviously, the comparison is based on samples obtained using two different sampling protocols.

We will assess the impact of these differences below, but it is important to note the following similarities between the two approaches: 1) the volumes sampled by the two nets - Manta and Hydrobios - were fairly comparable (several tens of m3); 2) observations of microplastics in collected samples at the chlorophyll maximum layer (CML) with the Hydrobios (60 µm mesh size) was carried out on fractions sieved on 300 µm (to remain within the size range of the samples observed with the Manta), 3) the binocular loupe observation methods were similar.
The biggest problem in the comparison, raised by the reviewer, concerns the types of net and mesh used.

Hydrobios vs Manta net

The two nets have different characteristics, but can be used to sample very similar volumes of water. The Manta net (net opening 60 cm x 20 cm, mesh size 300 µm, IFREMER / MSFD protocol following Hanke et al. 2013) surfs over the water with its wings and the rectangular opening of the net mouth is designed to filter the first 10 cm of the surface layer of water. For each 10-minute stroke at 2 knots, the net filters approximately 100 m^3. The Hydrobios type midi has an opening of 0.25 m2 and can be towed to collect tens of m3, with real-time on-board control of the filtration level by the difference measured by 2 volumeters, one outside and one inside the net, which prevents clogging.

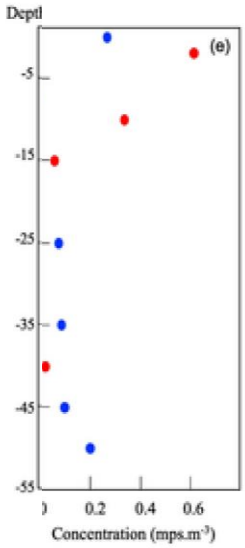
After the Hippocampe campaign (13 April - 14 May 2019), we organized a study in Marseille Bay to investigate the distribution of microplastics in the water column, using the same Multinet Hydrobios net and Manta net. This study, which was planned to take place over the different seasons of the same year (2020), was unfortunately halted by sudden restrictions on activities linked to the COVID pandemic. However, for two dates, we were able to obtain vertical distributions of microplastics under different wind conditions. These data supported simulations to investigate dynamic changes in the vertical distributions of microplastics and have been published (Chevalier et al., 2023 ; ; Chevalier, C., Vandenberghe, M., Pagano, M., Pellet, I., Pinazo, C., Tesán Onrubia, J.A., Guilloux, L., Carlotti, F., 2023. Investigation of dynamic change in microplastics vertical distribution patterns: The seasonal effect on

vertical distribution. Marine Pollution Bulletin, 189, 114674. https://doi.org/10.1016/j.marpolbul.2023.114674).

The sampling conditions, the microplastics concentrations observed and the physical and chemical variables in the water column are presented in Table 1, Figure 3 and Table 2 respectively of the cited article (Chevalier et al., 2023).

In the table below, we have reproduced the microplastics concentration values by adding the % of fibers to the total PMs.

Date (dd.mm.yyyy)	Depth (m)	MPs concentration (mps/m3)	% Fibers
03.02.2020	0 (surface)	0.27	84,6 %
(blue dots on fig.)	25	0.08	33,3 %
	35	0.09	85,7 %
	45	0.1	37,5 %
	50	0.2	37,5 %
Water column MPs abundance (mps/m2): 7.24			
10.02.2020	1	0.62	12,9 %
(red dots on fig.)	10	0.34	52,9 %
	15	0.06	100 %
	40	0.02	100 %
Water column MPs abundance (mps/m2): 7,90			



On 03.02.2020, with strong wind conditions (10 m/s) inducing mixing, due to turbulent vertical diffusivity (see Chevalier et al., 2023)., the highest microplastics concentration was at the surface sampled with the 300 µm mesh Manta net, and 84.6% of the MPs collected were fibers. Thus, the Manta net can collect fibers quite effectively (it sampled the highest percentage of fibers observed among the 9 analyzed samples). In the water column, the highest percentage of fibers was obtained at 35 m in an area where turbulent vertical diffusivity was low. On 10.02.2020, wind conditions were calm, and turbulent vertical diffusivity was low and homogeneous vertically. The MPs profile decreased drastically with depth, but the fiber contribution increased to reach 100% at 15m and 40m.

These data were used to calibrate and validate the model of the vertical distribution of microplastics as a function of wind conditions and made it possible to deliver consistent simulations that explain the observed distributions (see Chevalier et al., 2023).

Conclusion on the comparison of the two gears: On this basis, we do not consider that the use of the Hydrobios midi net versus Manta net induced major differences in the sampling process. In any case, at present, no other better combination of nets allows the surface and deep layers to be sampled horizontally with such precision, and with comparable collected samples in terms of filtered volumes. (Note that on 10.02.2020, the Hydrobios net was tested close to the surface (1m), but could not sample just the water surface interface.)

300 µm mesh nets vs. 60 µm mesh nets.

We used 300 µm mesh for the Manta net and 60 µm mesh for the Hydrobios during the Hippocampe cruise. However, our observation of the microplastics in the Hydrobios samples only concerned the fractions sieved above 300 µm, in order to be able to compare them with the surface microplastics content in the Manta.

The effect of the mesh may have a different impact on the types of MPs depending on their shape, from round (such as pellets) to tapered (such as fiber-type).

Non-tapered microplastics (pellets, fragments, films, etc.) were retained either by the 300 µm mesh of the Manta, or by the 300 µm sieve of the samples collected from the 60 µm Hydrobios. It may be considered that that the collection efficiency in the two cases is very similar. Questions may arise for fibre-type microplastics: 1) Can fibers be correctly collected using a Manta net with a mesh size of 300 µm mesh? 2) Does collection by the Hydrobios with a net of 60 µm mesh allow more microplastics fibers to be collected, even those obtained after sieving with a 300 µm mesh?
The size of microplastic fibres (MFs) is highly variable due to their myriad sources, material types and degradation history. However, their diameter ranges from 10 to several tens of µm (average around 25-30 µm), and their length is usually 1 to 2 orders of magnitude greater than the diameter (Cole, 2016) (although the theoretical minimum length should be at least 3 times the value of the diameter). It is therefore conceivable that isolated fibers could almost all pass through a 300 µm mesh and most through a 60 µm mesh. In reality, because of the aggregation of microfibers with particles (phytoplankton aggregates, TEP, others) (Galgani et al. 2022), the retention of microfibers by a mesh depends more on the size of the particle in which the microplastics fiber is embedded.

In the observations made in the bay of Marseille in February 2020 and described above, fibers represented 84.6% of the microplastics collected at the surface with the Manta net on 3 February, and the surface had the highest concentration of microplastics among the layers sampled.

Conclusion on the effectiveness of the net meshes used: The 300 µm mesh Manta net is able to collect microplastics fibers with diameters much smaller than 300 µm, because they are most often found with marine snow, or aggregated with the plankton collected by the net. The 300 µm sieve used to fractionate the samples obtained with the Hydrobios 60 µm mesh net retains microplastics fibers in the same way.

Conclusion on this question: We believe that the methodology used enables us to propose a valid comparison of surface data and the CML microplastics content collected above a 300 µm mesh. However, a new dedicated experiment should focus on the use of nets with identical mesh sizes. It may be added that a true comparison would probably require more than just a comparison between nets and meshes, but also for different wind and vertical hydrodynamic conditions, and for different particle and microplastics distributions, and for different particle sizes.

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Supplementary Material 2: **Examples of photographs of fibers, fragments and films found in our samples at the CML during the Hippocampe cruise**

Plate with examples of photos taken with a binocular magnifying glass to identify and measure microplastics in plankton samples collected at the CML.