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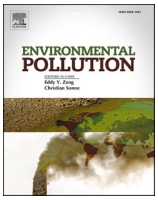

Microplastics evidence in yolk and liver of loggerhead sea turtles (*Caretta caretta*), a pilot study.☆

Giulia Chemello [a,1], Erica Trotta [a,1], Valentina Notarstefano [a], Luana Papetti [b], Ludovica Di Renzo [c,d], Marco Matiddi [e], Cecilia Silvestri [e], Oliana Carnevali [a], Giorgia Gioacchini [a,*]

[a] Department of Environmental and Life Science (DISVA), Polytechnic University of Marche, 60131, Ancona, Italy
[b] CRTM, TartAmare Onlus, 85100, Marina di Grosseto, Italy
[c] Istituto Zooprofilattico Dell'Abruzzo e Del Molise "G. Caporale", 64100, Teramo, Italy
[d] Centro Studi Cetacei, 65125, Pescara, Italy
[e] Centro Nazionale per La Rete Nazionale Dei Laboratori CN-LAB, Istituto Superiore per La Ricerca e La Protezione Ambientale (ISPRA), 00144, Roma, Italy

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ABSTRACT

The potential toxicity of microplastics is a growing concern for the scientific community. The loggerhead sea turtle (*Caretta caretta*) is particularly inclined to accidently ingest plastic and microplastic due to its long-life cycle features. The possible transfer of microplastics from the female to the eggs should be investigated.

The present study investigated the presence of microplastics in yolk and liver samples evaluating the number of melanomacrophages in the hepatic tissue as a possible biomarker of microplastics impact on the embryonic health status. The biometric parameters and liver histological analysis of 27 and 48 embryos (from two different nests respectively) at the 30 stage of development were analyzed. Raman Microspectroscopy was performed to identify the microplastics after alkaline digestion (10% KOH) of yolk and portion of liver from 5 embryos at the 30 developmental stage per nest. Microplastics were found in yolk and liver of loggerhead sea turtles at late embryonic stage for the first time. All microplastics were smaller than 5 μm and were made of polymers and colors suggesting their diverse origins. A total number of 21 microplastics, with dimensions lower than 5 μm, were found between the two nests (11 and 10 microplastics respectively). Only two shape categories were identified: spheres and fragments. The most frequent polymers observed were polyethylene, polyvinyl chloride and acrylonitrile butadiene styrene (31.5%, 21.1% and 15.8% respectively). Despite the eggs showing a higher number of microplastics in yolk samples than liver (15 and 6 microplastics in yolk and liver respectively), a positive correlation was observed only between the number of melanomacrophages ($r = 0.863$ $p < 0.001$) and microplastics in the liver. This result may suggest that microplastics could exert some effects on the hepatic tissues. Future studies should investigate this aspect and the possible relation between microplastics and other stress biomarkers.

1. Introduction

Sea turtles have colonized different marine environments from tropical to temperate latitudes. During their lifecycle, they move between oceanic and neritic waters venturing into the beach to nest (Jensen et al., 2013). In particular, the loggerhead sea turtle (*Caretta caretta* Linnaeus, 1758) are widespread in the Mediterranean Sea which offers several foraging and nesting areas (Casale et al., 2018; Clusa et al., 2014). The Mediterranean subpopulation of loggerhead sea turtle is now classified as Least Concern (IUCN Red List) thanks to the success of

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* Corresponding author.
E-mail addresses: g.chemello@staff.univpm.it (G. Chemello), trotta.erica@gmail.com (E. Trotta), v.notarstefano@univpm.it (V. Notarstefano), info@tartamare.org (L. Papetti), l.direnzo@izs.it (L. Di Renzo), marco.matiddi@isprambiente.it (M. Matiddi), cecilia.silvestri@isprambiente.it (C. Silvestri), o.carnevali@staff.univpm.it (O. Carnevali), giorgia.gioacchini@staff.univpm.it (G. Gioacchini).
[1] Co-author equally contributed.

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numerous conservation programs realized during the last decades (Casale et al., 2018). However, the stability of this species is still jeopardized by several anthropogenic threats (Panigada et al., 2008). Due to their long-life cycle, the late reproductive maturity age and seasonal migrations between foraging and nesting areas, sea turtles are extremely vulnerable to fishing activities, collision with boats, exposure to contaminants, plastic debris ingestion and climate change effects (Báez et al., 2019; Casale et al., 2011; Fuentes et al., 2011). Despite several efforts, to mitigate the sea turtles' by-catch, have obtained promising results (Gilman et al., 2010, 2007; Wang et al., 2010), plastic ingestion is still of great concern (Camedda et al., 2022; Matiddi et al., 2017). Two factors are likely to increase the risk of plastic debris ingestion by sea turtles compare to other marine species: their visual feeding strategy which confuses soft floating plastics and analogous structures for jellyfish (especially during the juvenile pelagic phase), and backward-facing esophageal papillae which inhibit regurgitation and facilitate particle accumulation in the gut (Caron et al., 2016). Damage to the digestive system, alteration of swimming behavior, buoyancy difficulty and at worst animal death have been reported in sea turtles that ingested plastic debris (Nelms et al., 2016; Senko et al., 2020). Considering its tendency to ingest plastic, the loggerhead species was chosen as a bio-indicator in the European Union's Marine Strategy Framework Directive as well as in the Barcelona Convention for the Protection of the Mediterranean Sea (UNEP/MED, 2022). In addition to these effects, sea turtles are menaced by the ingestion of microplastics (MPs) and their possible consequences (Darmon et al., 2022). As widely stressed by the scientific community, the main concern about MPs diffusion in the aquatic environment is their interaction with the aquatic biota which could be exacerbated by their ability to adsorb, release, and transport chemicals (Di Renzo et al., 2021; Duncan et al., 2019; Meaza et al., 2021). Following (Matiddi et al., 2021) definition, the term "microplastics" identifies all the small solid plastic particles less than 5 mm in two of the three dimensions or diameter. They can be either of primary or secondary origin. Primary MPs are produced in their micro-size to find application in several industrial products. On the other hand, secondary MPs derive from the fragmentation of greater plastic debris which are subjected to the synergistic effects of chemical, physical, biological, photic and thermal degradation (Cincinelli et al., 2019).

Regardless of whether they are of first or second origin, information on the fate and effects of MPs once entered the organism, are still controversial and vary among the species. To date, MPs have been identified in the gut and abdominal tissues of *Chelonia mydas* and *Caretta caretta* (Caron et al., 2018; Di Renzo et al., 2021; Ostiategui-Francia et al., 2017). However, the possibility that MPs reach other target tissues should be investigated since in different fish species MPs were found in the fillet, liver, gills and brain (Ding et al., 2018; Karami et al., 2017; Lu et al., 2016; McIlwraith et al., 2021). The prospect of MPs moving within the organism supports the hypothesis of maternal transfer as a possible and relevant route of exposure to MPs.

A case study reported, for the first time, the maternal transfer of polystyrene nanoplastics in zebrafish (*Danio rerio*) (Pitt et al., 2018). The authors found nanoplastics in the embryo yolk (among other tissues) of maternally and co-parentally exposed embryos and larvae suggesting that the egg yolk could be a potential vehicle for this specific nanoplastic. More recently, in the mollusk *Sepia officinalis*, it was also proposed a maternal transfer of MPs through the egg yolk after their detection in the embryonic yolk of this species (Chemello et al., 2023).

In light of these results, if the maternal transfer of MPs occurred in sea turtles as well, adverse effects on the extremely vulnerable embryonic phase are realistic and represent a pollution legacy before real contact with the external environment.

Considering the conservation concerns related to the MPs maternal transfer due to its potential impacts on subsequent generations, the present study aimed to investigate the presence of MPs in the yolk and the liver of loggerhead sea turtle embryos. Differently from model organisms, commonly used to test the toxic effects of MPs, no official protocols and analyses have been assessed for application in sea turtles. Therefore, the second goal of this study was to evaluate the possible effects of MPs occurrence in the yolk and the liver on the embryonic health status considering as a biomarker the number of melanomacrophages (MMs) in the hepatic tissue (Agius and Roberts, 2003; Nowak et al., 2021; Qualhato et al., 2018; Widdicombe et al., 2020).

2. Materials and methods

2.1. Samples collection

Caretta caretta eggs were collected in collaboration with the "tartAmare" association from 2 nests located on the beach of the Tuscany coast (Tyrrhenian Sea, Italy) during the summer of 2020. Both beaches were characterized by a low rate of anthropization, far from residential areas, light sources and watercourses. Both beaches had medium-size sand with a modest slope (4–7%). The first nest of 96 eggs, was laid on August 10th on the Baratti beach (42°59′49.192″ N 10°30′54.764″ E). In the second nest, 64 eggs were laid on August 15th on the beach of Rimigliano (43°01′31.0″N 10°31′25.8″E). The nests were monitored by the volunteers to protect the eggs from any external threats such as predators, human activity and environmental events. The death of the entire clutch (100% mortality rate) was verified according to the guidelines by ISPRA (ISPRA, 2013) by the authorized staff of "tartAmare" (PROT No. 35848 May 18, 2020, issued by the Italian Ministero dell'Ambiente e della Tutela del Territorio e del Mare). According to Italian legislation (D.L. 04/04/14 N.26 art1 a.1), no ethical approval was required for experiments carried out on vertebrates at embryonic stages that are unable to feed themselves independently.

From both nests, only 51 and 52 eggs (from Rimigliano and Baratti nests respectively) were provided by "tartAmare" for the present study. Eggs were sampled at the Laboratory of Reproduction and Developmental Biology, at the Department of Life and Environmental Science (DISVA), Polytechnic University of Marche (Ancona, Italy). For each egg containing an embryo, the following biometric parameters were recorded: embryo weight (without yolk sac), yolk weight, liver weight, straight carapace length (SCL) and straight carapace width (SCW). Based on the parameters recorded, the developmental stage of each embryo was identified following the classification proposed by Miller et al. (2017). Only embryos at stage 30 of development (27 and 48 embryos from Rimigliano and Baratti nests respectively) were considered for further biometric and histological analyses since it was the most representative stage in both nests. A portion of liver was stored in a formaldehyde/glutaraldehyde solution (NaH_2PO_4–H_2O + NaOH + Formaldehyde (36.5%) + Glutaraldehyde (25%) + H_2O) at 4 °C to perform the histological analysis.

During sampling process, the integrity of each eggshell was evaluated, broken eggs or with the visible presence of mold on the surface and within the egg were discarded. Based on egg quality examination, 5 embryos from Rimigliano nest and 5 embryos from Baratti nest at stage 30 were suitable to perform MPs extraction (see Results section 3.2). For each embryo at stage 30, a portion of the liver and the entire yolk were weighed and properly stored to perform MPs identification analysis.

2.2. Microplastics extraction protocol

Samples processing and analysis were performed at the developmental and reproductive biology laboratory of the Polytechnic University of Marche, Ancona (Italy). Yolk and liver samples were digested in a pre-filtered 10% KOH solution at 40 °C for 48h (fiber-glass filter, 1.6 μm pore-size, Whatman GF/A) following the protocol described by Chemello et al. (2023).

2.3. Microplastics quantification and identification

Microplastics were quantified and characterized following Ragusa

et al. (2022) at the ARI Laboratory at Polytechnic University of Marche (Ancona, Italy). All filter membranes, including the procedural and the environmental blanks, were inspected to quantitatively and qualitatively assess MPs using the optical microscope of XploRA Nano Raman Microspectrometer (Horiba Scientific) using a 10× objective (Olympus MPLAN10x/0.25). The whole filter surface was covered by inspecting it moving laterally from the top to the bottom, from left to right, and in the opposite direction. Microparticles that were presumed by visible inspection to be MPs were photographed and recorded according to number, color, shape, and maximum diameter using a 100× objective (Olympus MPLAN100x/0.90). Then, Raman Microspectroscopy was performed by using an XploRA Nano Raman Microspectrometer (Horiba Scientific) to acquire the Raman spectra of each microparticle (spectral range 400–1800 cm^{-1}, 532 nm or 785 nm laser diode). Polynomial baseline correction and vector normalization were applied to raw Raman spectra for pre-processing (Labspec 6 software, Horiba Scientific). Several spectral libraries of polymers and pigments (KnowItAll software, John Wiley & Sons, Inc.) (Dong et al., 2020; Rochman Lab) were exploited to compare and identify the chemical composition of the detected particles. Similarities higher than 80 of Hit Quality Index (HQI) were taken into account.

2.4. Quality assurance and control (QA/QC)

To avoid microplastic contamination during sample collection, storage, processing, and analysis several strict procedures were applied. In this light, all the steps of the analysis were performed in a dedicated room, and a plastic-free protocol was always adopted. Sterilized glass tools were chosen over plastic ones. Cotton laboratory coats and single-use latex gloves were worn during all phases of the experiment. 1.6 μm pore-size filter membranes (Whatman GF/A) were employed to filter all liquids, such as ethanol and deionized water for cleaning and preparation of all solutions. Work surfaces were thoroughly washed with filtered 70% ethanol before starting all procedures. Glassware and instruments, like scissors and tweezers, were cleansed using dishwashing liquid, triple rinsed with filtered 70% ethanol and then rinsed with 1.6 μm filtered deionized water. Moreover, to detect microplastic contamination from the laboratory environment and/or other external sources, environmental and procedural blanks were prepared and thoroughly analyzed. As regards environmental blanks, a filter membrane soaked with 1.6 μm filtered deionized water was positioned into an uncovered Petri dish and located each day of work in the above-mentioned dedicated room. A procedural blank was prepared together with every batch of samples following the same procedure used for the samples. MPs detected in the procedural and environmental blanks, also found in the samples, were removed as environmental contamination from the counting of the MPs in samples.

2.5. Histological analysis

27 and 48 liver samples, from Rimigliano and Baratti nests respectively, stored at 4 °C in formol solution were processed following the protocol described in (Chemello et al., 2023). Samples embedded in paraffin (Bio-Optica) were cut into 5 μm sections using a microtome (Leica RM2125 RTS, Nussloch, Germany) and then stained with hematoxylin and eosin Y stain (Merck KGaA). Sections were observed with an optical microscope (Zeiss Axio Imager.A2, Oberkochen, Germany) with a 40× objective and images were acquired with a combined color digital camera Axiocam 503 (Zeiss, Oberkochen, Germany). Liver sections were analyzed to count the number of melanomacrophages (MMs) and to calculate the lipid percentage using the ImageJ software following the protocol described by Gioacchini et al. (2023). Briefly,the number of MMs was assessed in three distinct areas per liver section (3 sections per liver sample), results were presented as the mean number of MMs/area of the section (digital field area 36.457 μm^2) while the lipid area was analyzed in 6 sections per liver samples and described as the mean percentage of lipid area/area of the section (digital field area 36.457 μm^2).

2.6. Statistical analysis

Average values of embryos' biometric parameters and average values of melanomacrophages number and hepatic lipid % were analyzed through a two-tailed unpaired *t*-test following the assessment of data normality by Shapiro-Wilk test using the GraphPad Software Prism8 for Windows. For all analyses the significance was set at $p \leq 0.05$. Spearman's Correlation analyses were conducted following the assessment of data normality by Shapiro-Wilk test with JASP (version 0.16.1) software. The statistical significance was settled using Bonferroni correction's adjusted *p* values calculated for each correlation matrix.

3. Results

3.1. Developmental stages, biometric parameters and histological analysis

From the 51 and 52 eggs provided for this study, stage 30 was the most represented in both nests (Fig. 1a and b). All the other development stages were consistently less abundant in both nests. Moreover, stages 31 and 26 were absent in Baratti nest (Fig. 1b). Therefore, the following analyses of the biometric parameters were performed considering only stage 30.

The analysis of embryo and yolk parameters did not highlight any significant differences between the two nests (Supplementary Fig. S1).

Histological images representative of liver sections examined in both Rimigliano and Baratti nests are reported in Supplementary Fig. S2. The comparison between the number of melanomacrophages in liver samples from Baratti and Rimigliano nests did not detect any significant difference (Fig. 2a). Although, it is evident the high standard deviation in Baratti samples due to a high variability in the number of melanomacrophages observed. Similarly, the percentage of hepatic lipids showed similar values between the two nests (Fig. 2b).

3.2. Microplastics quantification and identification

Considering the embryos at stage 30, only 5 embryos per nest were considered suitable for the MPs analysis (with no visible presence of mold on the surface and within the egg).

All the embryos analyzed in this study were found to be contaminated by MPs. A total number of 21 MPs, with dimensions lower than 5 μm, were found between the two nests and a similar occurrence was found between the two nests (11 and 10 MPs in Baratti and Rimigliano nests respectively) (Fig. 3a). All the MPs observed were included in two shape categories: spheres and fragments. Fragments were the most abundant shape in both nests (Fig. 3b).

Concerning the plastic nature, seven different polymers were identified, the most frequent polymers were polyethylene (PE), polyvinyl chloride (PVC) and acrylonitrile butadiene styrene (ABS) (31.5, 21.1 and 15.8% respectively) (Fig. 4a) (Supplementary Fig. S3). PE and PVC were present in both nests while polyethylene terephthalate (PET) was observed only in Rimigliano nest. The remaining polymers were detected exclusively in samples from Baratti nest (Fig. 4b). MPs were of different colors, yellow, blue and magenta polymers were the most abundant (Fig. 5a) and they were found in both nests except for magenta MPs found only in Baratti nest (Fig. 5b). Likewise, black and light blue colors were observed only in Baratti nest while grey, green and orange MPs were present in samples from Rimigliano (Fig. 5b).

The majority of the MPs identified were found in the yolk of sea turtle embryos (Fig. 6a) (15 and 6 MPs in yolk and liver respectively) (Table 1). Between the two nests, samples from Rimigliano had more MPs in the yolk compared to the liver (9 and 1 MPs respectively) (Table 1), while in Baratti nest, a similar number of MPs was found in the liver and yolk (5 and 6 MPs respectively) (Fig. 6b) (Table 1). In 2

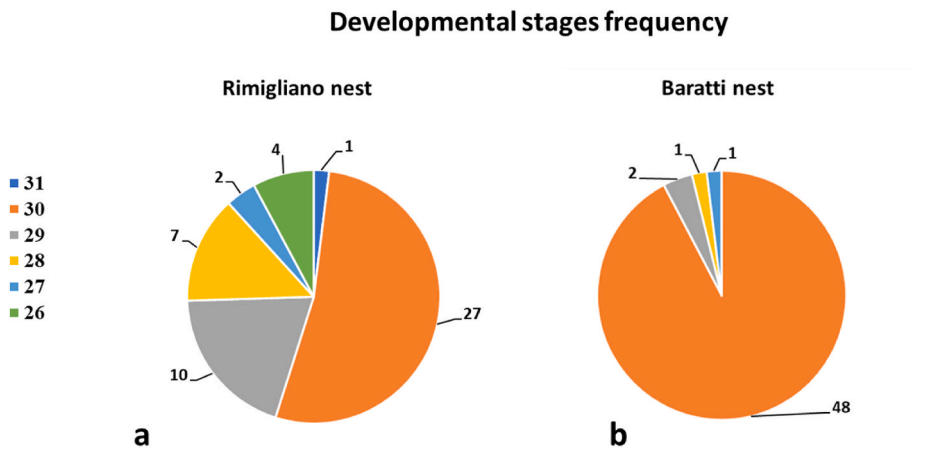

Fig. 1. Frequency of embryos' developmental stages in Rimigliano (**A**) and Baratti (**B**) nests.

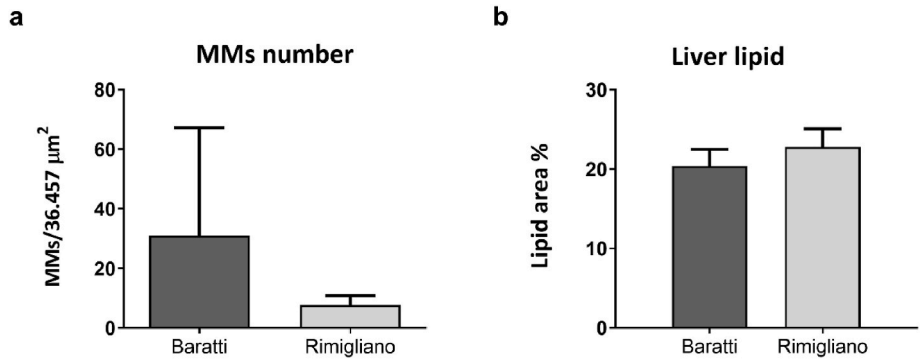

Fig. 2. Histological analysis of liver from embryos at stage 30. (**a**) number of melanomacrophages and (**b**) percentage of the lipidic area in the liver sections.

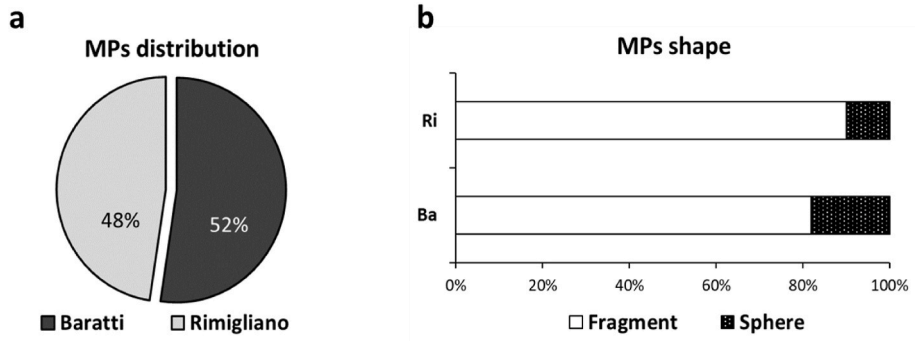

Fig. 3. Total MPs percentage distributed between Baratti and Rimigliano nests (**a**). MPs shape distribution between Baratti (Ba) and Rimigliano (Ri) nests (**b**).

samples (Ri1 and Ba1) the same polymer was detected in the yolk and liver collected from the same embryo (Table 1). The MPs were found only in the liver of 4 samples (Ri2, Ba1, Ba3, and Ba4), in the same embryos the highest number of MMs in the liver was observed.

3.2.1. Spearman's correlation

Spearman's Correlation analysis was performed on dataset presented in Table 1. The increase in the number of hepatic MMs was significantly correlated with the increase of MPs number in the liver (Table 2). Conversely, no significant correlation was observed between the number of MPs in the yolk and the number of hepatic MMs.

4. Discussion

Sea turtles' accidental ingestion of macro and microplastic has been largely documented to such an extent that loggerhead sea turtle (*Caretta caretta*) is considered the main target species for monitoring litter ingestion by marine organisms in the Mediterranean Sea (Matiddi et al., 2017; Solomando et al., 2022). However, up to now, embryos' exposure to MPs and its potential toxicity was still unexplored. The present pilot study produced results on the topic. For the first time, MPs were identified in liver and yolk of loggerhead sea turtle embryos. Previous studies, concerning MPs and embryonic development, merely examined sediment samples to assess the abundance, distribution and composition of MPs at nesting sites (Beckwith and Fuentes, 2018; Zhang et al., 2021). However, these studies only assumed MPs toxic effects on embryos

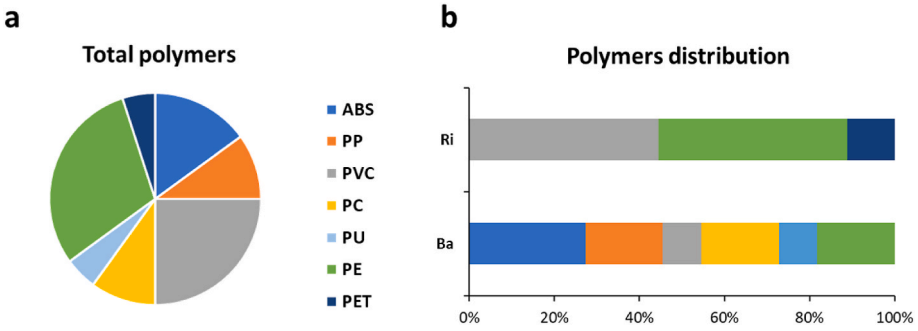

Fig. 4. Total (**a**) and nests (**b**) frequency of microplastic polymers (expressed in %). PET, polyethylene terephthalate; PE, polyethylene; PU, polyurethane; PC, polycarbonate; PVC, polyvinyl chloride; PP, polypropylene; ABS, acrylonitrile butadiene styrene. Ri, Rimigliano; Ba, Baratti.

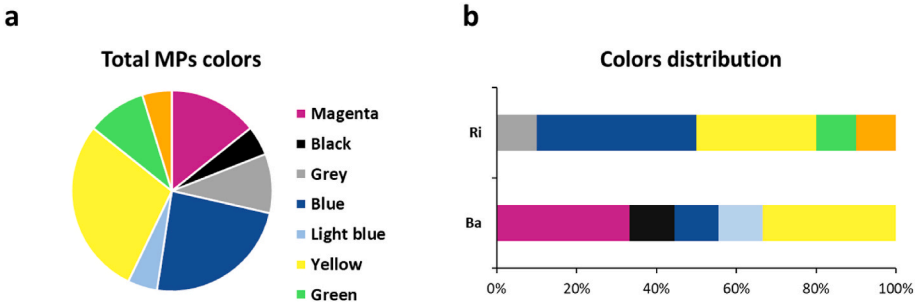

Fig. 5. Total (**a**) and nests (**b**) frequency of microplastic colors (expressed in %). Ri, Rimigliano; Ba, Baratti.

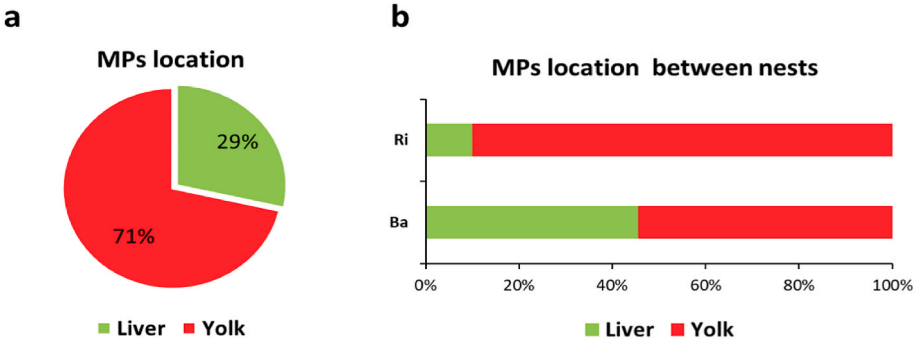

Fig. 6. Total (a) and nests (**b**) frequency of microplastics (expressed in %) detected in liver and yolk. Ri, Rimigliano; Ba, Baratti.

Table 1
Dataset of biometric, histological and microplastic parameters of the 10 embryos at stage 30. Embryo W, embryo weight expressed in g; Liver W, liver weight expressed in g; Yolk W, yolk weight expressed in g; #MPs/Yolk, number of MPs in yolk samples; MPs/Yolk, number of MPs per g of yolk; Yolk pol, polymers identified in yolk samples; #MPs/Liver, number of MPs in liver samples; MPs/Liver, number of microplastics per g of liver; Liver pol, polymers identified in liver samples; MMs, average number of melanomacrophages in liver samples in 36.457 μm^2; % Lipid, percentage of hepatic lipid area. PET, polyethylene terephthalate; PE, polyethylene; PU, polyurethane; PC, polycarbonate; PVC, polyvinyl chloride; PP, polypropylene; ABS, acrylonitrile butadiene styrene.

Samples	Embryo W	Liver W	Yolk W	#MPs/Yolk	MPs/Yolk	Yolk pol	#MPs/Liver	MPs/Liver	Liver pol	MMs	% Lipid
Ri 1	12.41	0.56	2.68	1	0.37	PE	0	0.00	nd	1.67	23.41
Ri 2	17.55	0.96	3.76	2	0.53	PVC	1	1.04	PVC	31.00	40.10
Ri 3	17.18	0.73	1.37	2	1.47	PVC; PE	0	0.00	nd	10.00	21.54
Ri 4	16.65	0.69	1.26	2	1.58	PET; PVC	0	0.00	nd	4.00	22.18
Ri 5	16.74	0.62	1.24	2	1.62	PE	0	0.00	nd	9.00	31.15
Ba 1	20.68	0.64	1.53	1	0.65	ABS	2	3.13	ABS; PP	65.00	23.96
Ba 2	15.83	0.68	3.92	1	0.26	PVC	0	0.00	nd	2.00	48.65
Ba 3	18.60	0.68	1.86	1	0.54	PP	2	2.94	PC	85.00	21.82
Ba 4	17.56	0.53	2.06	1	0.49	ABS	1	1.89	PU	29.00	20.29
Ba 5	18.32	0.75	2.85	2	0.70	PE	0	0.00	nd	2.00	29.92

Table 2
Spearman's correlation coefficients (n = 10). Liver W, liver weight expressed in g; % Lipid, percentage of hepatic lipid area; MMs, average number of melanomacrophages in liver samples in 36.457 μm^2; MPs/Yolk, number of MPs per g of yolk; MPs/Liver, number of microplastics per g of liver; Embryo W, embryo weight expressed in g; Yolk W, yolk weight expressed in g.

		Liver W	% Lipid	MMs	MPs/Yolk	MPs/Liver	Embryo W	Yolk W
Liver W	Spearman's rho p-value	–						
% Lipid	Spearman's rho p-value	0.304	–					
		0.393						
MMs	Spearman's rho p-value	0.058	−0.292	–				
		0.874	0.413					
MPs/Yolk	Spearman's rho p-value	0.261	−0.139	0.128	–			
		0.466	0.707	0.725				
MPs/Liver	Spearman's rho p-value	−0.195	−0.253	**0.863**	−0.218	–		
		0.589	0.481	**0.001**	0.544			
Embryo W	Spearman's rho p-value	0.146	−0.236	0.760	0.176	0.778	–	
		0.688	0.514	0.011	0.632	0.008		
Yolk W	Spearman's rho p-value	0.231	0.442	−0.237	−0.818	0.027	−0.067	–
		0.521	0.204	0.510	0.007	0.940	0.865	

without verifying their actual presence within the eggs. Differently, few studies have evaluated the presence of other contaminants in the eggs of different sea turtle species (De Andrés et al., 2016; Esposito et al., 2023; Muñoz and Vermeiren, 2020; Savoca et al., 2021). POPs and phthalates, which are recognized as widely used additives in plastic production, were found in the yolk, albumen and eggshell (De Andrés et al., 2016; Esposito et al., 2023; Muñoz and Vermeiren, 2020; Savoca et al., 2021). In all these studies contaminants' maternal transfer was suggested. Considering the significant release of these contaminants from plastic, the present study hypothesized that maternal transfer could be the more plausible route of exposure that explain the presence of MPs in both yolk and embryos' liver. Indeed, Cheloniidae embryos are surrounded by an eggshell made of crystalline calcium carbonate in form of aragonite characterized by a selective permeability to water and gases (Phillott and Permenter, 2006). This solid barrier, after a calcification process within the oviduct, prevents external material from entering the egg.

The MPs shape and the distribution of polymers and colors between the two nests also supported the maternal transfer theory. Based on a recent meta-analysis performed to identify the abundance of ingested plastic on a global scale, fragments resulted the most abundant shape found in seawater and the second most ingested shape by fish (Lim et al., 2022). Similar data were also collected along the Tyrrhenian Sea as well as other Mediterranean areas (Di Giacinto et al., 2023; Sbrana et al., 2020; Valente et al., 2022, 2019). Generally, fibers are reported as the most frequent MP types ingested by marine animals (Lim et al., 2022). However, their absence in this study should not be surprising since their mobilization within the biological compartments, when at a greater size than 100 μm, is unlikely (Kolandhasamy et al., 2018; Rebelein et al., 2021). Moreover, all laboratory experiments performed did not observe any fiber translocation within the hematic systems (Rebelein et al., 2021). Indeed, in this study, only fibers >100 μm were observed in few samples analyzed and the same fibers were also detected in environmental and procedural blanks.

The three polymers found in both nests (PET, PE and PVC) are some of the most common constituents of MPs and unfortunately of marine plastic pollution (Horton and Dixon, 2018). On the other hand, the difference in MP polymers and colors between the two nests (at only 5 km apart) suggested diverse MPs origins and could reflect the females' foraging sites where they spend the majority of their lifespan. In these areas, sea turtles are exposed to MPs that could be assimilated by accidental ingestion and inhalation. Indeed, even if MPs tend to distribute along the water column on a density base, with low-density polymers floating close to the surface and high-density polymers sinking, their distribution is strongly influenced by the combination of several factors (Erni-Cassola et al., 2019; Sbrana et al., 2022). MPs buoyancy and sink could be altered by biofouling plastic degradation and leaching of additives (Cincinelli et al., 2019). Therefore, several types of MPs such as the dense plastics PVC and PET found in the present study are commonly found at the same depth of floating lower-density polymers (such as PE and PP also herein detected) after experiencing degradation in smaller particles (Horton and Dixon, 2018). Other than density-based distribution, also MPs shape influences their occurrence in the aquatic environment highlighting the complexity of predicting MPs fate and transport (Horton and Dixon, 2018). Finally, should be considered that MPs ingested could be produce by the fragmentation of large items during the turtle's feeding activity.

Concerning the route that led MPs into the egg, the vitellogenesis process should be considered. This phase of oogenesis begins almost ten months before females move from foraging areas to reach the neritic waters for mating (Miller, 2017). As hypothesized in a previous study, a possible pathway for the mobilization of small-size MPs (<5 μm) within the organism is the circulatory system through which MPs are relocated in different target tissues such as the liver (Lu et al., 2016). Once in the liver, MPs could move to the egg yolk during vitellogenesis binding with protein or lipid molecules involved in the process such as the vitellogenin (a yolk precursor protein that stores lipids in the growing oocyte). A similar pathway has been suggested to validate the presence of persistent organochlorine pollutants (POPs) and neurotoxins in the yolk of sea turtles embryos (Alava et al., 2006; Muñoz and Vermeiren, 2020; Perrault et al., 2016). MPs involvement during vitellogenesis and follicles maturation has also been proposed to justify the presence of MPs in the yolk and embryos of *Sepia officinalis* (Chemello et al., 2023).

Finally, the presence of the same polymer in the yolk and liver of two embryos analyzed, suggested that MPs could be internalized during the yolk absorption. However, the high number of MPs found in the yolk of embryos at the ultimate developmental stage could indicate a late absorption of MPs.

The mechanism through which MPs are moved from the yolk requires in-depth studies as well as the possibility of MPs accumulating in a free form (after degradation of their carrier or selectively binding to some molecules). One possibility suggested by the authors is the affinity of MPs to the lipidic component of the yolk which is almost completely absorbed after hatching. Indeed, it has been demonstrated that in the flatback sea turtle, *Natator depressus,* the 73–74% of the yolk lipid content is not mobilized during the embryogenesis to be successively internalized by the hatchling (Hewavisenthi and Parmenter, 2002). Although, it has to be considered that chemical nature of MPs polymer together with shape and size could influence the pathway of internalization. These features could explain the different number of MPs observed in liver samples between the two nests.

Whether or not of maternal origin, the effects caused by a possible accumulation of MPs in embryos are still unknown. The organization of an organism's structure which occurs during the embryonic development represents a crucial life stage highly influenced by external perturbations. According to the biometric analysis embryos reaching stage 30 showed similar growth parameters between the two nests therefore,

embryos were considered for the correlation analysis independently of their origin.

Worthy of note was the positive correlation between the number of MPs in the liver and the number of the hepatic MMs. For the first time, the presence of MPs was associated with a physiological alteration in sea turtle embryos. MMs are pigmented cells present in the hematopoietic and other soft tissues of lower vertebrates, they appear dark brown due to their pigments content such as lipofuscin, hemosiderin and melanin (Hur and Lee, 2010). In normal physiological conditions, MMs are involved in melanin synthesis, free radicals' capture, removal and autolysis of red blood cells and internalization of prokaryotic organisms. Conversely, when abnormal conditions occur, due to disease or stress, the number of MMs increases and their size and shape vary (Agius and Roberts, 2003). An increased number of MMs in the liver has been already associated with methylmercury exposure in a tropical fish species *(Hoplias malabaricus*) (Mela et al., 2007). MMs hyperplasia and hypertrophy are associated with nonspecific responses such as emaciation, stress and chronic pathological processes (Flint et al., 2009). The increase in number of hepatic MMs where MPs presence was greater could suggest the activation of defense mechanisms possibly due to MPs or the release of contaminants absorbed on them. Greater knowledge on this correlation could be generated by investigating molecular mechanistic information (key events, key events relationship and adverse outcome pathways).

The correlation between MMs count and MPs presence in the liver, obtained in this pilot study, is a promising result that should be confirmed by increasing the number of embryos analyzed and the number of nesting sites. The use of MMs count as a response to microplastic presence in the liver should be examined in depth also for adult turtles and applied to monitoring marine litter ingestion as, up to now, different kinds of biomarkers have not given satisfactory results.

5. Conclusions

The loggerhead sea turtle (*Caretta caretta) is considered worldwide as* a bio-indicator of plastic pollution. Nevertheless, the effects of microplastic exposure on the health status of this species are still unclear and the consequences on reproduction and conservation are only hypothesized. The first obstacle to overcome is the difficulty to recognize a clear physiological response to the presence of MPs since their effects are probably species-specific and depend on the plastic features. Moreover, only dead specimens are available to be analyzed, therefore molecular investigations, usually performed to investigate toxicants' effects, are prohibitive due to animals' conservation status. Two main goals were achieved in the present study, firstly the presence of MPs was recorded, for the first time, in loggerhead sea turtle embryos. Successively, the correlation observed between the number of melanomacrophages in the liver and MPs contained in the same tissue suggested that MPs may exert their toxic effect in the embryonic tissues, and they could be used as biomarkers for MPs presence in the liver of sea turtle embyos. These findings represent the first step to improve the knowledge of MPs effects on embryo development and their mobilization during the oogenesis process.

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Giulia Chemello: Conceptualization, Methodology, Investigation, Data curation, Formal analysis, Writing- Original draft, Visualization, Validation. **Erica Trotta:** Conceptualization, Methodology, Investigation, Data curation, Formal analysis, Writing- Original draft, Visualization, Validation. **Luana Papetti:** Resources, Supervision. **Valentina Notarstefano:** Investigation, Validation, Writing- Original draft. **Ludovica Di Renzo:** Conceptualization, Writing-review & editing. **Marco Matiddi:** Conceptualization, Writing-review & editing. **Cecilia Silvestri:** Writing-review & editing. **Oliana Carnevali:** Writing - review & editing. **Giorgia Gioacchini:** Conceptualization, Supervision, Project administration, Data curation, Writing-review & editing.

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.

Data availability

Data will be made available on request.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.envpol.2023.122589.

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