frontiers in Marine Science

ORIGINAL RESEARCH
published: 19 February 2021
doi: 10.3389/fmars.2021.637030

Impact of Plastic Debris on the Gut Microbiota of *Caretta caretta* From Northwestern Adriatic Sea

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Plastic pollution is nowadays a relevant threat for the ecological balance in marine ecosystems. Small plastic debris (PD) can enter food webs through various marine organisms, with possible consequences on their physiology and health. The loggerhead sea turtle (*Caretta caretta*), widespread across the whole Mediterranean Sea, is a "flagship species," useful as indicator of the general pollution level of marine ecosystems. Ingested PD accumulate in the final section of turtles' digestive tract before excretion. During their transit and accumulation, PD also interact with the residing microbial community, with possible feedback consequences on the host's health. To explore the possible relationship between fecal microbial composition and PD ingestion, we collected fecal samples from 45 turtles rescued between 2017 and 2019 in the Northwestern Adriatic Sea (Italy), assessing occurrence and content of PD in the samples and in parallel the microbiome structure by 16S rRNA gene sequencing. According to our findings, almost all samples contained PD, mirroring the high level of plastic pollution in the area. We identified phylotypes associated to a high amount of PD, namely *Cetobacterium somerae* and other taxa, possibly responding to contamination by plastic-associated chemicals. Furthermore, putative marine pathogens were found associated to higher plastic contamination, supporting the hypothesis that PD can act as a carrier for environmental pathogenic bacteria into marine organisms. Besides confirming the role of the sea turtle as relevant flagship species for plastic pollution of the marine environment, our study paves the way to the exploration of the impact that PD ingestion can have on the microbial counterpart of large marine organisms, with potential feedback consequences on the animal and ecosystem health.

Keywords: loggerhead sea turtles, plastic litter, microbiome, Mediterranean Sea, plastic pollution

OPEN ACCESS

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Specialty section:
This article was submitted to Marine Pollution, a section of the journal Frontiers in Marine Science

Received: *02 December 2020*
Accepted: *01 February 2021*
Published: *19 February 2021*

Citation:
Biagi E, Musella M, Palladino G, Angelini V, Pari S, Roncari C, Scicchitano D, Rampelli S, Franzellitti S and Candela M (2021) Impact of Plastic Debris on the Gut Microbiota of Caretta caretta From Northwestern Adriatic Sea. Front. Mar. Sci. 8:637030. doi: 10.3389/fmars.2021.637030

INTRODUCTION

With a global mass production of more than 350 million tons per year (PlasticsEurope, 2019), plastic is nowadays one of the major emerging pollutant in marine environments (UNEP, 2014). Indeed, it has been estimated that more than 10 million tons of plastic enter the oceans every year (Jambeck et al., 2015), becoming responsible for more than 80% of the total marine litter

(European Parliament, 2019). Such amount of litter especially harms species that are not able to discriminate marine litter or confuse plastic debris (PD) with preys (Barnes et al., 2009; Schuyler et al., 2014a,b). Up to now, it has been estimated that around 260 species, including marine mammals, birds and sea turtles, are threatened by PD, via entanglement and/or ingestion (Caron et al., 2018; Isangedighi et al., 2018). Moreover, breaking down into smaller fragments and filaments (Rocha-Santos and Duarte, 2015; Peng et al., 2017), plastics enter the marine food webs through ingestion by a large number of fish and shellfish species (Barboza et al., 2018), progressively accumulating across the food chain up to the top predators, including humans (Rochman et al., 2015; Nelms et al., 2018; D'Souza et al., 2020).

Sea turtles are among the wildlife groups most impacted by plastics (Gall and Thompson, 2015), with more than half of the total individuals worldwide predicted to ingest plastics during their lifetime (Schuyler et al., 2016), making these large vertebrates important flagship species for plastic pollution of the marine environment (Foti et al., 2009). Once plastics are ingested by sea turtles, either actively (i.e., by mistaking plastic residues for pray), indirectly (feeding on animals which previously ingested plastics), or accidentally (Schuyler et al., 2016; Nelms et al., 2018), they mainly accumulate within the gastrointestinal tract, due to the inability of the animal to regurgitate items (Matiddi et al., 2017; Wilcox et al., 2018; Santos et al., 2020). Several studies have already investigated the presence of plastic pollution in different species of sea turtles, but the vast majority of them were performed on gastric contents taken from dead animals (Caron et al., 2018; Duncan et al., 2019; Digka et al., 2020; López-Martínez et al., 2020). Besides leaving a considerable gap of knowledge in monitoring the actual plastic contamination of live animals, it has been pointed out that the sole observation of the upper part of the intestinal tract, taken during necroscopy, can underestimate the real magnitude of plastic ingestion by the animal (Bjorndal et al., 1994; Pham et al., 2017). Indeed, the amount of PD is reported to increase progressively from the esophagus to the stomach and the intestine, and that debris can remain in the last part of the gut for more than 40 days before being defecated (Hoarau et al., 2014), gradually accumulating if the ingestion is not sporadic. This highlights the need to include feces as target samples in studies focused on the plastic ingestion by sea turtles (Pham et al., 2017).

The PD accumulation in the gastrointestinal tract is reported to exert sub-lethal effects on the sea turtle health, i.e., reproduction and endocrine systems dysfunctions, gastrointestinal blockage, injuries, reduced feeding, and absorption of toxic compounds (reviewed in Franzellitti et al., 2019). However, scarce knowledge is available on the mechanisms that cause those effects, calling for further investigation.

Dwelling in the last part of the intestinal tract, PD can interact with the residing microbial community, possibly influencing its compositional structure and functional properties. Gut microbiota, i.e., the bacterial community inhabiting the gastrointestinal tract of all vertebrates, is well known to play a crucial role in maintaining host physiobiological homeostasis and health, being important for food digestion, metabolism regulation, immune system functionality, and defense against pathogens' colonization (Hooper et al., 2012; Semova et al., 2012; Godon et al., 2016). The gut microbiome is known to change its composition in relation to the presence of environmental pollutants (Evariste et al., 2019) but it has also been proposed as a site for selection of metabolic function related to the detoxification of chemical pollutants, e.g., heavy metals and pesticides, that enter the gastrointestinal tract from the environment (Itoh et al., 2018; Duan et al., 2020). Indeed, bacteria recovered from the gut microbiota of Mediterranean loggerhead sea turtles have been pointed at as putatively capable of metabolizing pesticides (Arizza et al., 2019).

Research connecting microplastics ingestion with the gut microbiome is in its infancy (Fackelmann and Sommer, 2019). Studies mostly focused on model organisms and have been performed mostly in laboratory conditions, exposing model organisms at plastic concentrations and types that might not reflect the actual exposure. The mostly used model organisms incudes mouse (Lu et al., 2018; Jin et al., 2019) and zebrafish (Qiao et al., 2019; Gu et al., 2020; Kurchaba et al., 2020), or filter feeders, like mussels (Auguste et al., 2020; Li et al., 2020). In these studies, it was frequently reported that exposure to microplastics led to microbial communities distinct from the controls without microplastic treatments. This is likely due to the ability of plastic particles to be colonized by microbes on their surface (i.e., the so called "plastisphere") (Amaral-Zettler et al., 2020), thus acting as potential carriers of microbial pathogens (Keswani et al., 2016). Moreover, PD can cause epithelial damages (Fackelmann and Sommer, 2019), that, in turn, may promote local inflammation and a possible response in the gut microbiome structure, that become more prone to colonization by opportunistic bacteria. Moreover, plastics can be vehicle of chemical pollutants, both adsorbed on their surface because of their hydrophobic nature and added to the plastic material itself during manufacturing (Galgani et al., 2014; Campanale et al., 2020), which can act as stressors, forcing changes on the composition of the intestinal microbial ecosystem.

In this scenario, the fecal microbiome emerges as a potential bioindicator for the impact of PD contamination on the physiology and health of living individuals of important marine flagship species, such as sea turtles (Foti et al., 2009; MSFD Technical Subgroup on Marine Litter Group et al., 2013; Galgani et al., 2014; Matiddi et al., 2017), allowing for an effective assessment of overall animal health.

To shed light on this perspective, we explored the amount of plastic debris found in the feces of live loggerhead sea turtles (*Caretta caretta*) rescued from the Northwestern Adriatic Sea, along with possible relationships between occurrence of PD and changes in the animal gut microbiota. Fecal samples were collected from sea turtles after their arrival at a rescue center, where veterinary attention and rehabilitation are provided to stranded, drifted or accidentally captured animals.

Loggerhead sea turtle is widely distributed in coastal tropical and subtropical waters around the world and quite common in the Mediterranean Sea (Márquez, 1990). Due to the great availability of food and warm shallow waters, the North Adriatic Sea is an important foraging and over-wintering area for Mediterranean loggerhead turtles (Franzellitti et al., 2004;

Lucchetti and Sala, 2010). Although loggerheads in this area appear primarily threatened by the high rate of incidental bycatches during fishing activity (Lucchetti et al., 2017), pollution may represent an additional potential threat, as suggested by several evidence reporting well detectable tissue levels of widespread contaminants (Bucchia et al., 2015; Cocci et al., 2018, 2019, 2020). Here, the average benthic litter density has been estimated in 913 ± 80 items/km^2 (Pasquini et al., 2016), ranking the Adriatic Sea as one of the most affected basin by plastic pollution worldwide. The selection of this site provides an exceptionally interesting model for studying the effect of plastic pollution on important flagship species such as sea turtles, considered as a holobiont, i.e., the animal and the microbes that live in a symbiotic relationship, in its whole complexity.

MATERIALS AND METHODS

Study Animals and Samples Collection

The present study was conducted in the Northwestern Adriatic area, semi-enclosed shallow basin linked to the Mediterranean Sea, characterized by low salinity and strongly influenced by the Po river inputs. High temperature variations throughout the year are typical in this basin with an average depth of 35 m. The loggerhead sea turtles rescued in this area and involved in the present study, were found, stranded or captured by fishery nets, during winter and spring months from 2017 to 2019. Turtles were then temporarily hosted at the Sea Turtles Rescue Center "Ospedale delle Tartarughe–Fondazione Cetacea," Riccione, Italy (43.99444°N; 12.673889°E). Turtles were kept in the center for cure and rehabilitation, hosted in single fiberglass tanks or tanks separated by a septum, fed twice a week with fishery products (small fishes such as pilchards, anchovies, or herrings, crustaceans, and mollusks provided by local fishermen active in the same Northwestern Adriatic area in which the turtles dwelled before being rescued), until release. The amount of fishes administered to each turtle is calculated by the veterinary personnel according to the animal size (0.11–0.12 kcal per g of individual weight for turtles with CCL < 30 cm, 0.04–0.05 kcal per g of weight for larger turtles).

A total of 45 sea turtles were sampled for the present study. Turtle name, size (Curved Carapace Length, CCL), days of hospitalization at the sampling date, as well as samples ID and references are reported in **Table 1**. CCL was employed to determine life stage category of each individual, each corresponding to a preference for habitats (Casale et al., 2008, 2009, 2011): (i) size class = 28 cm (CCL range 0–28 cm): juveniles in the oceanic life stage; size class = 40 cm (CCL range 29–40 cm): juveniles undergoing through the transition from oceanic to neritic life; size class = 60 cm (CCL range 41–60 cm): sub-adults in oceanic and neritic stage; size class = 80 cm (CCL range 61–80 cm): sexually mature adults. CCL of assessed turtles ranged from 18 to 78 cm, and size class distribution is reported in **Supplementary Table S1**.

The first feces produced by each turtle after their arrival were collected from the tanks, using a metallic net that was washed each time twice using ultrapure Milli-Q water, and placed into sterilized glass containers. Feces were collected as soon as possible after production, trying to avoid prolonged contact with water, especially for what concern animals hosted in connected tanks. Volunteers working at the Rescue Center were trained on the importance to perform sampling correctly. Sampling was performed over a period of 5 months (January–May) in 2017 and in 2019. Because of the environmental and physiological stress that the enrolled turtles were enduring, they usually refused to eat for a variable length of time (as reported by Biagi et al., 2019). As a consequence, the first feces production happened over a very wide range of time after arrival, sometimes even weeks or months after hospitalization; this is highlighted in **Table 1** where days of hospitalization at the time of fecal sampling is reported for each turtle. Samples were immediately frozen at −20°C, then transported to the laboratory using coolers with ice packs; samples were then stored at −80°C until further analysis, i.e., microbiota characterization and PD analysis. As highlighted in **Table 1**, for the 22 turtles sampled in 2017, 16S rRNA sequencing data were already available (Biagi et al., 2019, MG-Rast, https://www.mg-rast.org/linkin.cgi?project==mgp84794).

PD Extraction and Identification

After a literature research on the plastic debris recovery from animal fecal samples (Reynolds and Ryan, 2018; Hudak and Sette, 2019; Le Guen et al., 2020) in order to set up the best protocol, plastic particle extraction from turtles fecal samples was performed following the procedure reported by Valente et al. (2019). Briefly, a subsample of 0.2 g fecal material was weighed and placed in a glass beaker with 40 mL of 10% KOH for the degradation of the organic matter. The beaker was covered with a glass cap. Samples were incubated overnight at 40°C under continuous stirring. To minimize contaminations, the different steps of the protocol were carried out under a fume hood and all the surfaces were wiped down with ethanol, cotton lab coat and gloves were worn. All equipment used during the experiments were of glass rinsed with 10% HCl solution. Despite these precautions, contaminations could not be excluded. For this reason, a blank control sample containing only the extraction solution (40 mL of 10% KOH) was run in parallel to every set of analysis. Furthermore, filaments supposed to be of textile origin have been excluded from the analysis. Samples were pre-filtered using 1 mm sieves, and then filtered under vacuum filtration system through Whatman® glass microfiber filters, with 1.2 μm pore size. During the filtration, the filtration system was sealed with a glass dish to avoid contamination. Filters were placed in closed double glass dishes and covered with aluminum to protect them from light, because light exposure can lead to fragmentation of the polymers (Rios et al., 2007). Each filter was left to dry for 24 h at room temperature under the fume hood and inspected using a Nikon Eclipse 80i digital microscope. The entire surface was photographed with 4, 10, and 20X magnification with a digital camera (Digital Sight DS-2Mv, Nikon). Pictures were subsequently employed for particle counting and their classification according to shape and

TABLE 1 | Features of target animals (*Caretta caretta*) and fecal sampling.

Turtle name	Sample ID	CCL (cm)	Date of sampling	Days of hospitalization at sampling time	16S rRNA analysis	PD analysis
Andrea	AN	62	21/01/19	1	This study	This study
Babi	BA	34	12/04/19	47	This study	This study
Big	BI3	62	22/02/17	42	Biagi et al., 2019	This study
Benedetta	BN	32	29/05/19	32	This study	This study
Clara	CL	62	24/04/19	106	This study	This study
Danilo	DA3	48	06/02/17	60	Biagi et al., 2019	This study
Edi	ED13	18	22/02/17	13	Biagi et al., 2019	This study
Enzo	EN	75	24/04/19	117	This study	This study
Franklin	FK3	39	07/02/17	195	Biagi et al., 2019	This study
Francesca	FR	33	06/04/19	14	This study	This study
Gaia	GA3	64	18/03/17	60	Biagi et al., 2019	This study
Gabriele	GB	41	24/04/19	154	This study	This study
Gelsomina	GE3	54	11/02/17	123	Biagi et al., 2019	This study
Gilda	GL	75	12/05/19	31	This study	This study
Gina II	GN3	26	25/02/17	13	Biagi et al., 2019	This study
Gelsa	GS	51	24/04/19	149	This study	This study
Giulio	GU3	67	11/02/17	78	Biagi et al., 2019	This study
Guizzo	GZ3	26	06/02/17	30	Biagi et al., 2019	This study
Indio	IN	60	07/04/19	104	This study	This study
January	JA	62	25/04/19	97	This study	This study
Koby	KO3	31	08/02/17	51	Biagi et al., 2019	This study
Larisa	LA	33	12/04/19	88	This study	This study
Leonardo	LE3	53	22/02/17	33	Biagi et al., 2019	This study
Lido	LI	78	05/05/19	18	This study	This study
Livia	LV	36	05/05/19	3	This study	This study
Marta	MA3	34	08/02/17	88	Biagi et al., 2019	This study
Matilde	MD	63	06/04/19	144	This study	This study
Mary	MR	42	29/05/19	8	This study	This study
Matteo	MT3	54	11/02/17	76	Biagi et al., 2019	This study
Max	MX3	63	09/03/17	51	Biagi et al., 2019	This study
Nicole	NI	58	05/04/19	71	This study	This study
Nunù	NU	23	01/05/19	66	This study	This study
Petra	PR3	32	08/02/17	58	Biagi et al., 2019	This study
Priscilla	PS	44	16/03/19	20	This study	This study
Pietro	PT	40	07/04/19	42	This study	This study
Petunia	PU3	74	08/02/17	20	Biagi et al., 2019	This study
Peggy	PY3	22	08/02/17	31	Biagi et al., 2019	This study
Rina	RI3	24	06/02/17	17	Biagi et al., 2019	This study
Silas	SI3	26	07/02/17	28	Biagi et al., 2019	This study
Speranza	SP	25	05/03/19	9	This study	This study
Uga	UG3	34	06/02/17	68	Biagi et al., 2019	This study
Victoria	VC	62	05/05/19	137	This study	This study
Viola	VI3	44	16/02/17	6	Biagi et al., 2019	This study
Viola2	VL	44	06/04/19	82	This study	This study
Zenone	ZE3	62	08/02/17	48	Biagi et al., 2019	This study

PD, Plastic Debris; CCL, Curved Carapace Length.

color. Particle size (maximum linear dimension for filaments or irregular shapes; maximum diameter for rounded and angular shapes) was assessed using the ImageJ image analysis software. Negative controls were compared with the samples, thus allowing to detect possible contaminations within each set of analysis.

Microbial DNA Extraction

Total DNA extraction from fecal samples was carried out using the DNeasy Blood & Tissue Kit (QIAGEN, Hilden, Germany) with a modified protocol as previously reported by Biagi et al. (2019). DNA was then quantified by using NanoDrop ND1000 (NanoDrop Technologies, Wilmington, DE) and stored at −20°C

for subsequent processing. Extracted DNA was diluted to the final concentration of 5 ng/μl using PCR grade water, immediately before performing PCR amplification. The V3–V4 hypervariable region of the 16S rRNA gene was PCR-amplified using the 341F and 785R primers with added Illumina adapter overhang sequences, as previously described (Barone et al., 2019). Five microliter of diluted DNA were used as template for PCR in a final volume of 50 μl. PCR reactions were purified by using Agencourt AMPure XP magnetic beads (Beckman Coulter, Brea, CA). Indexed libraries were then prepared by limited-cycle PCR reaction, using Nextera technology (Illumina, San Diego, CA). Libraries were normalized to 4 nM and pooled, after a further clean-up step as described above. The sample pool was finally denatured with 0.2 N NaOH and diluted to 6 pM with a 20% PhiX control. Sequencing was performed on Illumina MiSeq platform using a 2 × 250 bp paired end protocol, according to the manufacturer's instructions (Illumina, San Diego, CA). Sequencing reads were deposited in SRA-NCBI (project number PRJNA679693).

Bioinformatics and Statistics

For PD occurrence and distribution among animals, the datasets were analyzed permutation multivariate analysis of variance (PERMANOVA) using PRIMER v6 (Anderson et al., 2008). Log-transformed data were used to calculate similarity matrices based on the Euclidean distance (999 permutations; P(perm) < 0.05). Correlation analyses (Spearman's test), data visualization, and graphics were obtained with the GraphPad Prism software ver 9. In any case, statistical differences were accepted when $P < 0.05$. PD size distribution among different animal size classes and different PD shapes was analyzed by non-parametric one-way ANOVA (Kruskal-Wallis test) followed by the Mann-Whitney *U*-test, after deviations from parametric ANOVA assumptions being assessed (Normality: Shapiro-Wilk's test; equal variance: *F*-test). These statistical analyses were performed using the GraphPad Prism 9 software (GraphPad Inc.).

Raw sequences were processed using a pipeline combining PANDAseq (Masella et al., 2012) and QIIME (Caporaso et al., 2010). High-quality reads were binned into operational taxonomic units (OTUs) according to the taxonomic threshold of 97% using UCLUST (Edgar, 2010). The 97%-similarity threshold allowed us to obtain groups of sequences, possibly ascribable to species or small group of species, that could play specific ecological roles in the ecosystem, as previously reported (Biagi et al., 2020). The taxonomy was assigned using the RDP classifier against the Greengenes database (release May 2013). Unassigned sequences and those assigned to chloroplasts and mitochondria were discarded. The PD amount found in each fecal sample was normalized to number of debris per 10 g of stool (Schwabl et al., 2019). Samples were then split into 3 groups according to the normalized PD content: PD < 100, PD 101–300, and PD > 300 counts/10 g of stool. Samples included in each group and average features of the corresponding animals are reported in **Table 2**. Statistical analyses were performed using R version 3.5.1[1] and the packages stats, gplots, vegan, and made4. The similarity percentage (SIMPER) analysis (Clarke, 1993) was carried on to determine the contribution from individual OTUs to the dissimilarity between the three groups of samples (PD < 100, PD 101–300, PD > 300), using the function simper in the vegan package of R. OTUs with a *p*-value < 0.05 in at least two of the contrasts (PD < 100 vs. PD 101–300, PD < 100 vs. PD > 300, PD 101–300 vs. PD > 300) were retained for the subsequent clustering analysis in order to focus on the microbiome features possibly responding to a PD dose effect. Representative sequences of the OTUs of interest were identified as the highest score alignment to the NCBI bacterial 16S rRNA gene database (release September 2019) using BLASTn (version 2.9.0). Prevalence of each OTU in the groups of samples was calculated as percentage of samples in each group in which the reads assigned to the selected OTU were detected (relative abundance > 0). Correlation between OTUs relative abundance and continuous variables (i.e., normalized PD amount, CCL values) was calculated as Kendall correlation tau values; significance of the correlation was calculated using the function cor.test in the package stats in R. Sample clustering was performed accordingly to the selected OTUs relative abundance, adopting Spearman correlation coefficients as metric and Ward-linkage method. Heatmap was produced using the function heatmap.2 in R.

RESULTS

PD Occurrence, Concentration, and Size Distribution

All individuals except one (MT; **Figure 1**) showed particle items in the feces, for an average value of 6 ± 6.09 particles/sample

[1]https://www.r-project.org/

TABLE 2 | Groups of samples defined on the bases of the PD count per 10 g of stools.

	Number of samples	Include samples (Sample ID)	CCL (cm)*	Hospitalization (days)*	PD counts per 10 g of stools*
PD < 100	16	AN, CL, GB, GZ3, JA, LA, MA3, MT3, PS, PT, PY3, SI3, SP, VC, VL, ZE3	43.7 ± 15.2	64.8 ± 45.5	41.9 ± 15.9
PD 101–300	13	BA, BN, DA3, EN, FR, GL, GU3, IN, LE3, LI, LV, NI, PU3	55,6 ± 17.6	48.3 ± 35.5	171.3 ± 55.3
PD > 300	16	BI3, ED13, FK3, GA3, GE3, GN3, GS, KO3, MD, MR, MX3, NU, PR3, RI3, UG3, VI3	41.9 ± 15.9	66.5 ± 57.1	889.2 ± 861.7

**mean ± standard deviation. PD, Plastic Debris; CCL, Curved Carapace Length.*

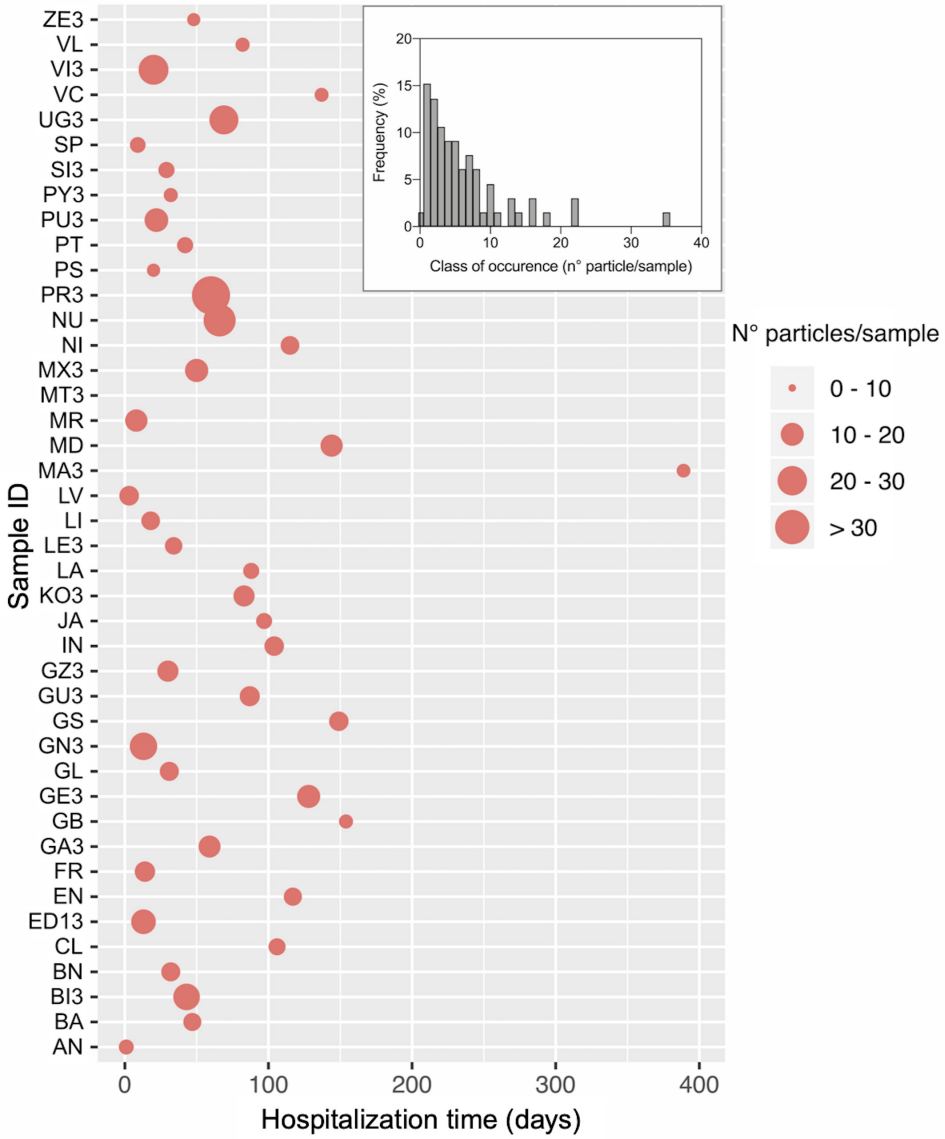

FIGURE 1 | Number of plastic particles isolated from fecal samples of loggerhead sea turtles rescued in the Northwestern Adriatic Sea. The main graph shows the number of particles for each in individual and the day within the hospitalization period at which the sample was collected. The Insert reports the frequency distribution of the number of isolated particles. Sample ID and total hospitalization period are as reported in **Table 1**.

of feces (0.2 g) (**Figure 1**). The maximum number of particles found in a single individual is 34. Frequency distribution of the different classes of particle occurrence shows that the most frequent number of items per sample was 1 (n = 15) and 2 (n = 14) (Insert in **Figure 1**). No significant relationship was observed between particle sample content and hospitalization time, i.e., the time during the hospitalization at which the sample was collected ($r = -0.32$, $P > 0.05$; Spearman correlation test).

Particle classification according to color and shape is reported in **Figure 2**. In all size class categories, data show the prevalence of filaments, followed by unclassified shapes, angular fragments, and finally round items (**Figure 2H**). As to colors, the most represented classes are transparent/white and red particles (**Figure 2G**). There were no statistical differences for shape or color category frequencies amongst different size classes (P(perm) > 0.05; **Supplementary Table S2**). PD size ranged between 11 and 889 μm with a mean value of 198 μm. No macrodebris (PD > 1 mm) was detected in our samples, likely due to the animal digestive processes. PD size distribution between turtle size classes was not statistically significant (**Figure 2J** upper panel), whereas within different particle shape category (**Figure 2J**, lower panel), filaments resulted significantly longer than angular or other shape PD [(mean, min-max range): filaments = 304 μm, 63–889 μm; angular = 59.6 μm, 12–153 μm; others = 86.4 μm, 11–212 μm; $P < 0.05$].

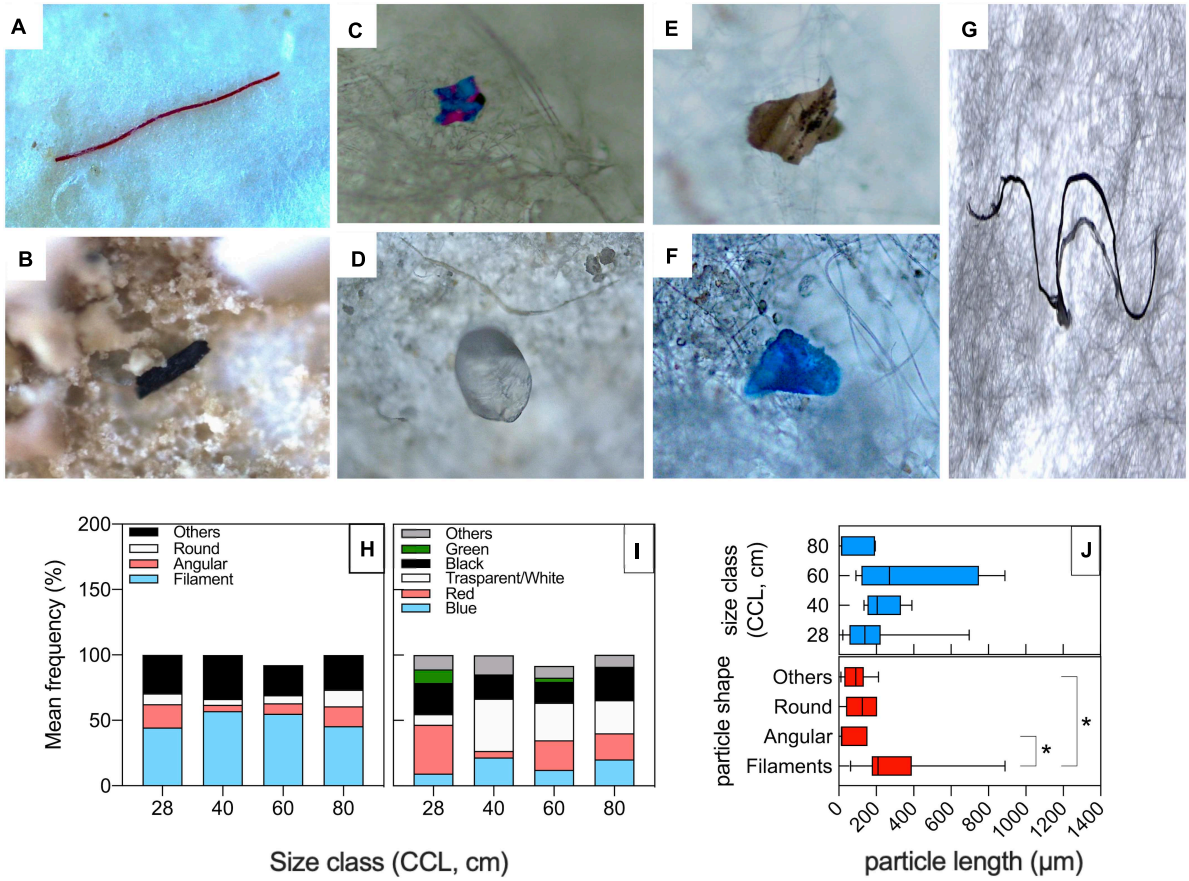

FIGURE 2 | Shapes and colors of particles isolated from fecal samples of loggerhead sea turtles from the Northwestern Adriatic Sea. **(A–G)** Microscope pictures showing representative particle items isolated from the sea turtle fecal samples: **(A)** Red filaments; **(B)** Black angular fragments; **(C)** Angular fragments with unclassified color (others); **(D)** Transparent round fragments; **(E)** Unclassified shape and color; **(F)** Blue fragment with unclassified shape; **(G)** Black filaments. Average frequency classification by shape **(H)** and by color **(I)** across different turtle size classes. **(J)** PD size distribution among turtle size classes (upper panel) and particle shape category (lower panel). *$P < 0.05$.

Impact of PD on the Sea Turtle Gut Microbiota

The fecal microbiota structure of the 23 sea turtles sampled in 2019 was profiled by NGS of the V3–V4 hypervariable region of the 16S rRNA gene. Reads from the present study were analyzed together with those obtained from the 22 sea turtles available from Biagi et al. (2019). A total of 1,884,864 paired-end sequences passed quality filtering (mean per sample ± SD, 41 886 ± 17 972). High-quality reads were clustered into 19 252 operational taxonomic units (OTUs).

To explore peculiarities of microbiota composition in sea turtles related to the PD content, samples were grouped according to PD amounts normalized to 10 g of stool, as previously reported (Schwabl et al., 2019): group "PD < 100" included 16 samples with a PD content < 100 counts per 10 g of stool, group "PD 101–300" included 13 samples with a PD content ranging from 101 to 300 counts per 10 g of stool, and group "PD > 300," with the remaining 16 samples counting > 300 PD per 10 g of stool (**Table 2**). The distribution of turtles' size was homogeneous among PD content groups (Kruskal-Wallis test, $P > 0.05$). Also, groups based on PD content did not differ significantly in terms of days of hospitalization at the time of sampling ($P > 0.05$).

Similarity percentage (SIMPER) analysis was carried out to identify the best subset of OTUs contributing to the dissimilarity in microbiota between groups of samples with different PD content. In order to focus our analysis on those microbiome features whose abundance increased or decreased in association to the increasing concentration of PD in the feces, we selected OTUs significantly (simper analysis P-value < 0.05) contributing to the dissimilarity between groups in at least two of the contrasts (PD < 100 vs. PD 101–300, PD < 100 vs. PD > 300, PD 101–300 vs. PD > 300). Fifty-eight OTUs emerged from this selection, 48 with an increasing abundance and/or prevalence in association with the normalized PD amount, and 9 showing an opposite trend (**Table 3**). The cumulative contribution of the selected OTUs to the dissimilarity between groups was 0.6% for PD < 100 vs. PD 101–300 contrast, 1.6% for PD < 100 vs. PD > 300 contrast, and 1.5% for PD 101–300 vs. PD > 300, showing that the PD content accounted for a minor quota of the

TABLE 3 | Taxonomy, abundance, and prevalence of OTUs significantly contributing to the dissimilarity among PD < 100, PD101–300, and PD > 300 groups of samples.

			Simper (%)[b]			Prevalence (%)[c]			Mean OTU Rel.ab.			Kendall correlation[d]
	OTU ID	Taxonomy - suggested name at the lower taxonomic level[a]	PD < 100 vs. PD 101–300	PD < 100 vs. PD > 300	PD 101–300 vs. PD > 300	PD <100	PD 101–300	PD > 300	PD <100	PD 101–300	PD > 300	OTU rel.ab. vs. normalized PD content
Increasing in prevalence and/or abundance	9510	*Fusobacterium varium*	0.05	0.19**	0.19**	20	43	73	<0.01	0.09	0.35	0.47***
	17389	*Cetobacterium somerae*	0.04	0.15**	0.15**	40	50	73	<0.01	0.07	0.27	
	10905	*Cetobacterium somerae*	0.04	0.14**	0.15**	13	43	73	<0.01	0.07	0.26	0.50***
	564	*Vibrio fluvialis*	0.04	0.14*	0.15**	40	50	73	0.01	0.08	0.26	0.40***
	9606	*Cetobacterium somerae*	0.04	0.12**	0.13**	20	43	73	<0.01	0.07	0.22	0.44***
	10661	*Cetobacterium somerae*	0.02	0.1**	0.1**	13	36	67	<0.01	0.04	0.19	0.44***
	14150	*Cetobacterium somerae*	0.01	0.07**	0.08**	13	36	60	<0.01	0.03	0.13	
	9697	*Cetobacterium somerae*	0.02	0.07**	0.08**	27	43	67	<0.01	0.03	0.13	0.41***
	1749	*Cetobacterium somerae*	0.01	0.06**	0.06**	7	36	73	<0.01	0.02	0.1	0.49***
	1782	*Psychrobacter aquaticus*	0.009	0.05**	0.05**	7	36	73	<0.01	0.02	0.09	0.50***
	8972	*Cetobacterium somerae*	0.004	0.04**	0.04**	0	36	67	0	<0.01	0.07	0.50***
	17723	*Cetobacterium somerae*	0.002	0.02*	0.02**	7	21	53	<0.01	<0.01	0.03	0.44***
	9964	*Cetobacterium somerae*	<0.001	0.02**	0.03**	0	14	53	0	<0.01	0.05	0.44***
	4323	*Cetobacterium somerae*	0.001	0.01*	0.01**	0	21	47	0	<0.01	0.02	0.41***
	4176	*Cetobacterium somerae*	<0.001	0.008*	0.009**	0	14	40	0	<0.01	0.01	
	443	*Romboutsia timonensis*	0.002	0.008*	0.009**	7	29	40	<0.01	<0.01	0.01	
	5611	*Cetobacterium somerae*	0.001	0.008**	0.009**	7	14	53	<0.01	<0.01	0.01	0.40***
	14553	*Cetobacterium somerae*	0.001	0.007*	0.007**	7	14	40	<0.01	<0.01	0.01	
	15621	*Cetobacterium somerae*	0.001	0.007**	0.008**	0	14	60	0	<0.01	0.01	0.46***
	16759	*Cetobacterium somerae*	<0.001	0.007**	0.007**	0	7	47	0	<0.01	0.01	0.40***
	10046	*Cetobacterium somerae*	<0.001	0.006*	0.006**	0	14	47	0	<0.01	0.01	0.42***
	5386	*Cetobacterium somerae*	0.001	0.006*	0.006**	0	21	47	0	<0.01	0.01	0.43***
	10219	*Cetobacterium somerae*	0	0.005**	0.005**	0	0	40	0	0	<0.01	
	1032	*Romboutsia sedimentorum*	0.001	0.005*	0.005**	7	29	47	<0.01	<0.01	<0.01	0.40***
	16025	*Cetobacterium somerae*	<0.001	0.005*	0.006**	0	14	33	0	<0.01	<0.01	
	16446	*Cetobacterium somerae*	<0.001	0.005*	0.005**	0	7	33	0	<0.01	<0.01	
	10872	*Desertihabitans aurantiacus*	<0.001	0.004*	0.004**	7	7	40	<0.01	<0.01	<0.01	
	12899	*Cetobacterium somerae*	0.002	0.004*	0.004*	13	7	53	<0.01	<0.01	<0.01	
	14457	*Cetobacterium somerae*	0	0.004**	0.005**	0	0	40	0	0	<0.01	
	15927	*Cetobacterium somerae*	0	0.004**	0.004**	0	0	40	0	0	<0.01	
	17039	*Cetobacterium somerae*	<0.001	0.004**	0.004**	0	14	40	0	<0.01	<0.01	

(Continued)

TABLE 3 | Continued

	OTU ID	Taxonomy - suggested name at the lower taxonomic level[a]	Simper (%)[b]			Prevalence (%)[c]			Mean OTU Rel.ab.			Kendall correlation[d]
			PD < 100 vs. PD 101–300	PD < 100 vs. PD > 300	PD 101–300 vs. PD > 300	PD <100	PD 101–300	PD > 300	PD <100	PD 101–300	PD > 300	OTU rel.ab. vs. normalized PD content
	3280	*Cetobacterium somerae*	0	0.004**	0.004**	0	0	40	0	0	<0.01	
	11438	*Cetobacterium somerae*	<0.001	0.003**	0.003**	0	7	47	0	<0.01	<0.01	0.46***
	1548	*Staphylococcus equorum*	0	0.003**	0.003**	0	0	33	0	0	<0.01	
	15929	*Cetobacterium somerae*	0	0.003**	0.003**	0	0	40	0	0	<0.01	0.42***
	15944	*Cetobacterium somerae*	0.001	0.003*	0.004*	21	21	53	<0.01	<0.01	<0.01	
	16923	*Cetobacterium somerae*	<0.001	0.003**	0.003**	7	14	47	<0.01	<0.01	<0.01	
	17758	*Terrisporobacter petrolearius*	<0.001	0.003*	0.004**	7	14	47	<0.01	<0.01	<0.01	
	6186	*Cetobacterium somerae*	0	0.003**	0.003**	0	0	40	0	0	<0.01	0.40**
	9294	*Cetobacterium somerae*	<0.001	0.003*	0.003*	7	0	33	<0.01	0	<0.01	
	9700	*Cetobacterium somerae*	0	0.003**	0.003**	0	0	33	0	0	<0.01	
	12913	*Cetobacterium somerae*	<0.001	0.002*	0.002**	7	7	40	<0.01	<0.01	<0.01	
	16503	*Cetobacterium somerae*	0	0.002**	0.002**	0	0	33	0	0	<0.01	
	1702	*Cetobacterium somerae*	0	0.002**	0.002**	0	0	40	0	0	<0.01	
	2397	*Cetobacterium somerae*	<0.001	0.002*	0.002*	7	0	33	<0.01	0	<0.01	
	3291	*Cetobacterium somerae*	0	0.002**	0.002**	0	0	33	0	0	<0.01	
	6541	*Cetobacterium somerae*	0.001	0.002*	0.003*	13	21	53	<0.01	<0.01	<0.01	
	718	*Gordonibacter pamelaeae*	<0.001	0.002*	0.002*	13	7	47	<0.01	<0.01	<0.01	
Decreasing in prevalence and/or abundance	12774	*Faecalicatena orotica*	0.13*	0.13*	0.05	87	57	67	0.24	0.02	0.09	
	5736	*Rikenella microfusus*	0.05*	0.05*	0.004	40	14	13	0.08	<0.01	<0.01	
	8565	*Clostridium baratii*	0.02*	0.02*	0.008	67	43	40	0.04	<0.01	<0.01	
	16407	*Pseudoflavonifractor phocaeensis*	0.01*	0.01*	0.003*	40	14	20	0.02	<0.01	<0.01	
	4024	*Clostridium perfringens*	0.02**	0.01**	0.007	73	36	40	0.03	0.01	<0.01	
	9974	*Clostridium methylpentosum*	0.007**	0.007**	0.001	53	21	7	0.01	<0.01	<0.01	
	13664	*Romboutsia sedimentorum*	0.005**	0.005*	0.002	53	29	13	<0.01	<0.01	<0.01	
	9538	*Clostridium tarantellae*	0.004**	0.004*	<0.001	33	7	0	<0.01	<0.01	0	
	19007	*Cloacibacillus porcorum*	0.003*	0.003*	<0.001	27	7	7	<0.01	<0.01	<0.01	
	16905	*Akkermansia muciniphila*	0.002*	0.002*	<0.001	33	7	0	<0.01	<0.01	0	

[a]*highest score alignment to the NCBI bacterial 16S rRNA gene database (release September 2019).*
[b]*Contribution (%) to the dissimilarity between groups; *P < 0.05, **P < 0.01.*
[c]*Percentage of individuals in each group in which reads assigned to the OTU were detected.*
[d]*Kendall correlation coefficients (tau) >0.40 and <−0.40 are reported; **P < 0.01, ***P < 0.001.*

microbiome dissimilarity among the animals. To confirm the association of the identified OTUs to the PD content, Kendall correlation coefficient (tau) was calculated between the relative abundance of each OTUs and the normalized PD content of the corresponding samples; correlation tendencies (tau > 0.40 or < −0.40) with *P*-values < 0.01 are shown in **Table 3**. Since PD content may be affected by feeding preferences of sea turtles, that largely depend on life stage (i.e., size class category as reported in **Supplementary Table S1**), we sought for a possible relationship between PD content, size and OTU distribution. Kendall tau was also calculated using CCL measurements as reference variable, as control of specificity for the selected OTUs as PD content responders. None of the selected OTUs was significantly correlated to the CCL of the corresponding animal (**Supplementary Table S3**).

The 58 OTUs, mostly contributing to the dissimilarity among PD content groups were identified down to species level as the highest score alignment to the NCBI bacterial 16S rRNA gene database (**Table 3**). Thirty-nine out of 48 OTUs showing an increasing trend in association to the PD content were assigned to the species *Cetobacterium somerae*. However, among the most contributing OTUs we found also two sequences assigned to animal pathogens, namely *Fusobacterium varium* (Duangurai et al., 2019) and *Vibrio fluvialis* (Arab et al., 2020). Other OTUs in this subset were assigned to the following genera: *Psychrobacter*, *Romboutsia*, *Desertihabitans*, *Staphylococcus*, *Terrisporobacter,* and *Gordonibacter*.

The 9 OTUs that showed a decreasing trend associated with the increasing PD content in feces were assigned to different microorganisms, several of which belonging to the genus *Clostridium*, with representation of different species. The one contributing most (0.13%) to the dissimilarity between PD < 100 samples and the other two groups was assigned to the species *Faecalicatena orotica*, an animal gut isolate previously classified as *Clostridium oroticum* (Sakamoto et al., 2017). Other genera represented in this group of OTUs were *Akkermansia, Rikenella, Cloacibacillus, Pseudoflavonifractor,* and *Romboutsia*, all previously identified in vertebrate gut bacterial ecosystem.

To confirm that the identified OTUs were discriminant for the content of PD in corresponding fecal samples, we performed a cluster analysis based on Spearman correlation between the abundance profiles of these selected 58 OTUs (**Figure 3**). Even if a certain level of dispersions was maintained, samples showed an overall tendency toward the segregation between groups, defining two major clusters, with samples from the PD > 300 group preferentially assigned to the cluster A, particularly enriched in those OTUs identified by the simper analysis as significantly contributing to the dissimilarity between PD > 300 samples and both the other groups. On the contrary, PD < 100 samples grouped together in cluster B, characterized by an overall higher abundance of the OTUs identified by the simper analysis as significantly contributing to the dissimilarity between PD < 100 samples and both the other groups (lower part of the heatmap). Samples from the intermediate group (PD 101–300) were distributed equally across both clusters.

DISCUSSION

The present study explored the plastic debris (PD) amount in the feces of wild captured loggerhead sea turtles (*C. caretta*), dwelling in the Northwestern Adriatic Sea, collected after their arrival at a local rescue center for their rehabilitation. Large marine vertebrates, such as cetaceans and sea turtles, are ideal sentinels to monitor plastic pollution in marine environment but, thus far, data on the impact of plastic ingestion on such wild species have been necessarily provided mostly through sampling of gastrointestinal contents from dead animals (López-Martínez et al., 2020). By employing fecal samples, our approach attempts to assess the actual distribution of PD in the gastrointestinal tract of live animals at different life stages, although it is challenging to compare our results with the available literature. The number of plastic particles found in the feces of the turtles involved in our study can be considered as quite high, with respect to data generally reported for the gastrointestinal content of dead stranded turtles. For instance, Duncan et al. (2018) found an average plastic micro-particles content ranging between 10 and 15 per 100 ml of gastric content of both *C. caretta* and *Chelonia mydas* from the Mediterranean Sea. Such number, even if higher than those reported for the same species of turtles recovered from Atlantic and Pacific oceans in the same paper, is still very low in comparison to the PD counts reported in our work, normalized to 10 g of solid feces. The comparison between the present study and previous ones is challenging because of the different nature of the matrix (liquid gastrointestinal contents vs. solid feces) and the different counting approach (normalization to 10 g of feces vs. counting of plastic items per samples or percentage of samples contaminated by plastic debris (Camedda et al., 2014; Hoarau et al., 2014). However, it is tempting to hypothesize that the peculiar location of our study, the Northwestern Adriatic Sea, might be partially responsible of the discrepancy between results, and for the fact that all, but one, of the 45 enrolled turtles provided fecal samples containing PD. The Adriatic basin is, in fact, one of the most polluted marine sites across the globe, due to its high productivity and anthropic impact, with an average concentration of > 400,000 plastic particles up to 5 mm per km^2 (MSFD Technical Subgroup on Marine Litter Group et al., 2013; Alessi and Di Carlo, 2018; Llorca et al., 2020). Renzi et al. (2018) assessed the presence of PD in *Mytilus galloprovincialis* bred in a mussel farm off Cesenatico (Northwestern Adriatic Sea, Italy), in comparison with mussels bred in other Italian coastal areas and with natural populations of the Tyrrhenian Sea. The concentration of PD in animals from the Northwestern Adriatic resulted higher than that of other areas, including individuals residing in nature, with an average of 12.4 items/mussel. All the materials found in the sampled individuals were filaments, with a dominance of blue and black colors. Bivalve mollusks are one of the main components of the loggerheads diet, and it was demonstrated that they can transfer particles to other invertebrates such as crabs (Farrell and Nelson, 2013), which are also a prey of *C. caretta*. Therefore, showing a relevant level of plastic pollution in fecal material of Adriatic Sea turtles—and in the light of the recognized relevance of sea turtle as flag species for the health status of the marine environment—these

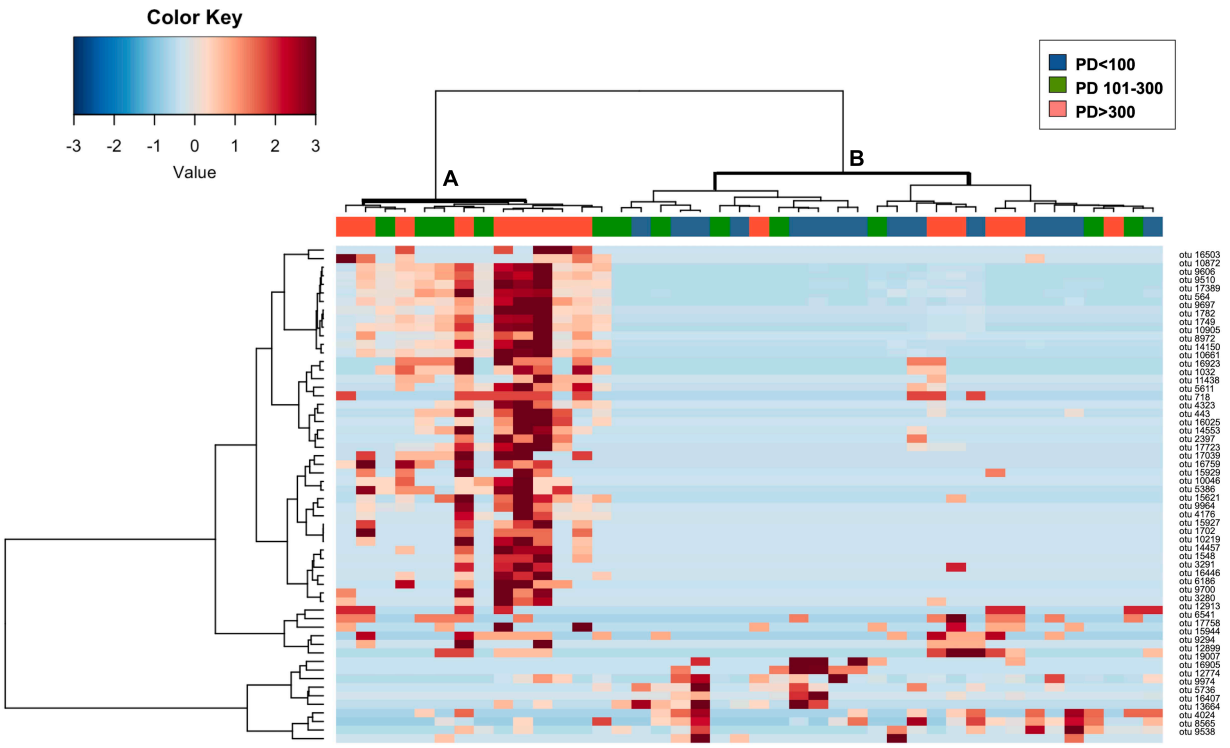

FIGURE 3 | Hierarchical clustering and relative abundance of OTUs significantly contributing to the separation of samples in PD < 100, PD 101–300 and PD > 300 groups. The heatmap shows Ward-linkage clustering based on the Spearman correlation coefficients of the available sea turtles fecal samples and 58 fecal OTUs that were found significantly contributing to the separation of samples into the three PD content groups according to the simper analysis. Samples are shown column-wise; color code (blue, green, and orange) is provided for identifying samples belonging to groups PD < 100, PD101–300, and PD > 300. OTUs relative abundances are reported on the rows. OTUs are identified as numbers; taxonomical assignation based on the highest score alignment to the NCBI 16S rRNA gene database is provided in **Table 3**. The two identified samples clusters (clusters **A** and **B**) are highlighted in bold.

results indicate and confirm the high level of plastic pollution in Adriatic ecosystems.

Besides representing a flagship species for the levels of environmental contamination, sea turtle health may be directly impacted by plastic ingestion. Larger debris have macroscopic effects, for instance by inducing gastric blockage and injuries, but also small fragments are known to affect turtle physiology (Bugoni et al., 2001; Derraik, 2002; Kühn et al., 2015; Nicolau et al., 2016). The impact of PD on the turtle health can also be mediated by the interaction with the intestinal microbial community, the composition of which can be altered by plastic-associated chemicals (i.e., adsorbed pollutants or plastic additives), and/or biological contaminants (i.e., plastisphere microbial components) (Amaral-Zettler et al., 2020; Campanale et al., 2020). Probably because of the difficulty in obtaining samples from wild animals, studies connecting microbiome composition and plastic gastrointestinal contamination have been mostly limited to model animals or easily accessible productive species, such as bivalves of interests for food consumption (Lu et al., 2018; Jin et al., 2019; Qiao et al., 2019; Auguste et al., 2020; Gu et al., 2020; Kurchaba et al., 2020; Li et al., 2020). Interestingly, in our observational study on wild capture animals only one of the turtles provided a sample free of fecal PD, mirroring the pervasive real world plastic contamination in the particularly polluted geographical location. Consequently, we choose to focus our attention on the effect of the PD amount on the fecal microbiota composition. Indeed, our statistical approach based on the analysis of similarity percentages (SIMPER) identified several microbial sequences whose abundance significantly contributed to the dissimilarity between groups of samples with different PD content. The cumulative contribution of the identified OTUs to the dissimilarity between groups of samples was expectedly small, since other variables (e.g., diet, health, age, individual physiology, history of environmental exposition) are known to play a relevant role in defining the overall structure the microbiome in animals. Still, we were able to define two groups of PD-responding microbiome OTUs that contributed to effectively separate the samples with the highest PD content from those with the lower one. We defined a first group of 48 OTUs, mainly composed by sequences assigned to the species *Cetobacterium somerae*, which showed an increasing trend, in terms of relative abundance and/or prevalence in the sample groups, along with the increasing PD content in feces. Conversely, a second, more diverse OTUs list was obtained showing an opposite trend.

C. somerae belongs to the Fusobacteria phylum and has already been reported as abundant in the gut microbiota of Adriatic loggerhead sea turtle (Biagi et al., 2019). In

particular, this species is known to ferment amino acids and peptides into short-chain fatty acids (SCFAs) that can be absorbed and utilized by both bacteria and the host (Tsuchiya et al., 2008; Olsen, 2014) and it also benefits the host by producing vitamin B12 and antimicrobial peptides (Ahasan et al., 2018). The reason why the highest PD content was associated to higher abundance of 16S reads assigned to this species is to be explored further in future studies. Recent studies on fish models (marine manaka and crucian carp) showed that *Cetobacterium* amount responds peculiarly to increasing exposure to organic chemical pollutants (perfluorobutanesulfonate and ammonia, respectively): exposure to low concentration of pollutants caused an increase in *Cetobacterium*, while its abundance dropped dramatically after exposure to high concentration (Qi et al., 2017; Chen et al., 2018). Perfluorobutansulfonate (PFBS) is used as processing aids in the manufacture of fluoropolymers and as flame retardants in polycarbonate materials (ECHA, 2019). It is not unthinkable that, even if it is usually considered a symbiont for marine carnivorous species, the increase in bacteria assigned to the genus *Cetobacterium* in our study could be actually related to some contaminants added in low concentration on the plastic materials. Moreover, microplastic contamination has been associated to an increase ammonia excretion in a model fish (Yin et al., 2019). Alongside the majority of *Cetobacterium*-assigned sequences, the OTUs showing an increasing pattern associated with the increasing PD content in feces also included some sequences assigned to bacterial species previously isolated from petroleum-polluted environmental sites, i.e., *Terrisporobacter petrolearius* (Deng et al., 2015), as well as potentially pathogenic species (i.e., *Vibrio fluvialis* and *Fusobacterium varium*) (Duangurai et al., 2019; Arab et al., 2020). The latter observations can support the hypothesis that PD can act as carriers for pollutant-selected and potential pathogenic species, easing their entrance and colonization of the gastrointestinal environment of marine animals (Fackelmann and Sommer, 2019).

The OTUs showing higher abundance or prevalence in the group of samples with lower PD content constituted a more diverse collection, all assigned to genera that were isolated from and/or are commonly found in vertebrate gastrointestinal tract, such as *Akkermansia*, *Rikenella*, *Faecalicatena*, *Cloacibacillus,* and *Clostridium* (Costello et al., 2010; Looft et al., 2013; Sakamoto et al., 2017; Biagi et al., 2019; Lin et al., 2019). It might be postulated that an increased amount of PD accumulating in the lower gastrointestinal tract might result in a less favorable environment for the survival of some endogenous symbiotic species, possibly because of the augmented local inflammation caused by small epithelial injuries or through effects of plastic-associated contaminants (Fackelmann and Sommer, 2019).

In conclusion, our observational study represents a step forward into the research field of evaluating the impact of plastic contamination in the marine environment. Indeed, beside involving ecologically relevant, wild, alive animals that did not undergo controlled plastic contamination, as reported in very few previous studies (Camedda et al., 2014; Hoarau et al., 2014), our work has the unprecedented feature of contextually exploring both PD content and microbiome composition of sea turtles fecal samples. Even if a partial influence of the controlled environment in which turtles were temporarily kept cannot be completely ruled out, the level of plastic contamination of the turtles is likely to reflect that of the marine food web of the Adriatic Sea. Indeed, the amount of PD found in the turtle feces was not statistically correlated to the number of days the turtles spent in captivity before sampling, possibly confirming that plastics are highly persistent and accumulative in the digestive tract of these animals, an aspect that could not be explored in previous studies on dead stranded animals. To date, standard methodologies and criteria of quantification have been provided to correlate the plastic litter found in dead turtles to the environmental status (Matiddi et al., 2019). The results provided by our analysis cannot be compared to such standard guidelines because of the differences in methodological approach. However, the detection and quantification of PD in feces of rescued, live animals, as presented in our work, highlighted that loggerheads' fecal samples might become a good proxy to evaluate the level of threat constituted by the PD in the Adriatic Sea, to be used together with other standardized surveys. Future research involving sea turtles from rescue centers located in different, and differently polluted, geographic area could provide data to sustain the potential of this approach.

For what concern the relationship between fecal microbial composition and PD concentration in fecal material, the detected "dose effect" of fecal plastic contamination regarded, as foreseeable, minor components of the microbial community. Still, the data presented here identified some putative gut microbiome biomarkers of plastic contaminations, such as a selection of microbiome components whose abundance increases in association to plastic-associated chemical pollutants (plastic flame retardants or other plastic components) and the presence of potential environmental pathogens potentially enriched on the plastisphere. Further, PD contamination was also associated with the depletion in several putative mutualistic components of the gut microbiome. Our findings open the way to future studies exploring the impact of the observed gut microbiome changes in terms of mechanism of action and consequent effects on the animals' health and physiology.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: https://www.ncbi.nlm.nih.gov/ PRJNA679693.

ETHICS STATEMENT

Ethical review and approval was not required for the animal study because Loggerhead sea turtles feces were collected from animals temporarily hosted at a local, authorized Rescue Center for veterinary attention before being released in the sea.

Fecal samples were collected from tank water without touching nor disturbing the animals in any way.

AUTHOR CONTRIBUTIONS

SF and MC: conceptualization, writing—review and editing, and supervision. EB, SR, SF, and MC: methodology. EB, MM, GP, VA, CR, DS, and SF: investigation. EB, MM, SP, SR, and SF: formal analysis. EB, MM, CR, and SF: data curation. VA, SP, SF, and MC: resources. EB, MM, and SF: writing—original draft preparation. SP, SF, and MC: funding acquisition. All authors contributed to the article and approved the submitted version.

FUNDING

This project has received funding from the European Union's Horizon 2020 Research and Innovation Programme under grant agreement No. 818290. This publication reflects only the author's view and the Agency is not responsible for any use that may be made of the information it contains.

ACKNOWLEDGMENTS

This study represents partial fulfillment of the requirements for the Ph.D. thesis of MM, GP, and DS at the Ph.D. course of Innovative Technologies and Sustainable Use of Mediterranean Sea Fishery and Biological Resources (FishMed—University of Bologna, Italy).

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmars.2021.637030/full#supplementary-material

Supplementary Table S1 | Size classification and distribution of the loggerhead sea turtles assessed in this study.

Supplementary Table S2 | Results of PERMANOVA pairwise comparisons of particle shape/color distribution among different sea turtle size classes.

Supplementary Table S3 | Kendall correlation coefficient (tau) and associated *P*-values calculated between the relative abundance of each OTU (**Table 1**) significantly contributing to the samples grouping and the normalized PD content and CCL of the corresponding turtles.

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.