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Microplastics and their associated organic pollutants from the coastal waters of the central Adriatic Sea (Italy): investigation of adipogenic effects *in vitro*

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Abstract

Even though microplastic (MP) pollution in aquatic environment is nowadays widely studied, a huge gap of knowledge exists on their actual biological effects. In this study we first reported environmental baseline data on the occurrence and characterization of floating MPs in Italian coastal waters of the Central Adriatic Sea by using a standardized monitoring protocol. Further, we analyzed the concentrations of MP-associated chemicals and evaluated their potential adipogenic effects using 3T3-L1 preadipocytes. MPs were found in each sampling stations showing the highest abundance (1.88±1.78 items/m^3) in the sites more distant from the coast with fragments as the most common shape category. All targeted organic pollutants (*i.e.* polychlorinated biphenyls - PCBs, polycyclic aromatic hydrocarbons -PAHs, organophosphorus - OP, and organochlorine - OC pesticides) have been detected on the surface of the collected MPs. The highest concentrations of PAHs were found on MPs from inshore (*i.e.* <1.5 NM) surface waters with low-ring PAHs as dominant components. Similarly, MPs from inshore waters had higher ΣPCB concentrations (64.72 ng/g plastic) than those found in offshore (*i.e.* >6 NM) waters (10.37 ng/g plastic). Among pesticides, all measured OPs were detected in each sample analyzed with pirimiphos-methyl as the most representative compound. For OCs, the sum of all concentrations of congeners was higher in coastal with respect to offshore waters. Moreover, *in vitro* 3T3-L1 screening of MP extracts indicated potential metabolic effects resulting in both adipogenesis and lipid uptake/ storage.

Keywords: Adriatic Sea; Microplastic; Biomonitoring; Endocrine disruptors; Metabolic disruptors**;** Adipocytes

1. Introduction

Microplastics (MPs, ≥0.001mm and<5mm) (Shim et al., 2018) are a well known global issue for marine and coastal ecosystems (Browne et al., 2011; UNEP, 2016) due to their ubiquitous presence throughout the marine environment compartments, as surface and water column, sediments, and biota. In this regard, the Mediterranean Sea with a mean density of floating microplastics of about 100,000 items/km^2 represents one of the basin most affected by MP litter (Fossi et al., 2012). Within the Mediterranean Sea, however, very few studies have investigated the occurrence of floating plastic debris in the Adriatic Sea that, due to its features (*e.g.* semi-closed conformation, oceanographic conditions and heavy anthropogenic pressure), can be considered a plastic pollution hot spot (Gomiero et al., 2018). In this regard, recent data on the Mediterranean Sea (partially including the Adriatic area) reported a MP range of 0.4 - 1.8 items/m^3 with polyethylene (PE) as the most common polymer composition types (Suaria et al., 2016). Similarly, a clear prevalence of MPs (65.1% of sampled debris) was demonstrated in marine sediments from the Central Adriatic (Munari et al., 2017). MPs were also found in a wide range of marine species collected along the Adriatic Sea showing highest concentrations (*i.e.* 1.0–1.7 items/specimens) in fish (Avio et al., 2015; Pellini et al., 2018). These findings indicate that small size debris are available to marine vertebrates and invertebrates, resulting in both physical and chemical impacts (Fossi et al., 2012; Fossi et al., 2014; Gall and Thompson, 2015). MPs can be translocated to the lymphatic and/or circulatory system, and accumulated in organs as the digestive and the respiratory tracts (Barnes et al., 2009; Cole and Galloway, 2015; Mazurais et al., 2015; Besley et al., 2017; Lusher et al., 2017a; Lusher et al., 2017b).

Nowadays the research on the ecological impact of microplastics is not limited to evaluate their accumulation in the marine environment and consequently in living organisms, but is now moving on to investigate the role of MPs as potential vehicles of a wide range of toxic chemicals (Teuten et al., 2007; Engler, 2012). Recent data indicate that hydrophobic organic contaminants (HOCs), including endocrine disrupting chemicals (EDCs), have been detected on the surface of MPs (Mato

et al., 2001; Ogata et al., 2009; Rios et al., 2010; Hirai et al., 2011; Rochman et al., 2014a). Indeed, due to the physical and chemical properties of plastic polymers, MPs can quickly accumulate and concentrate HOCs present in the surrounding water. It should also be taken into account that MP-associated HOCs have to be added to the chemicals already included as additives during plastic manufacturing (*e.g.* bisphenol A or nonylphenol) (Ogata et al., 2009; Hirai et al., 2011; Engler, 2012; Mai et al., 2018; Alimba and Faggio, 2019; Chen et al., 2019), making the potential of chemicals release from MPs to the marine environment and wildlife particularly harmful. In addition, ingestion of MPs by aquatic animals may result in increased bioavailability of HOCs, bioaccumulation and both potential toxicity and transfer along the trophic web (Avio et al., 2015; Batel et al., 2016).

It has been demonstrated that among HOCs, EDCs are commonly found on MPs, including abrasion beads, and easily released from plastic debris due to lower partition coefficients between plastic and water (Liu et al., 2016). EDCs accumulation in tissues of marine organisms, following their MP-mediated release, can thus exert adverse effects ranging from disruption of endocrine-based reproductive pathways to metabolic alterations (Franzellitti et al., 2019). In this last regard, a group of endocrine disruptors, named obesogens or metabolic disruptors, have been found to affect adipogenesis by perturbing peroxisome proliferator activated receptor (PPAR) signaling pathways (Grün and Blumberg, 2009). Recently, the obesogenic potential of these pollutants were assayed *in vitro* using 3T3-L1 adipocytes and primary cultures of sea bream hepatocytes (Cocci et al., 2017; Pomatto et al., 2018).

Thus, our first objective in this study was to collect environmental baseline data on the occurrence and characterization of floating MPs in Italian coastal waters of the Central Adriatic Sea by using a standardized monitoring protocol. Second, we aimed to examine the concentrations of chemicals adsorbed on MPs sampled from the same study area and to test their adverse effect by *in vitro* bioassays. In particular, we used 3T3-L1 preadipocytes to investigate the possible adipogenic

effects of these plastic extracts in order to provide preliminary insights on potential ecotoxicological risk of MPs as vehicles of chemical pollutants.

2. Materials and Methods

2.1 Sites and water surface sampling

Samples were collected inshore and offshore along the western (Italian) coasts of the Central Adriatic Sea (Province of Ascoli Piceno, Marche Region - Italy). The sampling operations were conducted during summer season 2018, following the microplastics monitoring methods applied by the Italian Ministry of the Environment and Land and Sea protection (Minambiente, 2018) and the requirements indicated by the ministerial protocol within the MSFD monitoring program (MSFD, 2013). Briefly, transects were outlined perpendicular to the coast line along the municipalities of San Benedetto del Tronto (SBT) and Grottammare (GRT), respectively (Figure 1). Three sampling stations were located at different distance from the coast (0.5, 1.5 and 6 nautical Miles, NM) along each transect. An additional sampling station was located inside the San Benedetto del Tronto harbor. Floating samples were collected from surface sea water, using a manta trawl with mesh size of 300 µm (30 x 15 x 200 cm) equipped with a mechanical flow meter (Hydro-Bios). The manta net was towed on the water surface for 20 minutes at 2 knots and was kept at a distance far from the boat to avoid turbulence by the waking of the ship. Samples were rinsed from the outside to the end of the net, placed in glass containers and immediately stored at 4 °C until the sorting using a stereomicroscope. To prevent external contamination during the analysis, the laboratory procedures were performed according to Baini et al. (2018).

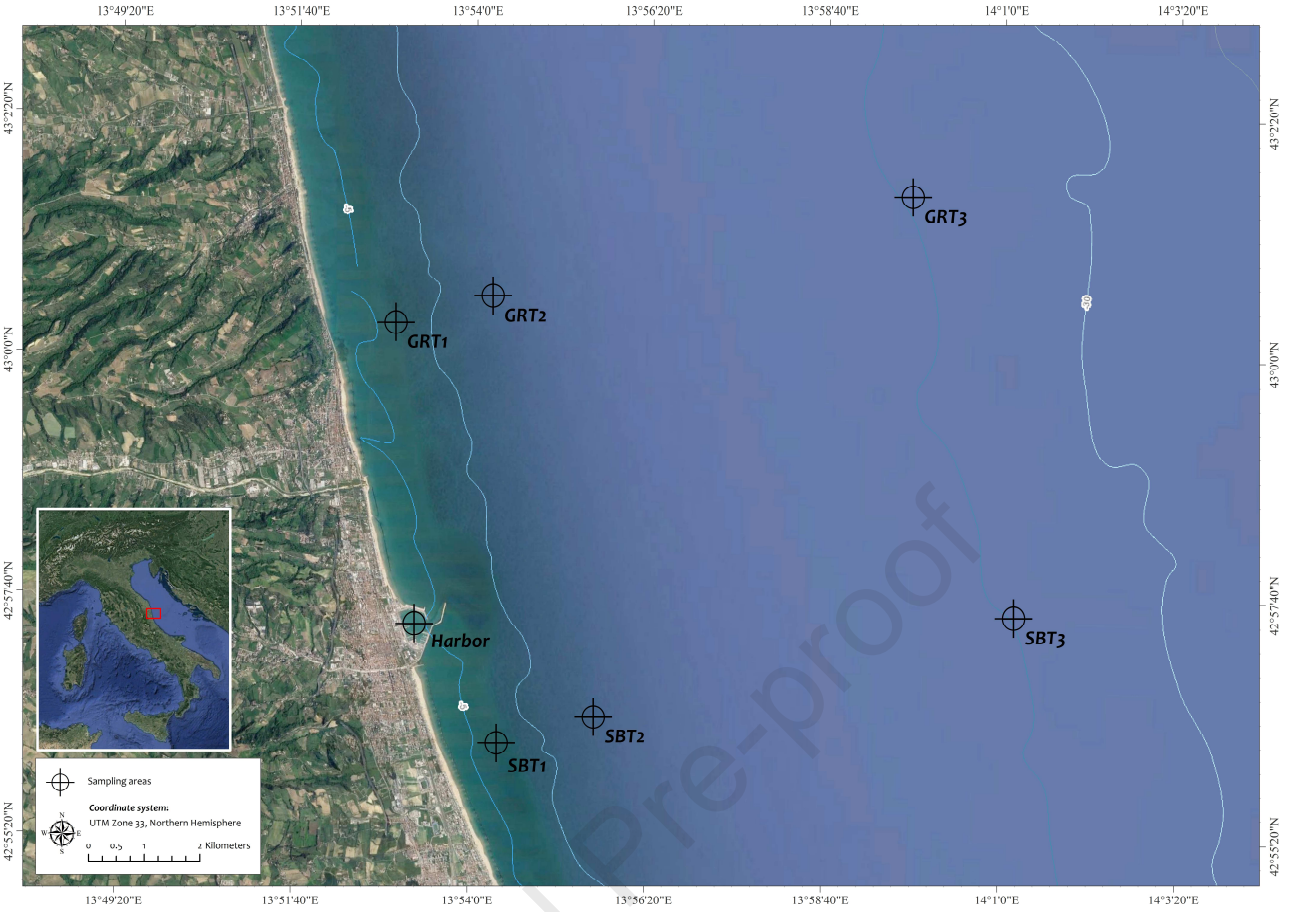

Figure 1. Map of the study area. Sampling stations were located at different distance from the coast (0.5, 1.5 and 6 nautical Miles, NM) along each transect.

2.2 Microplastic identification and count

Samples where dried at room temperature and weighted in OHAUS Explorer analytical balance. For microplastics identification, samples were observed under a stereomicroscope (Carl Zeiss Stemi^{TM}) and images have been examined by a USB Camera (Optika B Series) using the Optika ProView software. Plastic particles were sorted based on their colour (blue, red, black, white, transparent, green, other colour), size (<5, 5–25, >25 mm) and shape (spherical, filament, fragment, sheet, other shape) according to the MSFD guidelines and Imhof et al. (2017). As reported by Baini et al. (2018), the number of microplastic was normalized to the total water volume filtered (V) and expressed as items/m^3.

2.3 Determination of PAHs, PCBs, Organophosphorus and organochlorine pesticides

Due to limited amount of particles to be provided for both bioassay and chemical analysis, MPs collected within the study area were pooled according to distance from the coast in order to obtain 3 type of samples: offshore waters (*i.e.* 6 NM), inshore waters (*i.e.* <1.5 NM, corresponding to local areas of touristic and commercial traffics) and the harbor of San Benedetto del Tronto as expected polluted area.

Hydrophobic organic contaminants were isolated from marine debris following the protocol described by Chen et al. (2019) with slight modifications. Briefly, 4 mL of dichloromethane was added to each microplastic sample (400 mg) and incubated for 30 min at room temperature, vortexing every 10 minutes. The procedure was repeated for 3 times and finally the supernatant was evaporated to 500 µl using a Rotavapor R-300 (Buchi). The PCB mix standard (PCB 28, PCB 52, PCB 95, PCB 99, PCB 101, PCB 105, PCB 110, PCB 118, PCB 138, PCB 146, PCB 149, PCB 151, PCB 153, PCB 170, PCB 177, PCB 180, PCB 183, PCB 187) at a concentration of 10 mg/L in iso-octane, the organochlorine pesticides OC1 (six congeners, *i.e.* α-HCH, β-HCH, γ-HCH, HCB, heptachlor and heptachlor epoxide) mix standard at a concentration of 10 mg/L in cyclohexane and the organochlorine pesticides OC2 (six congeners, *i.e.* 2.4-DDT, 4.4-DDT, 2.4-DDE, 4.4-DDE, 2.4-DDD, 4.4-DDD) mix standard at a concentration of 10 mg/L in cyclohexane were supplied by Dr. Ehrenstorfer (Ausburg. Germany). Standard working solutions at various concentrations were prepared daily by appropriate dilution of the stock solutions with hexane. The organophosphorus pesticides (four congeners, *i.e.* chlorfenvinphos. chlorpyrifos. malathion. pirimiphos-methyl) mix standard were supplied by Fluka (Milano. Italy). Individual stock solutions of organophosphorus pesticides at concentrations of 1000 mg/L were prepared by dissolving pure standard compounds in HPLC grade methanol and then stored in glass-stoppered bottles at 4 °C. Afterwards standard working solutions at various concentrations were prepared daily by appropriate dilution of the stock solution with methanol. A gas chromatograph/mass selective detector (GC/MSD) (Hewlett Packard. Palo Alto. CA. USA; HP-6890 with HP 5973) was used. Separation was performed on an HP 5 MSI

column (30 m X 0.25 mm X 0.25 µm film thickness). An HP Chem workstation was used with the GC/MS system. All injections were splitless and the volume was 1 µl. The flow rate (He) was 0.8 mL/min. The injector temperature was 270 °C. The column temperature program used for PCB and for the second group of organochlorine pesticides, *i.e.* dichlorodiphenyltrichloroethane (DDT) and its metabolites (OC 2 subgroup) analyses was from 60 °C (3 min) to 190 °C at 8 °C min^{-1}, from 190 °C (18 min) to 300 °C at 15 °C min^{-1}, then at 300 °C for 1 min. The column temperature programme used for the first group of organochlorine pesticides, *i.e.* α-HCH, β-HCH, γ-HCH, HCB, heptachlor and heptachlor epoxide (all included in the OC 1 subgroup) was from 60 °C (3 min) to 190 °C at 8 °C min^{-1}, from 190 °C (13 min) to 300 °C at 15 °C min^{-1}, then at 300 °C for 1 min. The column temperature programme used for organophosphorus pesticides analyses (chlorfenvinphos, chlorpyrifos, malathion, pirimiphos-methyl) was from 50 °C (5 min) to 320 °C at 15 °C min^{-1}, then at 320 °C for 4 min. Data were acquired in the electron impact (EI) mode (70 eV) using the selected ion monitoring (SIM) mode. For this purpose, a gas chromatograph/mass selective detector (GC/MSD) (Hewlett Packard. Palo Alto. CA. USA; HP-6890 with HP 5973) was used. Extracts were analyzed for concentrations of 15 of the most environmentally relevant PAHs (Naphthalene, Acenaphthene, Fluorene, Chrysene, Phenanthrene, Fluoranthene, Anthracene, Pyrene, Benzo[a]anthracene, Benzo[b]fluoranthene, Benzo[k]fluoranthene, Benzo[a]pyrene, Dibenz[a,h]anthracene, Benzo[g,h,i]perylene and Indeno[1,2,3-c,d]pyrene). The detailed methodology for separation and quantification of target analytes was published previously (see "Supplementary data" of Cocci et al., 2017).

2.4 3T3-L1 cell culture and quantification of adipocyte lipid accumulation

3T3-L1 preadipocytes (ATCC® CL-173™; Lot No 70009858, ATCC, Manassas, VA, USA) were cultured in high-glucose (4.5 g/L) Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% bovine calf serum, 2 mM L-glutamine, 50 IU/mL penicillin, and 50 µg/mL streptomycin (Sigma Aldrich, St. Louis, MO, USA).

For experiments, 5×10^3 cells/well were seeded in 96-black well clear bottom plates (Greiner Bio-One, Frickenhausen, Germany). Two days after reaching confluence (day 0), cells were incubated with differentiation medium (MDI; DMEM containing 10% fetal bovine serum, 1 µg/mL insulin, 0.5 mM isobutylmethylxanthine), containing different concentrations of the various microplastic organic extracts. In particular, inshore, offshore and harbor microplastic extracts were dried and redissolved in 30 µl DMSO; then scalar dilutions, ranging from 10^{-3} to 10^{-6}, were added to the MDI used for adipocyte differentiation. Two days later (day 2), MDI medium was replaced with maintenance medium (MM; DMEM 10% FBS, 1 µg/mL insulin), always containing the various dilutions of microplastic extracts. Negative controls (solvent only) were set up using MDI and MM added with 0.1% DMSO, corresponding to the maximum solvent concentration present in microplastic extract dilutions; as a positive control, 100 nM Rosiglitazone (Sigma Aldrich) was added to MDI and MM. Fresh medium was provided every two days; experiments ended after 9 days from the beginning of the differentiation (day 9). Lipid accumulation was quantified by using AdipoRed™ assay reagent (Lonza, Walkersville, MD, USA), while the DNA content (that correlates with cell number) was estimated by NucBlue™ staining (Invitrogen, Carlsbad, CA, USA). Briefly, medium was removed from 3T3-L1 cultures and cells were rinsed with PBS, subsequently replaced with a dye mixture containing AdipoRed™ and NucBlue™ assay reagents diluted in PBS (25 µl and 1 drop, respectively, per mL of PBS). After 40 min of incubation at room temperature in the dark, fluorescence was measured with Filtermax F5 microplate reader (Molecular Devices, Sunnyvale, CA, USA) with excitation at 485 nm and emission at 535 nm for AdipoRed™ and excitation at 360 nm and emission at 460 nm for NucBlue™ quantification. Experiments were repeated three times (four wells for each condition), using cells at different passage numbers (p3-p5). Data were referred as % of the control (0.1% DMSO) condition (set = 100%).

2.5 Statistical analysis

Data are expressed as mean ± standard error of the mean (SEM). Statistical analysis was performed using ANOVA (one-way analysis of variance) followed by Bonferroni's multiple comparison test. Differences with $p<0.05$ were considered statistically significant.

3. Results

3.1 Microplastics in water surface samples

Particles isolated from surface seawater were visually counted and sorted based on their dimension, shape and color (Table 1). Among the total particles sampled, microplastics (<5mm) constituted the most representative size class (n=401.08, 95%), and were detected in each transect representing the totality of plastic litter (100%) in GRT1, GRT2 and SBT2 stations and showing the highest abundance in the sites more distant from the coast (SBT3: 1.88±1.78 and GTR3: 3.42±2.28 items/m^3) or inside the San Benedetto del Tronto harbor (0.91±0.58 items/m^3). Marine debris larger than 5 mm were recorded in harbor (16%), open waters (SBT3, 6% and GRT3, 12%), and also at 0.5 NM along the SBT transect (2%).

Table 1. Particles abundance (items/m^3) and size (mm) in water surface samples collected in three different sampling sites; mean values ± s.d. for each transect have been reported.

Sampling site	Distance (nM)	Items/m^3	Micro- (< 5 mm)	Meso- (5 to 25 mm)	Macro- (> 25 mm)
GRT1	0.5	0.17 ± 0.02	12.33 ± 2.51 (100%)	-	-
GRT2	1.5	0.11 ± 0.05	9.34 ± 4.01 (100%)	-	-
GRT3	6	3.42 ± 2.28	240.33 ± 160.55 (88%)	38.33 ± 33.29 (10%)	1.67 ± 1.53 (2%)

SBT1	0.5	0.17 ± 0.11	15.75 ± 10.59 (98%)	0.50 ± 1.00 (2%)	-	235
SBT2	1.5	0.04 ± 0.01	3.00 ± 0.00 (100%)	-	-	236
SBT3	6	1.88 ± 1.78	66.00 ± 8.54 (94%)	3.33 ± 0.58 (5%)	1.0 ± 1.00 (1%)	237
Harbor		0.91 ± 0.57	54.33 ± 24.11 (84%)	11.67 ± 15.30 (11%)	4.33 ± 4.51 (5%)	238 239

240

241 Analyzing the overall study area by combining the values measured at two sampling stations at the
242 same distance (along both transects), 75% of the total MPs were collected from sampling sites at 6
243 NM, 3% at 1.5 NM, 8% at 0.5 NM from the coastline and finally the 13% inside the harbor.
244 Fragments were the most common shape category observed in each sampling station (Figure 2a),
245 reaching the 74.7% (n= 28.4) of all the MP types sampled in coastal sites (<1.5 NM). Films were
246 the second most abundant group of the plastic items collected with the highest number (n= 23.3,
247 42.9%) recorded in SBT harbor. On the other hand, spherical particles were commonly found in
248 o□shore waters (6 NM) showing a total number of 54 (25%, Figure 2a).
249 Analysis of MP color pattern revealed that transparent and white items accounted for 37% and 44%
250 of the particles collected respectively (Figure 2b).

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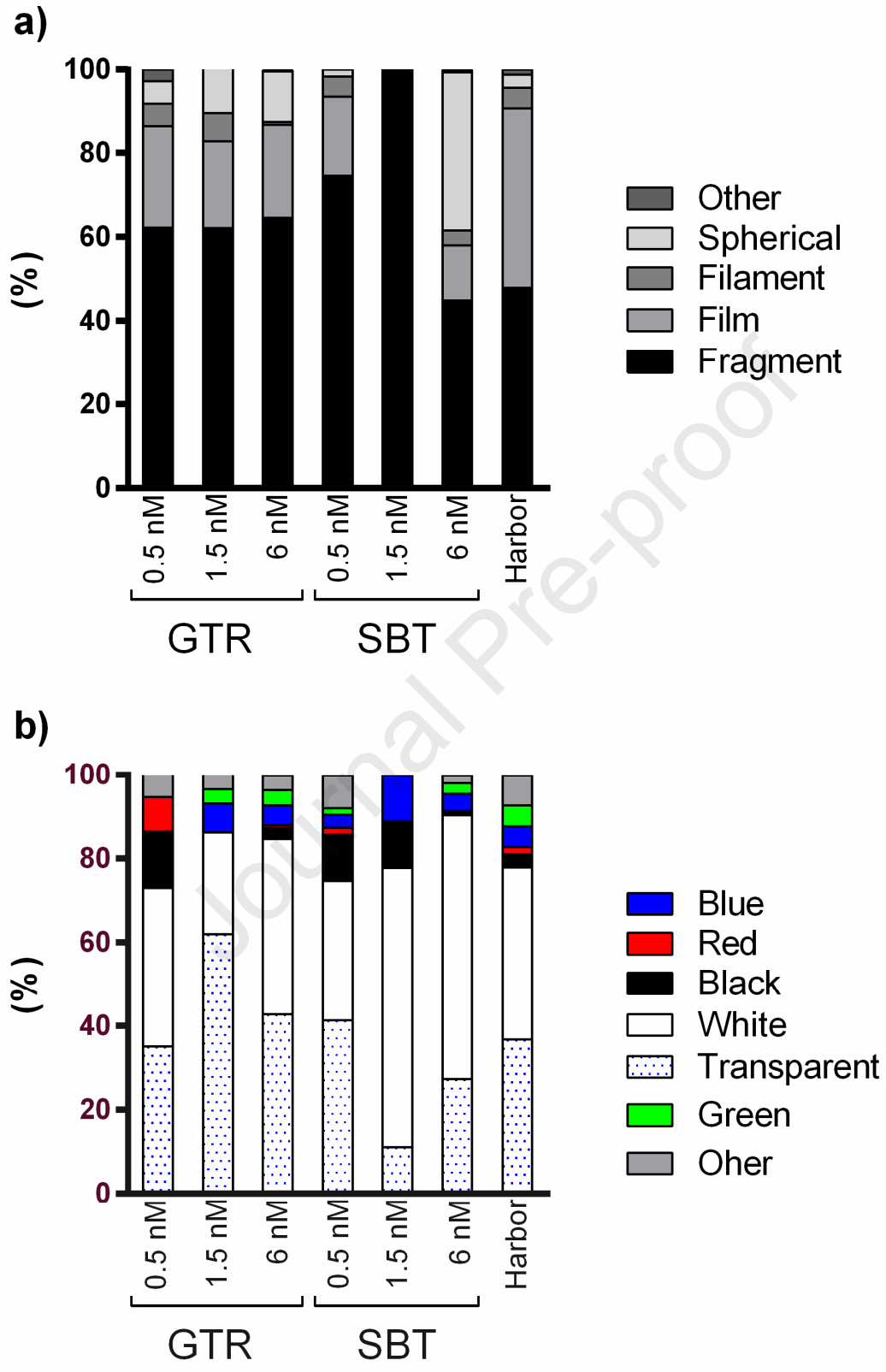

Figure 2. Sampling station-related abundance % of microplastics isolated from water surface samples in relation to shape (a) and color (b).

255 *3.2 Concentrations of pollutants on inshore and offshore microplastic samples*

256

257 MP extracts were analyzed for PCBs, PAHs, OPs, and OCs (Table 2).

258

259 *Table 2. Concentration (ng/g plastic) of Polychlorinated biphenyls (PCBs), organophosphorus*
260 *(OPs), organochlorine pesticides (OCs), and polycyclic aromatic hydrocarbon (PAH) congeners*
261 *measured on microplastics from inshore (i.e. <1.5 NM), offshore waters (i.e. 6 NM), and harbor.*
262

POPs	**Inshore (ng/g plastic)**	**Offshore (ng/g plastic)**	**Harbor (ng/g plastic)**
PCB 28	n.d.	n.d.	n.d.
PCB 52	27.91	0.70	12.22
PCB 95	3.58	0.17	5.85
PCB 99	0.60	n.d.	n.d.
PCB 101	n.d.	0.08	0.52
PCB 105	n.d.	n.d.	n.d.
PCB 110	n.d.	0.08	0.34
PCB 118	n.d.	n.d.	n.d.
PCB 138	28.51	0.54	16.01
PCB 146	n.d.	n.d.	n.d.
PCB 149	5.07	0.29	2.84
PCB 151	n.d.	n.d.	n.d.
PCB 153	n.d.	n.d.	n.d.
PCB 170	n.d.	n.d.	n.d.
PCB 177	n.d.	n.d.	n.d.
PCB 180	n.d.	n.d.	n.d.
PCB 183	n.d.	n.d.	n.d.
PCB 187	n.d.	n.d.	n.d.
∑PCBs	65.67	1.86	37.78
Chlorfenvinphos	6.57	0.14	1.98
Chlorpyrifos	45.37	0.77	32.19
Malathion	17.31	0.22	7.23
Pirimiphos-methyl	78.36	3.59	32.70
∑OPs	147.61	4.72	74.10
α-HCH	95.22	0.84	27.02
β-HCH	0.37	0.02	0.86
γ-HCH	3.58	0.07	1.98
HCB	2.09	0.10	10.84
Heptachlor	n.d.	n.d.	n.d.
Heptachlor epoxide	n.d.	n.d.	n.d.
∑OC1s	101.26	1.03	40.70
2.4-DDE	10.15	0.16	2.41
4.4-DDE	8.66	0.19	n.d.

2.4-DDD	n.d.	1.32	8.00
4.4-DDD	15.52	n.d.	67.38
2.4-DDT	n.d.	0.59	n.d.
4.4-DDT	n.d.	2.79	n.d.
∑OC2s	34.33	5.05	77.79
Naphthalene	9.55	n.d.	6.71
Acenaphthene	n.d.	n.d.	n.d.
2-Bromonaphthalene	46.27	2.46	40.36
Acenaphthylene	109.40	1.27	100.00
Fluorene	n.d.	n.d.	n.d.
Phenanthrene	n.d.	n.d.	2.58
Anthracene	64.18	1.54	25.04
Fluoranthene	16.87	0.57	n.d.
Pyrene	n.d.	1.48	35.13
Benzo(a)Anthracene	67.31	2.64	40.36
Chrysene	81.64	2.28	47.93
Benzo(bFluoranthene	n.d.	n.d.	n.d.
Benzo(a)Pyrene	n.d.	n.d.	7.66
Indeno(1,2,3-cd)Pyrene	n.d.	n.d.	n.d.
DiBenz(a,h)Anthracene	n.d.	4.40	12.91
Benzo(g,h,i)Perylene	12.84	2.34	41.48
∑low-ring PAHs	229.40	5.27	174.69
∑high-ring PAHs	178.66	13.71	185.47
∑Total PAHs	408.06	18.98	360.16

n.d.: not detectable.

The highest concentrations of PAHs were found in microplastics collected from both inshore and harbor surface waters (Figure 3) with low-ring PAHs as dominant components (Table 2). On the contrary, high-ring PAHs were most abundant in offshore samples (Table 2).

Among the sixteen PCB congeners analysed, only seven, namely PCB 52, PCB 95, PCB 99, PCB 101, PCB 110, PCB 138 and PCB 149, were detected in the different samples (Table 2). The congeners 52, 95, 138 and 149 were found in MPs from each sampling area. If congeners were considered altogether, inshore waters had the highest ΣPCB concentrations (64.72 ng/g plastic) followed by SBT harbor (37.86 ng/g plastic). On the contrary, the lowest ΣPCBs was found in MPs from offshore waters (10.37 ng/g plastic) (Figure 3).

All measured OPs were detected in each sample analyzed with pirimiphos-methyl as the most representative compound. Generally, the highest ΣOP concentrations were found in samples from

inshore waters (Figure 3). For OC1 and OC2, the sum of all concentrations of congeners were higher in coastal waters with respect to offshore waters (Figure 3).

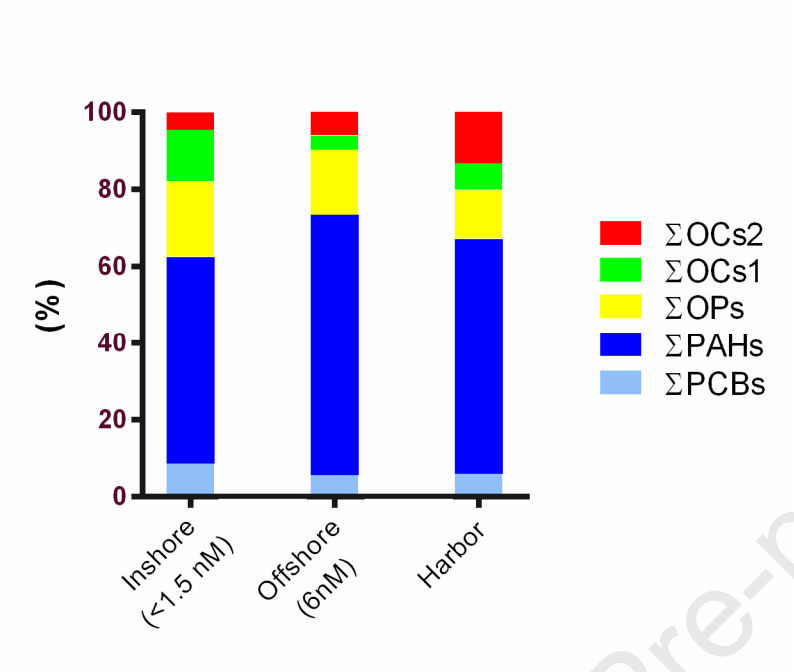

Figure 3. Microplastic-associated PAH, PCB, OP, OC1 and OC2 patterns across sampling sites.

3.3 Effects of microplastic extracts on 3T3-L1 adipocyte differentiation

The effect of MP extracts was assayed on the murine 3T3-L1 preadipocyte cell line, a commonly used cell model for adipose cell biology research. Since adipogenic effects can be exerted by increasing intracellular lipids (adipocytes hypertrophy) and/or adipocytes number (adipocytes hyperplasia), we assayed both triglyceride accumulation (AdipoRed™ assay) and total cell number (NucBlue™ staining, measuring DNA content). Confluent preadipocytes, cultured in 96-well plates, were induced to start adipogenic differentiation and were treated throughout the differentiation period (9 days) with solvent only (0.1% DMSO; negative control) or with different concentrations of microplastic extracts (serial dilutions ranging from 10^{-3} to 10^{-6}); Rosiglitazone, a well-known agonist of the adipogenesis master regulator PPARγ, was used as a positive control. All the tested concentrations of microplastic extracts did not exert cytotoxic effects, as demonstrated by

cell evaluation under the microscope and by NucBlue staining quantification, showing no difference in DNA content, and thus in cell number, in microplastic extract-exposed cells versus control adipocytes (Figure 4c). Interestingly, harbor, inshore and offshore microplastic extracts, at all the concentrations used, did induce an increase in triglyceride accumulation, in respect to control cells (Figure 4a, b). Differently from Rosiglitazone treatment, exposure to microplastic extracts induced no changes in total cell number (Figure 4c), thus indicating that microplastic extracts did not induce adipocytes hyperplasia. On the other hand, triglyceride accumulation per cell (i.e. triglyceride accumulation normalized for DNA content) resulted statistically increased after microplastic extracts exposure (Figure 4d), thus suggesting an induction of adipocytes hypertrophy.

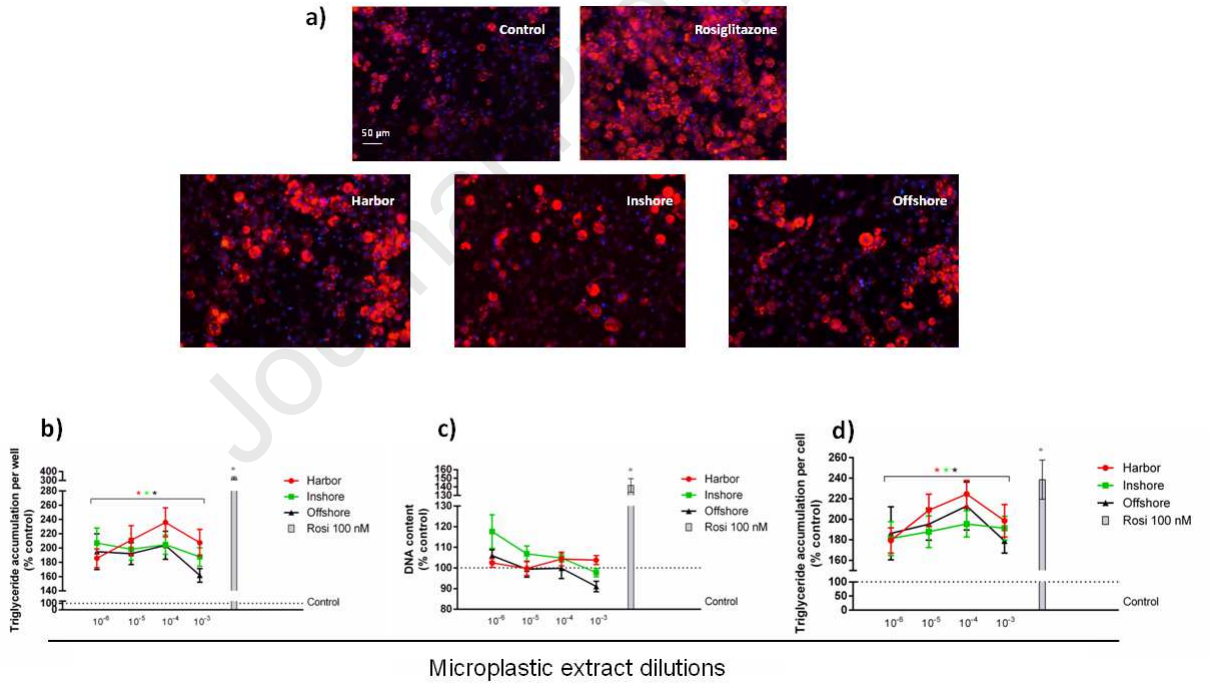

Figure 4. *(a) Representative micrographs of AdipoRed (red) and NucBlue (blue) staining of undifferentiated 3T3-L1 preadipocytes (Undiff.) and adipocytes after 9 days of differentiation in the presence of vehicle only (0.1% DMSO; Control), Rosiglitazone, or extracts obtained from microplastics collected at the different sampling stations (Harbor, Inshore, Offshore). Scale bar: 50 µm. (b) Graph summarizing AdipoRed staining experiments to assess lipid accumulation in differentiated 3T3-L1 adipocytes treated with 0.1% DMSO solvent (control) or cells treated with 100 nM Rosiglitazone (positive control) or serial dilutions (ranging from 10^{-3} to 10^{-6}) of the different microplastic extracts, showing triglyceride accumulation per well. (c) Graph summarizing NucBlue staining experiments to assess variations in the number of cells, showing DNA content per*

*well. (d) Graph showing triglyceride accumulation per cell (triglyceride accumulation normalized to DNA content), calculated as the ratio of AdipoRed and NucBlue staining. In b,c,d data are reported as % of control condition (solvent control values were set equal to 100%; horizontal dotted line) and represent the mean ± SEM of three independent experiments. *Indicates dilutions with significant increase over solvent control, p<0.05, within each sampling site.*

4. Discussion

Despite the growing monitoring of MP presence in the Mediterranean Sea, there is still a substantial lack of information on amount and distribution of MPs in the Adriatic Sea (de Lucia et al., 2014; Gomiero et al., 2018). In this respect, the present study was aimed to provide new insight on the occurrence of MPs in Italian coastal waters of the Central Adriatic Sea and on their capability to adsorb organic pollutants.

Our results revealed that the average amount of MPs (2.65 items/m^3) estimated in surface offshore waters was similar to that recorded within the Tremiti island Marine Protected Area (Central Adriatic Sea, Italy) in 2017 (2.2 items/m^3; Mezzelani et al., 2018). Interestingly, this last data was considerably increased with respect to the amount of 0.165 items/m^3 found by (De Lucia et al., 2018) in the same area during 2015. These findings suggest how the Adriatic Sea basin has been characterized by an exponential increase in plastic pollution over the last years. Recently, a range of 0.05 to 4.90 particles/m^3 was reported for quantification of floating plastics (<700 μm) in the southern Adriatic Sea by a large scale monitoring study (Suaria et al., 2018). The results of this study also showed that about 51% of the total recorded particles were smaller than 500 μm (Gomiero et al., 2018) thus supporting the key role of this size fraction within the plastic pollution affecting the Adriatic Sea (Mezzelani et al., 2018; Zeri et al., 2018). In addition, the high abundance of fragments, as previously observed in other studies (Isobe et al., 2014; Baini et al., 2018), suggests that fragmentation of large plastic objects could represent the main source of MPs in our study area.

In the present work, we observed that higher MP abundances were detected in offshore (6 nM) rather than in inshore (< 1.5 nM) sampling sites. These findings are consistent with the results of some previous studies which observed prevalence of plastic debris in areas 10-100 Km far from the coastline, indicating a progressive decrease in the number of plastic items from open sea to coastal waters (Pedrotti et al., 2016; Baini et al., 2018). In contrast, Zeri et al. (2018) have recently found a statistically significant increases in nearshore (≤ 4 km) microplastic concentration with respect to that of o□shore (> 4 km) Adriatic waters. These contrasting results could be attributed to the variability in observational conditions which are severely affected by the complexity of the study area circulation patterns, weather conditions and the heterogeneity in sources of marine litter.

A great body of studies is nowadays starting to focus on the potential toxicity of MPs and to investigate their role as vector of HOCs (Endo et al., 2005; Teuten et al., 2007; Bakir et al., 2014; Chen et al., 2019; Tang et al., 2020). In this study, the overall evaluation of pollutants carried on MPs provided a clear separation between in- and offshore waters, highlighting the highest abundance of these chemicals in the former. This finding is likely due to adsorption mechanisms from the surrounding environment, because these chemicals, especially PAHs and PCBs, show affinity with plastic particles (Teuten et al., 2009) and the tendency to accumulate in the organic phase of sediments (Leggett and Parker, 1994).

Among the great variety of organic pollutants which have been isolated on the surface of microplastics, PHAs (Teuten et al., 2007), PCBs (Endo et al., 2005) and pesticides (Bakir et al., 2014) represent the most common categories of contaminants. Indeed, PAHs are ubiquitous pollutants in waters, sediments and marine organisms from the Italian coasts of the Adriatic Sea (Cocci et al., 2017; Cocci et al., 2018; Frapiccini et al., 2018; Bajt et al., 2019). Due to their strong ability to bind to plastic particles (Liu et al., 2016), it's not surprising that PAHs are the most abundant contaminants found on the surface of MPs collected from our study area. The measured concentration of ∑PAHs is consistent with that found on plastic pellets (166.3 to 2,781 ng/g plastic) or fragments (85.9 to 2,841 ng/g plastic) beached along Crete shore (Karkanorachaki et al., 2018).

Additionally, the levels of PAHs were found to range from 35.1 to 17,023.6 ng/g in marine plastic debris from Canary Island beaches (Camacho et al., 2019) and from 3400 to 120,000 ng/g in microplastics from Chinese surface waters (Mai et al., 2018). Our findings are also comparable to the PAH concentrations (*i.e.* 136.3 to 1586.9 μg/kg plastic and of 397.6 to 2384.2 μg/kg plastic) recently found in microplastics from two beaches in China (Zhang et al., 2015). Low-ring PAHs were the most prominent components especially in inshore MPs thus suggesting that petroleum-derived contaminants are likely to be the main sources of MP contamination. Of the sixteen PAHs, Acenaphthylene (Acl) showed the highest abundance in each analyzed samples; interestingly, this finding parallels the highest Acl concentration found in surface coastal waters by our previous survey (Cocci et al., 2017).

PCBs were one of the most abundant group observed in our study showing higher concentrations in microplastics sampled from coastal areas than in those from offshore waters. These levels are substantially in line with the measures (*i.e.* 0.9 to 2285.8 ng/g plastic particles) recorded in plastic pellets and micro fragments beached along the coasts of Canary Islands (Camacho et al., 2019). PCB concentrations in coastal surface waters of the central Western Adriatic Sea were found to vary from 6,260 ng/L in areas interested by an intense maritime traffic to 10,650 ng/L in sea basin affected by riverine runoffs (Cocci et al., 2017). Interestingly, we observed similar patterns in PCB congener distribution between waters and plastics with highest levels of PCB 52 and 138 (Cocci et al., 2017). On the other hand, PCB congener 101 and 110 were exclusively identified in microplastics from the offshore sites or from the harbor, and no detectable concentrations were found in inshore samples.

Total concentrations of OCs (OCs1 and OCs2) were 130.62 ng/g plastic, 19.20 and 59.25 ng/g plastic in MP from coastal-, offshore waters and harbor, respectively. In the context of pesticides, there is considerable evidence that OCs are chemically stable under environmental condition, and their residues can be found in the water even years later then their ban (Breivik et al., 2004). Among OCs1, we observed that four of them (α-HCH, β-HCH, γ-HCH and HCB) were found in the

analyzed samples with α-HCH and β-HCH present in each sampling site. The detected concentration of OC1s (*e.g.* HCB) is consistent with levels found in pellets and plastic fragments from Camacho et al. (2018) but higher (*e.g.* α-HCH) than values measured in pellets sampled from beaches in China (Zhang et al., 2015). The α-HCH/γ-HCH ratio was higher than 1, which means that there has been no major recent input of HCHs and that the observed values are due to their persistence in the marine environment. After all, isomeric mixture of HCHs has been used as pesticides for a long time. Recent studies have shown higher concentrations of β-HCH with respect to the other isomers in tissues of mollusks collected at different areas including the Eastern Adriatic (Wang et al., 2008; Kljakovic-Gaspic et al., 2010). These findings are interesting because support the role of environmental conditions in determining the different behaviors of HCH contaminations in different matrices.

Regarding OCs2, 2.4-DDE was found at each sampling site while very high concentration of 4.4-DDD were detected exclusively in the harbor. In addition, high levels of 4.4-DDT were measured in pooled samples from offshore waters. The overall analysis of DDT input in terms of ratio of (DDD + DDE)/DDT (Hitch and Day, 1992) shows a value of >0.5 suggesting presence of DDT residues in the environment. It is not surprising that we found similar values since DDT in the environment is mostly a result of both past production and use (ATSDR, 2002).

OPs are the major representative groups of pesticides found on MPs. In particular, the Chlorpyrifos pesticide was carried by MPs sampled at each site and was found to show the highest concentration among OPs. This is an innovative finding due to the classification of chlorpyrifos as emerging contaminant but, at the same time, it is not surprising because Camacho and coworkers (2019) have recently found this pesticide bound to microplastics collected along the Canary Islands beaches. Chlorpyrifos, malathion and pirimiphos-methyl have been extensively used in agriculture during the last decade and therefore have been detected in biotic and abiotic marine compartments (Henriquez-Hernandez et al., 2017; Moreno-Gonzalez and Leon, 2017). In Italy, chlorpyrifos is largely employed on vineyards and despite the low persistence in the environment occasional

418 contamination of surface water can be related to point-source pollution (Carter and Capri, 2004;
419 Capri et al., 2005). In addition to these findings, starting in 2007, a particularly interesting massive
420 use of chlorpyrifos was made as part of the integrated pest management (IPM) strategy for *R.*
421 *ferrugineus* control in the Marche region (Central-Eastern Italy) (Nardi et al., 2011).
422 Several recent reports have provided evidences that marine microplastics can work as contaminants
423 vectors and that their leachates of chemical pollutants exert possible adverse effects on health
424 (Franzellitti et al., 2019). Although these studies have investigated the activation of both aryl
425 hydrocarbon-receptor (AhR)- and estrogen receptor (ER)-signal transduction pathways, little
426 research has been carried out to investigate the role of MP-associated micropollutants in altering
427 metabolic functions. In this regard, recent evidence proves that environmental pollutants can affect
428 lipid homeostasis promoting obesogenic effects (Cocci et al., 2015; Palermo et al., 2016; Cocci et
429 al., 2017; Cocci et al., 2019). The present study thus aimed to investigate the potential adipogenic
430 properties of MP extracts by using 3T3-L1 adipocytes, a long-established *in vitro* model to assess
431 adipocyte differentiation. Indeed, our results revealed that the pollutants extracted from
432 microplastics recovered in different sites of the Central Adriatic Sea are able to trigger obesogenic
433 effects *in vitro*. Comparatively, it is not possible to define the contribution of each toxicant to the
434 observed effect, because not all components present in the extract may induce the effect chosen for
435 analysis. We can, however, suggest a modulatory influence of pollutants on the effects of other
436 chemicals (found in the extract) that cannot be predicted by adopting the concept of additivity
437 (Kortenkamp, 2007). Our findings are in line with previous observations reporting obesogenic
438 effects of persistent organic pollutants including PCBs, PAHs and OC pesticides (Lee et al., 2012;
439 Rundle et al., 2012; Ferrante et al., 2014). *In vitro* data have demonstrated the obesogenic nature of
440 some PCBs which are likely to modulate adipogenesis via AhR-mediated mechanisms (Arsenescu
441 et al., 2008). Interestingly, two of the PCBs found in our plastic extracts, PCB101 and 138, were
442 able to promote adipogenesis in 3T3-L1 cells (Ferrante et al., 2014; Kim et al., 2017). In particular,
443 these studies suggest that the obesogenicity of PCBs seems to be congener specific.

It has also been reported that prenatal exposure to PAH mixture can be associated to obesity (Rundle et al., 2012). This finding was further confirmed following postnatal exposure to benzo(a)pyrene that was found to induce similar increases in fat mass (Irigaray et al., 2006). Also OC pesticides have been suggested as a possible causative agents of obesity due their role in modulating adipogenesis and lipid metabolism (Lee et al., 2012; Rosenbaum et al., 2017). Previous *in vitro* data found that both p,p′-DDT and DDE significantly enhanced differentiation of 3T3-L1 preadipocytes (Moreno-Aliaga and Matsumura, 2002; Mangum et al., 2015). For β-HCH, one of the most common OCs2 found in our plastic extracts, epidemiological data indicate the presence of a positive correlation between its serum levels and BMI (Jakszyn et al., 2009; Dirinck et al., 2011).

It is clear from the previous findings and the results presented here that the metabolic effects induced by MP-associated contaminants are due to altered expression of key lipogenic transcriptional factors such as the PPARγ. Indeed, the signaling cascades that provoke adipogenesis and lipid accumulation in adipocytes is mediated by the induction of PPARγ (Tontonoz and Spiegelman, 2008). Most of the chemicals found in the present work have demonstrated the ability to interact with nuclear receptors acting as agonist of the different PPAR isoforms in both *in vitro* and *in vivo* models. Previous data from our lab found that exposure to environmentally relevant doses of several plasticizers induce PPARγ-mediated lipid accumulation in 3T3-L1 adipocytes (Pomatto et al., 2018). Besides PPARγ, the other PPARs play also a role in tissue fatty acid metabolism. For example, Cocci et al. (2017) observed an upregulation of PPARα in fish hepatocytes exposed to seawater organic extracts from PAHs and PCBs contaminated areas of Adriatic Sea. Adeogun et al. (2016) found similar effects demonstrating a correlation between the chemical levels of PAHs and PCBs detected in Tilapia ssp sampled from African polluted rivers and the expression levels of PPARs genes. Therefore, PPARγ seems to be the principal driver of environmental pollutant-induced adipogenic effects and epigenetic mechanisms are most likely to be responsible for this action.

Although the present work doesn't investigate the potential transfer of MP-associated contaminants to marine organisms, our results provide further indication of the risk related to litter pollution. Ecotoxicological e□ects of microplastics have been demonstrated by Avio et al. (2015) showing bioaccumulation of PAHs in mussels exposed to pyrene-coated plastic. Similarly, ingestion of PVC with sorbed tricolosan was found to affect feeding behavior or cause mortality in lungworms (Browne et al., 2013). In fish, Rochman et al. (2013; 2014b) showed that long term exposure to plastics coated with a complex mixture of POPs and metals, caused liver toxicity, glycogen depletion, lipidosis, cellular death, tumor promotion, abnormal growth of germ cells in gonads. Overall these findings highlight the importance of combined chemical and physical impacts of microplastics to marine ecosystem.

Conclusions

In conclusion, this paper reports new preliminary monitoring data, obtained by a standardized sampling protocol, on the quantification of floating microplastics along the coastline in the southern Marche (middle Adriatic). We found a higher abundance of items in the offshore waters (3 - 10 km to the coast) and a general prevalence of fragments as shape category. Land-based sources and riverine inputs are most likely to influence the presence of microplastics, whose distribution depends on the complexity of the study area circulation patterns. There is overall consistency between our findings and those already published for the Adriatic basin. Chemical analysis showed that microplastics, mainly from inshore coastal surface waters, possessed relevant hydrophobic organic contaminants concentrations, including PAHs and pesticides. These findings clearly support the greater sorption ability of marine microplastics. Moreover, *in vitro* 3T3-L1 screening of MP extracts indicated potential metabolic effects resulting in both adipogenesis and lipid uptake/ storage. Although our results cannot represent a long-term weather exposure scenario, it is clear from this study and other works that special attention should be paid to the eco-toxicological impact of microplastics, including potential obesogenic effects. It is now evident that combined chemical

and physical effects of microplastics may pose health risks to marine organisms, especially when considering long term exposure in low-flow ecosystems. However, further investigations are needed to elucidate ecotoxicological models in order to ensure a suitable and detailed risk assessment.

Declaration of competing interest

The authors declare that they have no competing interests.

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Highlights

- Floating MPs were collected along the western coasts of the Central Adriatic Sea.
- MPs were found in all sampling stations showing the highest abundance in open waters.
- Chemical analysis showed relevant concentrations of pollutants onto MP surfaces.
- MP extracts induced triglyceride accumulation in 3T3-L1 preadipocytes.
- Our results provide further support for the eco-toxicological impact of MPs.

Declaration of interests

☒ 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. On behalf of all authors, the corresponding author states that there is no conflict of interest.

☐The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

Camerino, May 23rd, 2020

Sincerely yours,
Francesco A. Palermo