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Microplastic ingestion in reared aquaculture fish: Biological responses to low-density polyethylene controlled diets in *Sparus aurata*☆

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ABSTRACT

During the last years, ingestion of microplastics (MPs) has been quantified in marine species both with an ecological and commercial interest at sea and under experimental conditions, highlighting the importance to assess MP ingestion in commercially and aquaculture important species such as gilthead seabream (*Sparus aurata*) fish. In order to study the ingestion of MPs in a commercially valuable species, gilthead seabreams were exposed to an enriched diet with virgin and weathered low-density polyethylene (LDPE) pellets for three months followed by a detoxification period of one month of no exposure to MP enriched diets. Our results indicate that MP ingestion in these fishes increased with exposure time, and differences were found between treatments, showing the highest ingestion values after three months of exposure to MP enriched diets and in the weathered treatment. However, after one month of detoxification, no MPs were found in the gastrointestinal tracts of fish, reflecting no long-term retention of MPs in *Sparus aurata* digestive system. According to results from this study, exposure of fish to MP enriched diets does not affect fish size neither the Fulton's condition index as both parameters increased with time in all treatments (control, virgin and weathered). Both carbon and nitrogen isotopic signatures decreased with fish size in all treatments which could be related to an increase of nitrogen deposition efficiency in fish muscle with a high protein assimilation during the first months of *Sparus aurata*.

1. Introduction

It is widely known and accepted that ingestion of microplastics (MPs) might cause physical, physiological and ecotoxicological effects in marine species, including commercially important species such as *Sparus aurata* Linnaeus, 1758 (Solomando et al., 2020; Capó et al., 2021; Rios-Fuster et al., 2021). Physical effects considering abrasion and satiation are mainly caused by the type of particles ingested (Foekema et al., 2013; Ryan 1988) and weight loss and reduction of species condition index can be related to MP ingestion (Compa et al., 2018). Moreover, other effects such as toxicity due to bioaccumulation of persistent toxic substances (Koelmans et al., 2013), carcinogenesis and endocrine disruption, amongst others (Wright et al., 2013), are the outcome of pollutants being added to MPs during manufacturing processes (Teuten et al., 2009) or sorbed to their surface once in the marine environment (Rochman et al., 2013). Most demanded plastic polymers in the European Union include polyethylene (PE), polypropylene (PP), poly (vinyl-chloride) (PVC), polystyrene (PS), poly (ethylene-terephthalate) (PET) and polyurethane (PU) (Gewert et al., 2015). However, in marine habitats high-density polyethylene (HDPE), low-density polyethylene (LDPE) and polyethylene terephthalate (PET) are most commonly found (Suaria et al., 2016; Baini et al., 2018; Compa et al., 2020). Particularly, polymers of HDPE, LDPE and PP sorb higher concentrations of polychlorinated biphenyls (PCBs) and polycyclic aromatic hydrocarbons (PAHs) than other polymers such as PET and PVC (Rochman et al., 2013).

It is acknowledged that marine litter has its main origin from land based sources (80%) but sea-based sources such as marine aquaculture can also contribute to the input of litter, especially of the plastic fraction (Jambeck et al., 2015). Aquaculture is a fast growing industry, with a global production of 80.0 million tons of

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commercial fish in 2016, despite that during the 6 previous years (2000–2016) this sector experienced an average annual growth decrease of 5.8% (FAO, 2018). The Mediterranean Sea is an important producer of intensive open water fish culture, with the most important industries in Spain, France, Italy and Greece, which generated 2133 million Euros in 2008 (FAO, FishStat). A common point in this industry is plastic which is widely used in all aquaculture facilities due to its physical characteristics for multiple uses. For instance, tanks used for spawning, incubation and stock holding of cultivated species are made up of fiber-reinforced plastic (FRP) and HDPE which can fragment and break down into smaller plastics (secondary MPs) (Huntington, 2019). Moreover, pipes used for aeration and water supply purposes are also made up of PVC and the degradation of plastics into MPs can be fast (Huntington, 2019). Given that most of the material used in aquaculture facilities is made up of plastic polymers, impacts associated to these on the cultivated species should not be underestimated. Even though it is estimated that between 3000 and 41,000 tons of marine litter are lost every year to the environment (Sherrington et al., 2016), there is scarce information on the amount and contribution of meso- and microplastics from aquaculture practices along with the quantification on ingestion values in cultivated species.

In the marine environment, fish species from the pelagic realm and seafloor areas have been seen to ingest plastic polymers such as PP, PET and PVC (Alomar and Deudero, 2017; Compa et al., 2018). In the Mediterranean Sea in some cases, MP occurrence in the gastrointestinal tracts and stomachs of fish species are high, up to 60% and 100% of the sampled individuals (Avio et al., 2015, 2017; Nadal et al., 2016). However, variability amongst species and geographical areas is high (Rios-Fuster et al., 2019; Alomar et al., 2020) and can be attributed to both feeding behavior and strategies of species but also to a high variability in environmental abundances of plastic litter.

In aquaculture facilities, fish species are susceptible to MP ingestion from the surrounding environment. For example, a recent study conducted in the East China Sea quantified a high degree of MP ingestion, ranging from 22.21 ± 1.70 to 13.54 ± 2.09 items/individual, in fish captured at the vicinity of one of the most important aquaculture areas in China (Feng et al., 2019) and Ory et al. (2018) indicated that fish ingested MPs when floating close to pellet food under laboratory experiments. Moreover, Wu et al. (2020) studied MP ingestion in fish species from a typical aquaculture sheltered area in China detecting MPs in all sampled organisms and pointing out that ingestion rates were relative high when compared to previous studies in open waters. Additionally, a study in this region of the world, specifically in the Maowei Sea which is impacted by river discharge and mariculture activities, indicated that out of 66 analyzed fish, MPs were observed in all gastrointestinal tracts of fish and observed levels were yet again higher than previously reported values (Zhu et al., 2019). Even though at relatively low levels, a study conducted under laboratory conditions demonstrated MP translocation into the fillet of European seabass (*Dicentrarchus labrax*) juveniles which are consumed by humans, warning of possible potential effects to human beings (Zeytin et al., 2020). Regardless of the previous mentioned studies, up to date there is a knowledge gap concerning the response of organisms to MPs in aquaculture systems (Lyu et al., 2020), and from a review regarding MP ingestion on worldwide fish, only 14% of the sampled fish came from aquaculture highlighting the need to further assess plastic exposure in fish species linked to the aquaculture industry which represents 47% of fish for human consumption (FAO, 2018) (Sequeira et al., 2020).

Amongst the most important cultivated fish species, *Sparus aurata* has been indicated as a good sentinel species for aquaculture research (Seginer et al., 2016) and it has also been suggested as a valid indicator species for plastic pollution in the Mediterranean Sea (Bray et al., 2019; Fossi et al., 2018). According to the Food and Agriculture Organization (FAO) of the United Nations (UN), in 2016, its global aquaculture production was of 185,980 tonnes (FAO FishStat). In nature, gilthead seabream mainly feeds on molluscs, crustaceans and small fish. In aquaculture settings, the first feeding of gilthead seabream with synthetic food takes place when individuals weigh more than 10 mg (FAO, 2005). Experimental laboratory studies with this species, have demonstrated that the ingestion of MPs in *Sparus aurata* induces oxidative stress and a pro-inflammatory response in gut and liver related to a progressive increase in the activities of antioxidant enzymes, glutathione s-transferase and reduced glutathione (Solomando et al., 2020; Capò et al., 2021).

Effects of fish exposure to MP ingestion and associated contaminants can be assessed through biomarkers of oxidative stress and cellular damage in selected organism tissues (Guzzetti et al., 2018; Solomando et al., 2020). Indicators of enzymatic response of macromolecules in fish species have indicated alterations and inflammation in liver and intestine due to MP exposure under laboratory conditions (Lu et al., 2016; Pedà et al., 2016; Espinosa et al., 2019). On the other hand, other biomarkers such as Stable Isotope Analyses (SIA) have been analyzed in gilthead seabream demonstrating that they can be good indicators of feeding conditions (Serrano et al., 2007). In this sense, $\delta^{15}N$ values can be used to evaluate nutritional status of fish being a valuable and complementary tool to take into consideration when determining optimal nutritional conditions for farmed species (Martin-Perez et al., 2013). Moreover, the C:N isotopic ratio has been used as an indicator of nutritional status in marine organisms (Box et al., 2010) and given that nitrogen is mostly present as part of proteins, diets with a low C:N ratio contain a greater proportion of protein and are of higher quality. Consequently, stable isotopic signatures in fish soft tissues such as muscles could be investigated as an indicator to assess MP ingestion in fish species.

Given the importance of aquaculture production worldwide, which in 2018 accounted for almost 46% of fish consumption by humans (Zhou et al., 2021), and that plastics are present throughout the material and equipment used in aquaculture facilities (Lyu et al., 2020) as well as in the supplied fish food (Thiele et al., 2021), it is necessary to provide with empirical data on the impacts of a prolonged exposure to MPs in aquacultured-reared fishes. Therefore, this research will assess for the first time, MP ingestion in fish species within a temporal scale of four months and, consequently the main aims are to 1) analyze MP ingestion and associated biological parameters in *Sparus aurata* exposed to MP enriched diets with virgin and weathered LDPE pellets and 2) assess changes in the isotopic signature of *S. aurata* muscle associated to the ingestion of MP enriched diets.

2. Material and methods

2.1. Dietary exposure

In order to study microplastic ingestion in *Sparus aurata* exposed to an enriched LDPE diet, three treatment diets (control and two MP enriched diets; virgin and weathered) were prepared with commercial fish feeds. For the MP weathered treatment, virgin pellets of LPDE were deployed in an anthropogenized harbor within the facilities of the experimental aquaculture installations of LIMIA (*Laboratorio de Investigaciones Marinas y Acuicultura*) in Port d'Andratx (Balearic Islands, Spain) for 2 summer months (July and August 2018). For this, a total of 4.8 kg of LDPE pellets were placed in 24 individual nylon mesh bag, which in turn were placed into 3

mesh bags (0.6 kg per bag). The 3 mesh bags were deployed in a floating aquaculture cage at 0.5–1 m depth from the sea surface in order to sorb chemical compounds from the marine environment (Rochman et al., 2013). After the deployment, pellets were recovered, rinsed in ultrapure water, placed in a glass Petri dish, cleaned with acetone and dried in closed desiccators at room temperature. Afterwards, virgin and weathered pellets were sent to the University of Almeria, Spain, to be ground into smaller particles in preparation for the MP enriched fish diets. Immediately before grinding, pellets were dipped in liquid nitrogen, using a stainless steel mesh, to avoid loss of chemical contamination due to heat. Once grinned, plastics were sieved in pre-cleaned stainless steel metal sieves to collect fragments smaller than 0.5 mm. Pellets (thereafter microplastics, MPs) were ground into particles ranging from 500 to 200 μm (~30% of 500 μm, ~25% of 400 μm, ~25% of 300 μm and ~20% of 200 μm) using separate conical grinders for conventional use. MPs were mixed with standard fish feed ingredients at the University of Almeria. Three types of treatment diets were prepared: control (100% feed and 0% plastic), virgin (90% feed and 10% virginplastic) and weathered (90% feed and 10% of weathered plastic). Ingredients for the feeds consisted of 37 g of fishmeal LT94, 9.2 g wheat gluten, 21.5 g soybean meal, 8.3 g fish oil, 0.9 g soy lecithin, 9.1 g wheat flour, 0.5 g choline chloride, 0.1 g vitamin C, 0.9 g vitamin and mineral mixes, 1.9 g binders (½ guar gum + ½ carboxymethyl), 0.5 g amino acid mixture, mixture (methionine-lysine, 1: 1) and 10 g filler (sepiolite-cellulose-guar gum, 1:1:1) in 100 g dw. Diets containing plastic consisted of the same quantity of ingredients but 10 g of the filler were substituted by 10 g of virgin or weathered LDPE for every 100 g.

In June 2018, 2000 fishes of approximately 3 g each were placed into a large 5000 L tank and fed with 70–85 g of feed per day until October. After 4 months of acclimation at LIMIA facilities, fish increased their weight up to 40 g approximately. At that time 900 gilthead sea bream (7 months old and approximately 38–54 g in weight and 1.70–3.61 cm in length) were randomly placed into 9 tanks of 1000 L (100 fishes per tank and three tanks per treatment). The photoperiod was of 16:8 h cycle (light: dark in hours). The semi-closed system had a water flow-rate of approximately 1 L/minute to ensure 1.5 watercycles per day and the oxygen concentration was 5.9–6.1 ppm. The water which fed the experimental tanks came from the adjacent sea, outside of the aquaculture facilities, and was filtered through a diatom sand filter followed by a 1 μm polypropylene filter before running into the tanks. On average seawater surface temperature from the outside surroundings decreased from 22° to 17 °C from October to February while in our system it decreased from 23° to 17 °C.

The complete experiment lasted for four months and five sampling periods were carried out with one month difference (T0, T30, T60, T90 and T120). During the first three months (exposure period from T0 to T90) fish were fed with the three different types of diets (control, virgin and weathered) within 2% of their biomass per day meaning that fish from the virgin and weathered treatment were exposed to MPs at a concentration of approximately 0.1 g per kg body mass weight^{-1}. This MP concentration makes our study comparable to previous research (e.g. Jovanović et al., 2018; Rochman et al., 2013). During the last month (from T90 to T120), the detoxification period, all fishes were fed with the control feed diet. To assign daily feed supply, these were determined monthly from fish biological parameters (average body weight for 15 fish per tank) assessed during the euthanization process from the sampling procedures. In this sense, portions ranged from 65 to 100 g of feed. Fishes were fed twice a day using an automatic feeder to ensure that all fish in each tank were able to gain access to the food, minimizing the possible effects of feeding hierarchy formation (Jobling, 1994). In addition, the timeinterval between the two meals (7–8 h) allowed for good feed utilization by fish. Tanks were cleaned daily and observed floating MPs were removed using vacuum water siphons with a net of 63 μm to retain MPs.

2.2. Fish sampling

Every month, for each treatment 45 fishes, 15 individuals per tank were sampled after a 24 h period of feed deprivation. Fish sampling lasted for three days and in order to avoid contamination between treatments, fish from the control treatment were sampled first, followed by individuals from the virgin treatment and lastly those from the weathered treatment were sampled. During the sampling procedure, 15 fish from each tank were isolated from the experimental tanks and were placed in an individual tank to be euthanized with MS22-fenoxiethanol anesthetic (1 g MS22-fenoxiethanol in 10 L of water). Fish were weighed, measured and dissected by opening the abdominal cavity. The gastrointestinal tracts were extracted from each individual and placed into a zip lock bag and frozen at −20 °C for posterior MPs analysis at the laboratory. A piece of dorsal muscle (1 × 1 cm) was also extracted, rinsed thoroughly with distilled water and frozen at −20 °C for posterior SIA. Metal dissection material and working surfaces were carefully cleaned with alcohol 96° after the dissection of every fish and disinfected with acetone between tanks. During all the biological survey, nitrile gloves were used. Animal care, maintenance, handling and sampling followed protocols approved by the University of the Balearic Island, Animal Care and Use Committee (Reference CEEA 96/05/18).

2.3. Biological parameters

During each sampling process, for each individual, total length (TL, cm) of fish was measured from the tip of the snout to the tip of the longer lobe of the caudal fin and total fresh weight (in grams) was obtained. The Fulton's condition index (*K*) and the stomach Fullness Index (FI) were also calculated for each fish as:

- Fulton's condition index (*K*) = total weight(g)/(total length (cm)^3) *100
- Stomach Fullness Index (FI) = content weight (g)/eviscerated weight (g) *100

Due to sampling procedures, a mean hepatosomatic index (HSI) was calculated per tank. For this, the liver of three individuals in each tank were weighed and divided by the total weight of fish as following:

- Hepatosomatic index (HSI) = liver weight (g)/total weight (g) *100

2.4. Microplastic analyses

Gastrointestinal tract samples from 217 individuals were defrosted at room temperature and all instruments were cleaned and rinsed with alcohol before every analysis. Samples were transferred to glass Petri dishes and fat was removed to facilitate the posterior digestion and filtration of gastrointestinal tracts. For the chemical digestion of the gastrointestinal tracts, each sample was placed in an Erlenmeyer glass flask and potassium hydroxide (KOH) at 10% was added (20 ml of KOH/per gram of sample) following the procedure in Duflos et al. (2017). Erlenmeyer flasks were covered with aluminium paper to prevent airborne contamination and placed in a laminar flow cabinet. Digestion time was

sample size dependent and lasted between 48 and 96 h at room temperature. Erlenmeyer flasks were gently shaken once or twice a day to facilitate the digestion of organic matter. After this period, solutions were filtered using a glass funnel filtration and polycarbonate filters (20.0 μm, diameter 47 mm, FILTER-LAB). Filters were transferred to Petri dishes and dried at room temperature for 24 h and were placed under the stereomicroscope (Euromex NZ, 1903-S) for visual sorting of MPs with optical enhancement from 6.7x to 40.5x and with an attached CMEX 3.0 MP camera using a calibration software, ImageFocus® 4.0 (Euromex software).

2.4.1. Microplastic ingestion index

For MP ingestion analyses, each filter was placed in a glass Petri dish (to reduce airborne contamination) and was positioned under the stereomicroscope for visual sorting and MPs identification and quantification. Ingestion of MPs was estimated using an abundance-dominance covering index following the Braun-Blanquet scale (Boudouresque, 1971). A referral grid composed by 2.5 × 2.5 mm cells was used, resulting in a total of 175 cells. Each cell was examined separately in order to determine the percentage of MPs coverage (MPs coverage (%)) and was classified into five categories considering the whole cells' inner area covered by MPs as well as the MPs aggregations in each cell. The five categories for the MPs coverage were defined as: C+ for cells where the coverage was next to 1% of the total of the cell; C_1 for coverage lower than 25%; C_2 coverage lower than 50%; C_3 coverage lower than 75%; and C_4 coverage higher than 75% or fully covered (Fig. 1). In addition, three categories of aggregations were applied to those cells where MPs were disposed in three-dimensions: A_1 for cells where MPs reached up to 33% of thickness; A_2 up to 66% and A_3 up to 100%. The MP ingestion index was then calculated using the following equations:

$$MPs\ coverage\ (\%) = \frac{\sum C_+ *1 + \sum C_1 *25 + \sum C_2 *50 + \sum C_3 *75 + \sum C_4 *100}{Total\ cells}$$

$$MPs\ aggregations\ (\%) = \frac{\sum A_1 *33 + \sum A_2 *66 + \sum A_3 *100}{Total\ cells}$$

$$MP\ ingestion\ index\ (\%) = MPs\ coverage + MPs\ aggregations$$

2.5. Stable isotopes analyses (SIA)

For SIA, muscle samples from 135 individual were rinsed with distilled water, dried at 60 °C for 24 h and then ground to a fine powder using a mortar and a pestle. For each sample, 2 ± 0.1 mg of dried and powdered sample was placed into a tin cup and combusted for $\delta^{13}C$ and $\delta^{15}N$ isotopic analysis with a continuous flow mass spectrometer (Thermo Finnegan Delta x-plus). Vienne Pee Dee Belemnite (VPDB) standards for carbon and atmospheric nitrogen for nitrogen were used. In order to calibrate the system and to compensate for drift over time, one sample of an internal reference material, Bovine Liver Standard (BSL) was analyzed every eight samples (BLS; 1577 b; U.S Department of Commerce, National Institute of Standards and Technology, Gaithersburg, MD, 20899, USA). Analytical precision of SIA based on the mean standard deviation of replicates of internal BLS was 0.08‰ for $\delta^{13}C$ and 0.08‰ for $\delta^{15}N$. Stable isotopes ratios were expressed in δ notation with units of parts per thousands (‰) according to the equation, $\delta^{13}C$ or $\delta^{15}N = [(R\ sample/R\ reference) - 1] \times 1000$, where R is the corresponding $^{13}C/^{12}C$ and $^{15}N/^{14}N$ ratio for $\delta^{13}C$ and $\delta^{15}N$ respectively.

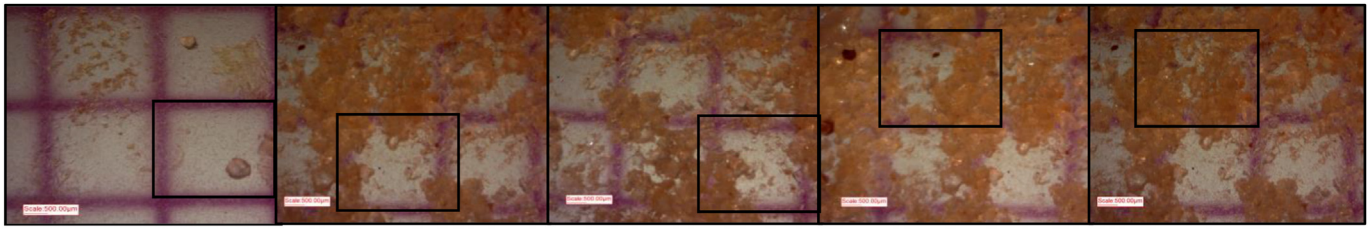
2.6. Statistical analyses

For all variables, normality and homogeneity was assessed by applying the Shapiro-Wilk test and Levene's test respectively. For the biological parameters, differences in size, Fulton's condition index (*K*), stomach Fullness Index (FI) and Hepatosomatic index (HSI) according to the MP ingestion index between treatments (control, virgin and weathered) and sampling period (T0, T30, T60, T90 and T120) were analyzed with a Kruskal-Wallis test with two factors: Treatment and Sampling period.

For MP ingestion analyses, differences in MP ingestion index between treatments (control, virgin and weathered) and sampling period (T0, T30, T60, T90 and T120) were analyzed with a Permutational multivariate analysis of variance (PERMANOVA) of two factors: Treatment as fixed with three levels (control, virgin and weathered) and Sampling period as fixed with five levels (T0, T30, T60, T90 and T120).

For stable isotopes analyses, also a PERMANOVA was applied for each isotopic signature ($\delta^{13}C$ and $\delta^{15}N$) and for the C:N ratio separately to test differences within treatments and sampling period. The PERMANOVA consisted also of two factors: Treatment as fixed with three levels (control, virgin and weathered) and Sampling period as fixed with five levels (T0, T30, T60, T90 and T120).

The correlation between biological parameters (size and Fulton's condition index) and the MP ingestion index, stable isotope signature ($\delta^{13}C$ and $\delta^{15}N$) as well as the C:N ratio was assessed through a Spearman's rank correlation. Significant level was established at $p = 0.05$ level.



A) C_+: -1% of MPs B) C_1: 25% of MPs C) C_2: 50% of MPs D) C_3: 75% of MPs E) C_4: 100% of MPs

Fig. 1. Five categories of MPs coverage were defined for every grid: C_+ for cells where MPs coverage was next to 1% (A); C_1 for MPs coverage lower than 25% of the cells (B); C_2 for MPs coverage lower than 50% of the cells (C); C_3 for MPs coverage lower than 75% of the cells (D); and C_4 for MPs coverage higher than 75% of the cells or all cells covered (E).

All statistical analyses were done with the R Core Team (2019) and Primer V6 and the add-on package PERMANOVA+ (Anderson et al., 2008).

3. Results

3.1. Biological parameters

A total of 352 individuals of *Sparus aurata* were sampled for microplastic and stable isotopes analyses during the four month experiment. Fish size and weight increased with time, from a mean value (±Standard Error (SE)) of 12 ± 0.12 cm and 46.18 ± 1.42 g in T0 to 16.93 ± 0.12 cm and 128.66 ± 2.79 g in T120 (Fig. 2a). Lowest values were given in T0 at the control treatment (11.33 ± 0.17 cm and 38.21 ± 1.73 g) and highest values in T120 at the weathered treatment (17.30 ± 0.16 cm and 135.09 ± 4.18 g) (Fig. 2a). Size of fish increased significantly with time ($p < 0.001$; Kruskal-Wallis; Fig. 2a) and fish from the virgin and weathered treatments were significantly larger than those from the control when considering all sampling periods ($p < 0.001$; Kruskal-Wallis; Fig. 2b).

According to the Fulton's condition index, this value increased from 2.62 ± 0.02 in T0 to 2.79 ± 0.02 in T90, and decreased to 2.62 ± 0.02 in T120, showing no significant differences between T0 and T120 (p > 0.05; Kruskal-Wallis; Fig. 2c). Lowest values in T0

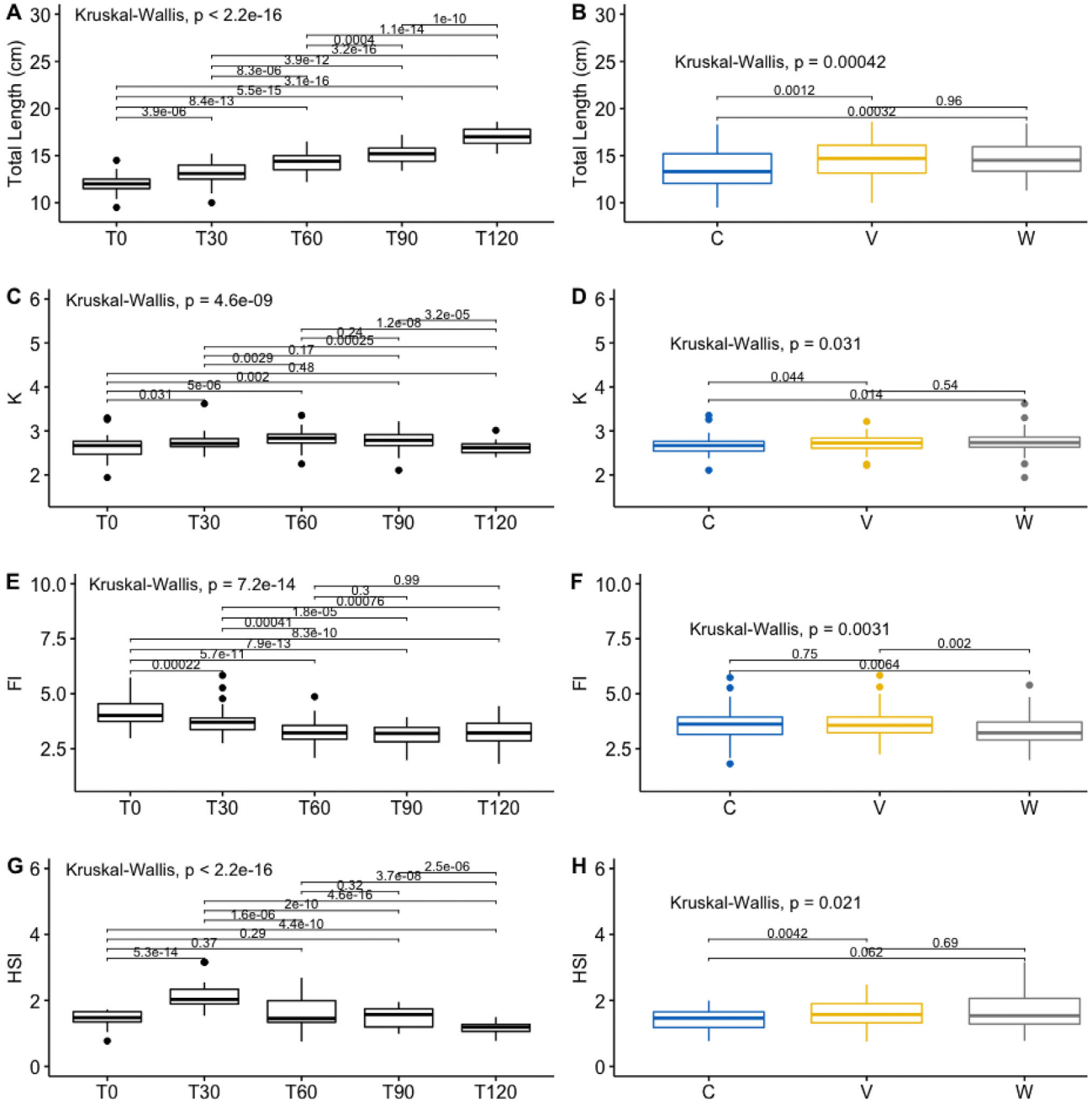


Fig. 2. Differences in biological values for *Sparus aurata* under experimental conditions assessed with the Kruskal-Wallis test (significance level at $p = 0.05$) according to sampling period (T0, T30, T60, T90 and T120) and treatments (Control-C, (blue), Virgin-V, (yellow) and Weathered-W, (grey)) for: fish size total length (A,B), Fulton's Condition Index (*K*) (C,D), Stomach Fullness Index (FI) (G,H) and Hepatosomatic Index (HSI) (E,F). Box plot represent the minimum, maximum, median, first quartile and third quartile of the data set and dots represent outliers. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

were significantly different to T30 ($p < 0.05$; Kruskal-Wallis; Fig. 2c), T60 ($p < 0.001$; Kruskal-Wallis; Fig. 2c) and T90 ($p < 0.01$; Kruskal-Wallis; Fig. 2c). Regarding treatments, a significantly higher Fulton's condition index was given in the virgin and weathered treatments compared to the control ($p < 0.05$; Kruskal-Wallis; Fig. 2d).

The stomach Fullness Index (FI) ranged from 4.14 ± 0.07 (T0) to 3.12 ± 0.06 (T90) and significant differences were observed between treatments in T0 and T30 and the rest of the sampling periods ($p < 0.001$; Kruskal-Wallis; Fig. 2g). Regarding treatments, mean FI values were always significantly lower in the weathered treatment compared to control and virgin treatments ($p < 0.01$; Kruskal-Wallis; Fig. 2h).

According to the Hepatosomatic index (HSI), this index ranged from 2.25 ± 0.10 in T30 to 1.16 ± 0.02 (T120). Highest (T30) and lowest (T120) values were significantly different to all sampling periods ($p < 0.001$; Kruskal-Wallis; Fig. 2e). According to treatments, HSI highest values were given in the virgin treatment and were significantly different to values from the control ($p < 0.01$; Kruskal-Wallis; Fig. 2f).

3.2. Microplastic ingestion index

For microplastic ingestion analyses, gastrointestinal tracts from 217 individuals of *Sparus aurata* were analyzed. After applying the Shapiro-Wilk test, the MP ingestion index was observed to follow a non-normal distribution.

As expected, mean MP ingestion index values were 0% for T0 and increased with time from 7.00 ± 1.85% (T30) to 32.14 ± 4.62% (T90) and after one month of detoxification, without exposure to MPs, mean MP ingestion index values were 0% again (Fig. 3a). The interaction of treatment and sampling period was statistically significant as well as differences according to treatment and sampling period when treated separately ($p < 0.01$; PERMANOVA; Table 1). Regarding MP ingestion within treatments and sampling period, highest mean values were observed in the weathered treatment (14.16 ± 2.88%) and in T90 (Fig. 3a). No significant differences were given between treatments for T0 and T120 ($p > 0.05$; PERMANOVA; Fig. 3a) due to plastic ingestion being 0%. However, for T30, T60 and T90, the control was significant differences to the weathered and virgin treatments ($p < 0.001$; PERMANOVA; Fig. 3a). No significant differences were found between weathered and virgin treatments throughout all sampling periods ($p > 0.05$; PERMANOVA; Fig. 3a).

The MP ingestion index was significantly and positively related to fish size for the control and virgin treatments ($p < 0.001$ Spearman's rank correlation; Fig. 3b). In addition, the MP ingestion index was also positively and significant correlated with the Fulton's condition index in the weathered ($R = 0.29$, $p < 0.01$, Spearman's rank correlation; Fig. 3c) and virgin treatments ($R = 0.25$, $p < 0.05$ Spearman's rank correlation; Fig. 3c).

3.3. Stable isotopes

For the Stable Isotope Analyses (SIA), the dorsal muscle of 135 individuals of *Sparus aurata* were sampled. Stable isotope signatures ($\delta^{13}C$, $\delta^{15}N$ and C:N) were found to follow a non-normal distribution after applying the Shapiro-Wilk test and showed a low variability within the four months of experiments.

Carbon isotopes ranged from −20.11 ± 0.10‰ (T0) to −20.99 ± 0.09‰ (T120) and significant differences were observed between the interaction treatment and sampling period ($p < 0.01$; PERMANOVA; Table 1) as well as treatment and sampling period independently ($p < 0.05$; PERMANOVA; Table 1). Lowest mean values were given in the weathered treatment (−20.64 ± 0.19‰) and significant differences were observed between values in this treatment and the control (−19.93 ± 0.10‰)

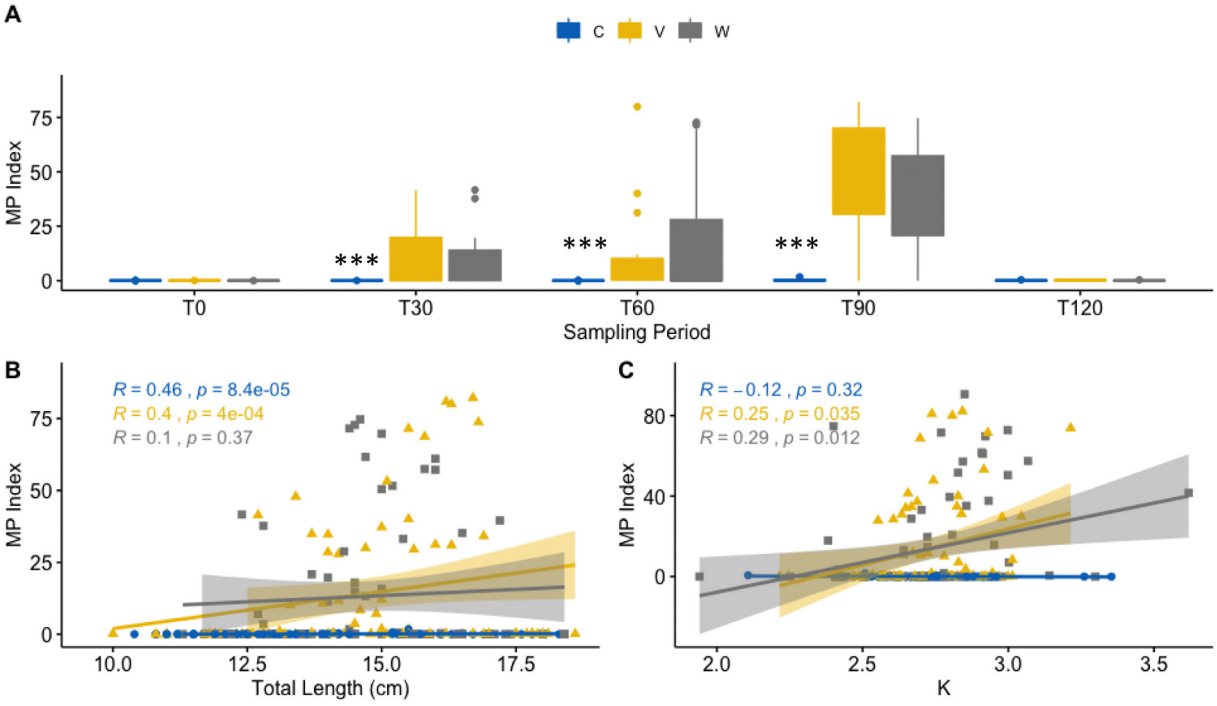


Fig. 3. Microplastic (MP) ingestion index for samples of *Sparus aurata* according to: Sampling period (T0, T30, T60, T90 and T120) and Treatments (Control-C, (blue), Virgin-M, (yellow) and Weathered-W, (grey)) (A) as well as the Spearman's rank correlation (significance level at $p = 0.05$) between the MP ingestion index and size of fish (total length) (B) and Fulton's Condition Index (k) (C). Significant differences assessed according the Permutational Multivariate Analyses of Variances (PERMANOVA), with a significance value established at $p = 0.05$; ***$p < 0.001$. Box plot represent the minimum, maximum, median, first quartile and third quartile of the data set and dots represent outliers. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

Table 1
Results of the PERMANOVA analysis applied for each isotopic signature ($\delta^{13}C$ and $\delta^{15}N$) and C:N ratio in muscle of *Sparus aurata* to test differences within sampling period and treatments. Df degrees of freedom, MS mean sum of squares, Pseudo-F value by permutation, asterisk indicates statistical significance $p < 0.05$ (*) and $p < 0.001$ (**); n.s = no significant differences. *P* values based on 999 permutations.

	MP ingestion index			$\delta^{13}C$ and			$\delta^{15}N$			C:N		
Source of variation	df	MS	Pseudo-F	df	MS	Pseudo-F	df	MS	Pseudo-F	df	MS	Pseudo-F
Sampling period	4	35.10	35.56**	4	3.36	31.94**	4	0.49	22.33**	4	0.03	0.39
Treatment	2	36.79	37.28**	2	0.88	8.34*	2	0.30	13.69**	2	0.20	7.33*
Sampling period x Treatment	8	8.20	8.30**	8	0.60	5.71**	8	0.07	3.35**	8	0.16	5.97**
Residual	202	0.10		119	0.10		119	0.02		119	0.07	
Total	216			133			133			133		

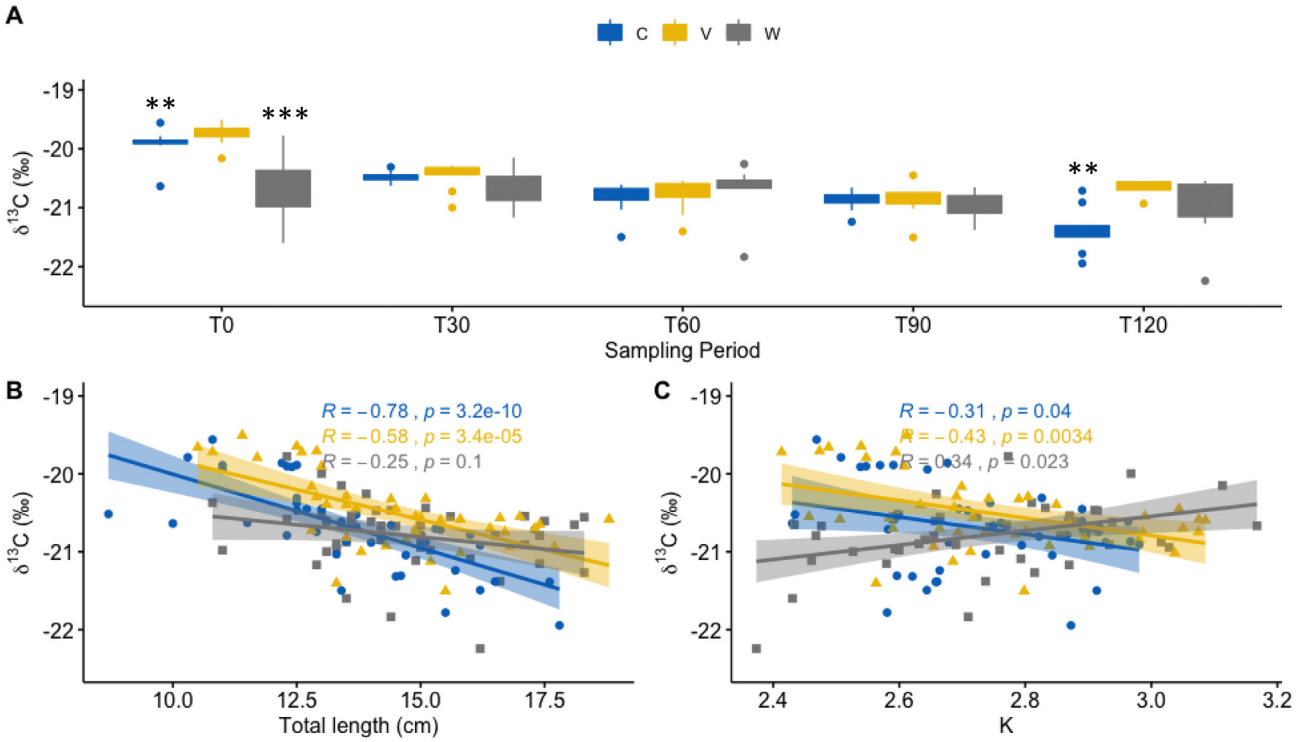


Fig. 4. Carbon isotopic signatures ($\delta^{13}C$) for samples of soft tissue of *Sparus aurata* according to: Sampling period (T0, T30, T60, T90 and T120) and Treatments (Control −C, (blue), Virgin-V, (yellow) and Weathered-W, (grey)) (A) as well as the Spearman's rank correlation (significance level at $p = 0.05$) between carbon isotopic signatures and size of fish (total length) (B) and Fulton's Condition Index (*K*) (C). Significant differences assessed according the Permutational Multivariate Analyses of Variances (PERMANOVA), with a significance value established at $p = 0.05$; *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$. Box plot represent the minimum, maximum, median, first quartile and third quartile of the data set and dots represent outliers. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

and virgin treatments ($p < 0.001$; PERMANOVA). For T30, T60 and T90, no significant differences were given between carbon isotopic values amongst the three treatments ($p > 0.05$; PERMANOVA; Fig. 4a). Carbon isotopic values were lowest in T120 compared to the rest of the sampling periods, and mean values were significantly higher in the virgin treatment (−20.65 ± 0.04‰) compared to the control treatment (−21.36 ± 0.13‰) ($p < 0.05$; PERMANOVA; Fig. 4a).

Carbon isotopic values decreased with fish size length in all treatments, however this decrease was only significant in the control ($R = -0.78$, $p < 0.001$ Spearman's rank correlation; Fig. 4b) and in the virgin treatments ($R = -0.58$, $p < 0.001$ Spearman's rank correlation; Fig. 4b). In the weathered treatment, carbon isotopic signatures were positively and significantly correlated to the Fulton's condition index ($R = 0.34$, $p < 0.05$ Spearman's rank correlation; Fig. 4c). However, for control and virgin treatments, carbon isotopic signatures significantly decrease with the Fulton's condition index ($R = -0.31$, $p < 0.05$ Spearman's rank correlation, and $R = -0.43$, $p < 0.01$ Spearman's rank; Fig. 4c).

Regarding nitrogen isotope values, as well as observed for carbon isotope values, significant differences were given at the interaction between treatment and sampling period as well as differences according to treatment and sampling period independently ($p < 0.01$; PERMANOVA; Table 1). Nitrogen values ranged from 10.12 ± 0.04‰ (T0) to 9.84 ± 0.03‰ and 9.84 ± 0.02‰ (T60 and T90, respectively). Values for period T0 were higher compared to the other sampling periods and values from the weathered treatment were significantly lower (9.92 ± 0.06‰) than values in the virgin and control treatments ($p < 0.01$; PERMANOVA). For T30 and T60, nitrogen isotopic values in the control were significantly higher than in the virgin and weathered treatments ($p < 0.01$; PERMANOVA; Fig. 5a). However, in T90 no significant differences were observed in nitrogen values from the three treatments ($p > 0.05$; PERMANOVA; Fig. 5a). After the detoxification period, nitrogen values were significantly lower again in the weathered treatment ($p < 0.05$; PERMANOVA; Fig. 6a) compared to both the

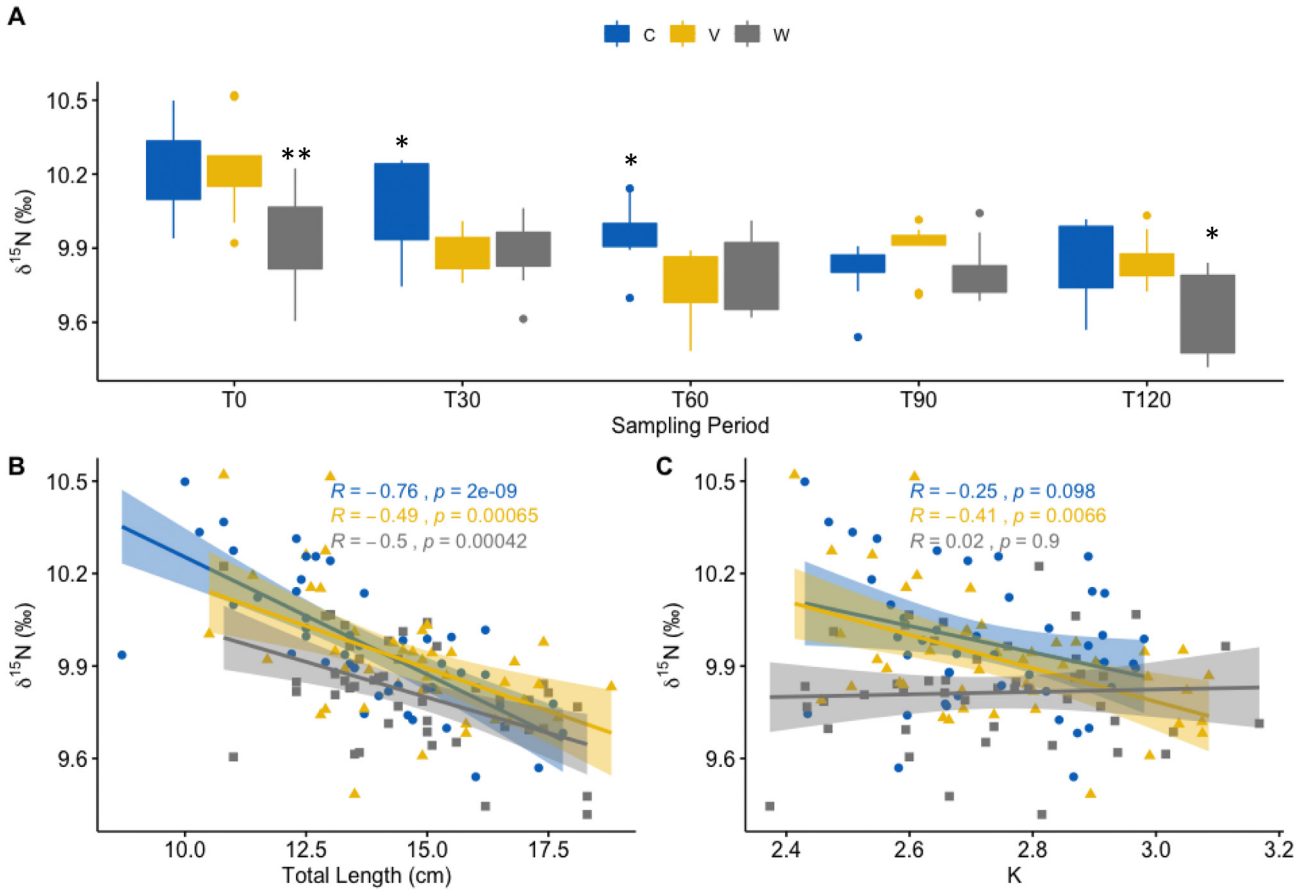


Fig. 5. Nitrogen isotopic signatures ($\delta^{15}N$) for samples of soft tissue of *Sparus aurata* according to: Sampling period (T0, T30, T60, T90 and T120) and Treatments (Control-C, (blue), Virgin-V, (yellow) and Weathered −W, (grey)) as well as the Spearman's rank correlation (significance level at $p = 0.05$) between nitrogen isotopic values and size of fish (total length) (B) and Fulton's Condition Index (k) (C). Significant differences assessed according the Permutational Multivariate Analyses of Variances (PERMANOVA), with a significance value established at p = 0.05; *$p < 0.05$; **$p < 0.01$. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

virgin and the control treatments.

Nitrogen isotopic signatures significantly decrease with fish size (R = − 0.76 (control); R = −0.5 (weathered); R = −0.49 (virgin), $p < 0.001$ Spearman's rank; Fig. 5b) and with the Fulton's condition index, but this decrease was only significant in the virgin (R = −0.41, $p < 0.01$ Spearman's rank; Fig. 5c).

The C:N value was also constant with values ranging from 2.09 ± 0.05‰ (T0) to 2.02 ± 0.02‰ (T30) and 2.02 ± 0.03 (T60) and significant differences were given regarding the interaction between treatment and sampling period ($p < 0.01$; PERMANOVA; Table 1) and treatment alone ($p < 0.05$; PERMANOVA; Table 1) but not for sampling period itself ($p > 0.05$; PERMANOVA; Table 1). According to sampling period, no significant differences were given throughout the experiment ($p > 0.05$; PERMANOVA), however the interaction between treatment and sampling period was significant ($p < 0.001$; PERMANOVA). According to sampling periods, in T0, C:N values in the weathered treatment were significantly higher to the virgin and control ($p < 0.05$; PERMANOVA; Fig. 6a). For T30, C:N values between the weathered and control treatments were significantly different ($p < 0.05$; PERMANOVA; Fig. 6a) but for T60 and T90, no significant differences were given between treatments ($p > 0.05$; PERMANOVA; Fig. 6a). At the end of the detoxification period, significant differences were given between mean values in the virgin (1.93 ± 0.02‰) and control (2.25 ± 0.06‰) treatments ($p < 0.001$; PERMANOVA; Fig. 6a) driven by the enrichment of carbon isotopic signatures in the virgin treatment at T120. The C:N ratio significantly decreased with fish size in the weathered treatment (R = −0.40, $p < 0.01$ Spearman's rank; Fig. 6b). On the contrary, in the virgin and control treatments, C:N ratio increased with fish size but this increase was only significant in the control (R = 0.31, $p < 0.05$ Spearman's rank; Fig. 6b). The C:N ratio decreased with the Fulton's condition index in the weathered treatment but this decrease was not significant (R = −0.27, $p > 0.05$ Spearman's rank; Fig. 6c). On the other hand, the C:N ratio increased with the Fulton's condition index in the virgin and control treatments, but it was only significant for the virgin treatment (R = 0.35, $p < 0.05$ Spearman's rank; Fig. 6c).

4. Discussion

Hitherto most of the studies have assessed short-term experimental responses from hours (Espinosa et al., 2018; Campos et al., 2021) to weeks (Jovanović et al., 2018; Batel et al., 2020). Therefore, this is the first study assessing MP ingestion in a fish species within a temporal scale of four months. Experimental results show that MP ingestion in *Sparus aurata* increases with time and ingestion values are highest after three months of exposure to MP enriched feed. Moreover, after one month of detoxification no MPs were observed in the gastrointestinal tracts of fish, reflecting no long-term retention of potential MPs in *Sparus aurata* as Jovanović et al. (2018) suggested.

In this study, microplastics were present in 38.71% of the gastrointestinal tracts of all analyzed fish which had been deprived from food for 24 h. Previous laboratory studies have reported rapid

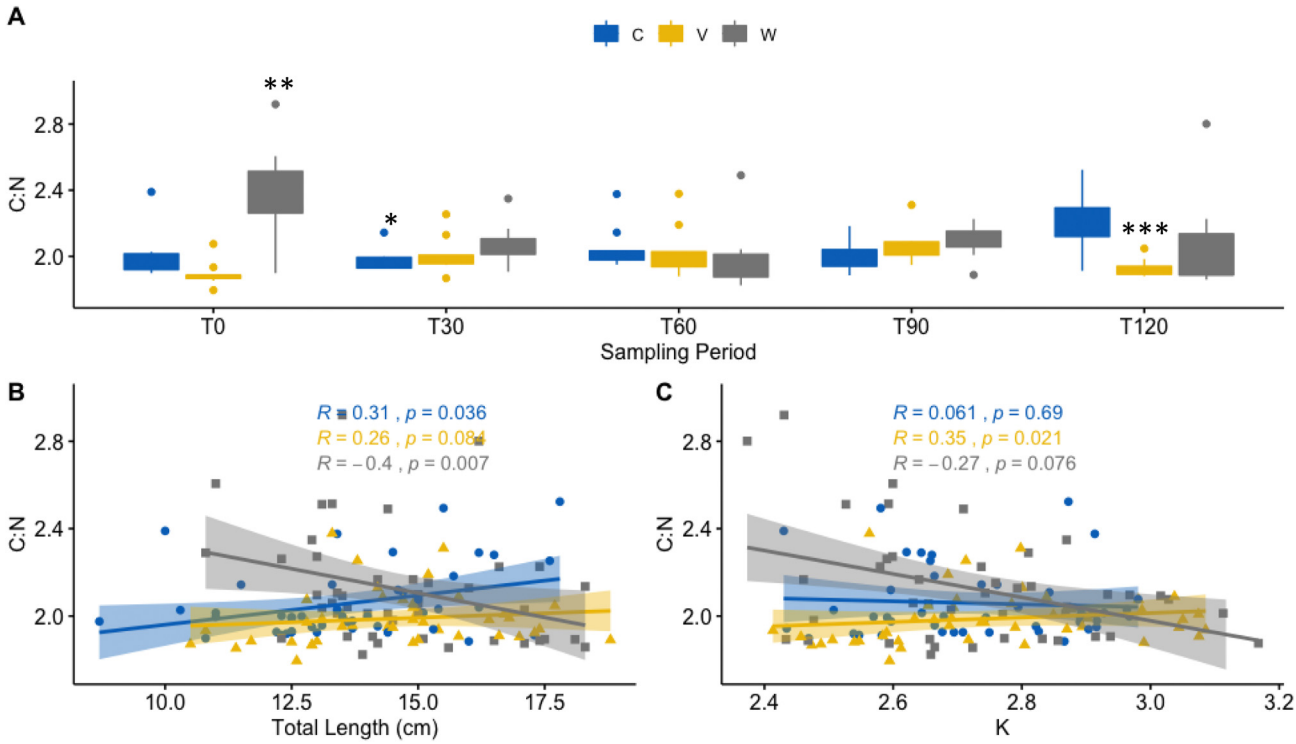


Fig. 6. C:N ratio for samples of soft tissue of *Sparus aurata* according to: Sampling period (T0, T30, T60, T90 and T120) and Treatments (Control-C, (blue), Virgin-V, (yellow) and Weathered-W, (grey)) as well as the Spearman's rank correlation (significance level at $p = 0.05$) between the C:N ratio and size of fish (total length) (B) and Fulton's Condition Index (K) (C). Significant differences assessed according the Permutational Multivariate Analyses of Variances (PERMANOVA), with a significance value established at $p = 0.05$; *$p < 0.05$; **$p < 0.01$; ***p < 0.001. Box plot represent the minimum, maximum, median, first quartile and third quartile of the data set and dots represent outliers. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

(Naidoo and Glassom, 2019) and complete egestion of microplastics in 24–48 h (Grigorakis et al., 2017). Due to high egestion times, MPs and their associated contaminants might not be retained in the gut for a long enough period for contaminants to transfer to the wall of guts and internal tissues and organs (Burns and Boxall, 2018; Naidoo and Glassom, 2019). However, a direct, constant and prolonged exposure to MPs (such as the one in this experiment) can cause an accumulative effect with time and a progressive increase in antioxidant enzymatic activities and glutathione S-transferase as well as an increase in the levels of reduced glutathione in MP enriched fed *Sparus aurata* (Solomando et al., 2020; Rios-Fuster et al., 2021). Additionally, an increase of the activity of the pro-inflammatory enzyme, myeloperoxidase, and the levels of oxidative damage markers -malondialdehyde and protein carbonyls-has also been detected with highest values after 90 days of MP exposure (Solomando et al., 2020), which coincides with the highest MP ingestion index values in our study.

According to our results, exposure of fish to MP enriched diets does not seem to affect fish size: fish size increased with time in all treatments and Fulton's condition index also increased during the feeding phase and decreased after the detoxification period to the same values as at the beginning of the experiment. This slight decrease of Fulton's condition index during the detoxification period was observed in all tanks and could be attributed to environmental conditions due to temperature of water being lower during the last months of the experiment (December–January) and affecting fish condition index. These results could corroborate that fish are not redirecting energy invested to MP depuration (Welden and Cowie, 2016) but are responding to changes in water temperature of the tank.

Contrary to our findings, the Korean rockfish (*Sebastes schlegelii* Hilgendorf, 1880) decreased its specific growth rate by 65.9% after exposure to 10^6 particles/L of polyester microplastics (Yin et al., 2018). Differences in the physiological response of fish to MP exposure could be related to feeding behavior of the study species (Markic et al., 2019) but also to MP exposure pathway as higher effects have been seen in fish which are exposed directly to microplastics in water than through diets (Wesch et al., 2016). Concentrations of MPs to which species are exposed can also determine the effects that MP ingestion has upon species, with higher concentrations of MPs being more likely to trigger effects (Liu et al., 2020). For example, a negative effect in medaka freshwater fish was observed in species exposed to 0.65 mg-MPs/L but not to lower concentrations (Chisada et al., 2021). In the present experiment, fish were exposed to MPs at a daily concentration of ~0.1 g per kg body mass weight^{-1} and a recent study suggested no imminent harm to adult gilthead seabream after being exposed 45 days (half of the time period of this experiment) to virgin enriched MP diet at the same concentration per body mass (Jovanović et al., 2018). In this study it is remarkable that fish are ingesting the supplied feed throughout the exposure period of the study, which emulates natural conditions of fish exposure at sea being in direct contact with plastic particles, suggesting that during the three months time-lag of the experiment there is no induced fish mortality due to MP ingestion in the applied MP concentrations. However, MPs at environment relevant concentrations do not always cause adverse effects (Liu et al., 2020). In this sense, independently of exposure route and MP size, no effects were observed in the zebrafish when exposed to environmental relevant MPs concentrations: MPs were ingested, transported through the intestinal and egested without adverse effects (Batel et al., 2020). Moreover, during the experimental phase of our study only one fish

died and no external injuries were observed during the sampling procedure. Previous experimental studies have also shown no affectation to hatching, growth and survival rates in the first developments stages of the sea trout (*Salmo trutta*) exposed to MP particles of PS, PET and PE (Jakubowska et al., 2020). These findings in juvenile individuals of fish species such as *Sparus aurata* are important given that at their early stages, fish are more vulnerable to perturbations which can affect their survival (Lima et al., 2016).

Regarding organic responses of *S. aurata* quantified in the fish hepatosomatic index, individuals from this study exhibited a decreasing pattern. In this sense, fish hepatosomatic index decreased in time during the exposure to MPs, and after the detoxification period ulterior the index decreased and did not recover to initial levels. A recent study has detected particles trapped in the liver of *Sparus aurata* (Jovanović et al., 2018) and consequently functioning of this organ could be thought to be affected by MP ingestion given that glycogen depletion, fatty vacuolation and single cell necrosis has already been observed in this tissue (Rochman et al., 2013). Moreover, this could lead to cellular damage or oxidative stress in fish liver which has already been observed in experimental studies with *Sparus aurata* (Rios-Fuster et al., 2020v; Capo et al., 2021) and in wild fish ingesting MPs (Alomar et al., 2017).

Given that $\delta^{13}C$ and $\delta^{15}N$ stable isotope signatures in species' muscles can give information on species food sources (DeNiro and Epstein, 1978; Vander Zanden and Rasmussen, 2001; Serrano et al., 2007) and nutritional assessment (Martin-Perez et al., 2013), it could be thought that plastic enriched feeds could have an effect on the isotopic signature of consumer tissues. Carbon and nitrogen stable isotopes have been analyzed in petroleum and plant-derived polymers indicating that $\delta^{13}C$ could be a possible tool for plastic polymer identification in the marine environment (Berto et al., 2017) but isotopic signatures in fish muscle from this study do not reflect MP ingestion values. During the enrichment phase of the experiment (T0, T30, T60 and T90) no significant differences were observed amongst carbon isotopic values of the three treatments which would suggest that signatures in muscle tissue are not indicating differences due to MP intake in the studied species as well as no change in fish's fat content through this phase (Martin-Perez et al., 2013). In light of these results, it could be thought that other tissues, such as liver with a higher metabolic activity, could be more useful short-term dietary indicators of MP ingestion (Schmidt et al., 2016). According to nitrogen isotopic signatures, values of fish from the weathered treatment were significantly lower than those from the virgin and control treatments which could be related to depletion in the food sources as it is known that consumers' isotopic signatures are strongly dependent on food quality (Hill and McQuaid, 2009). Moreover, both carbon and nitrogen isotopic signatures decreased with fish size in all treatments and even though this decrease was only significant in the virgin treatment it is rather surprising given that nitrogen and carbon isotopic values increase with fish size (Deudero et al., 2004). However, this decrease could be related to the increase of nitrogen deposition efficiency in muscle with a high protein assimilation (Martin-Perez et al., 2013) during the first live months of *Sparus aurata*. Regarding the C:N value, with exposure time, values of this ratio in fish muscle gained similarity (T60 and T90) probably reflecting similar nutritional status (Box et al., 2010). The fact that C:N ratio significantly decreased with fish size and with the Fulton's condition index only in fish from the weathered treatment, could be indicating changes in diet quality possibly linked to the ingestion of biofouling organisms adsorbed to pellets surface during the weathering of plastic polymers in the harbor area as previously documented for certain species of anchovies, copepods, corals and sea urchins (Vroom et al., 2017; Porter et al., 2019; Wright et al., 2020; Corona et al., 2020).

Translocation of MPs to tissues in organisms' body depends on particle size and this seems unlikely to occur for particles >100 μm (Asmonaite et al., 2018). In this experiment MP particles ranged in size from 500 to 200 μm making it difficult for translocation to take place from the gastrointestinal tract to other internal tissues (Elizalde-Velazquez et al., 2020). However, given that MPs can sorb contaminants from the marine environment (Rochman et al., 2013) and that MPs used in the weathered treatment had been exposed to anthropogenic pollutants from a harbor area, biochemical effects due to sorbed contaminants in target organs, such as liver, of individuals from the weathered treatment are expected (Guzzetti et al., 2018). Indeed, a recent study conducted under the same experimental conditions (Capò et al., 2021) documented a more significant antioxidant response of biomarkers (CAT, GRs, and GST) in liver of *S. aurata* exposed to weathered MPs than virgin MPs.

Studies on protein modulation after MP ingestion have indicated that the main pathways affected are related to energy metabolism, stress-related defense and cytoskeletal dynamic (Liu et al., 2020) but in our study no effect on fish size nor isotopic signatures were observed due to MP exposure and ingestion in *S. aurata.* Nevertheless, to have an integrated scope of the effects of MPs on fish physiology, as many authors have already suggested, experimental research under laboratory conditions should also consider different types of polymers and shape of particles (including fibers) simulating similar conditions to the those found out at sea (Wang et al., 2020) as well as assessing different target organs.

5. Conclusion

For the first time, microplastic ingestion of LPDE has been assessed in *Sparus aurata* under controlled diets for a temporal scale of three months followed by a detoxification period of one month. Results indicate that MP ingestion increased with exposure time, peaking after three months of exposure to MPs, although increased MP ingestion did not result in fish biological effects since neither fish size, Fulton's condition index nor stable isotopic composition in muscle tissues reflected this ingestion. After one month of no exposure diet to MPs, no long-term retention of MPs in gastrointestinal tracts of *S. aurata* was encountered. Future research investigating the effects of MP ingestion in commercial and ecological important species should attempt to target different organs at a monthly temporal scale including a wide spectrum of plastic polymers, shapes and concentrations reflecting environmental sea conditions.

Author statement

Carme Alomar, Conceptualization, Methodology, Investigation, Formal analysis, Writing, Funding acquisition. Marina Sanz-Martín, Methodology, Formal analysis, Writing, Montse Compa, Conceptualization, Methodology, Investigation, Formal analysis, Writing, Funding acquisition. Beatríz Rios-Fuster, Methodology, Formal analysis, Writing, Elvira Álvarez, Conceptualization, Methodology, Investigation, Vincent Ripolles, Methodology, José María Valencia, Conceptualization, Methodology, Investigation. Salud Deudero, Conceptualization, Methodology, Investigation, Formal analysis, Writing, Project administration, Funding acquisition.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have

appeared to influence the work reported in this paper.

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