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Licenciado em Engenharia do Ambiente

**Histopathological evaluation of two  
*Blennius* fishes exposed to microplastics  
via feeding**

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## **Abstract**

The microplastic particles have dramatically increased in marine environments, emerging the concern of their potential adverse effects on marine biota, among others. These biological adverse effects of microplastics may result not only in physical harm such as internal abrasions and blockages, but also in an entrance vector of contaminants into marine organism. Therefore, the overall aim of this study was to assess the health status of two gobies (*Blennius pholis* and *Blennius galerita*) exposed to microplastics, both coated and uncoated with antifouling paints (*i.e.* common source of metals), via feeding for a month. For that purpose, multi-organ histopathological assessment (*i.e.* gills, liver, kidney and digestive tract) was carried out qualitatively and semi-quantitatively in gobies, as well as histochemical evaluation. The results showed no sign of microplastics ingestion, suggesting that these gobies were not able to ingest used microplastic spheres. Accordingly, no gross histological alterations were recorded in both fish species exposed to microplastics. Similarly, regardless the pathway of contaminants exposure (*i.e.* by ingestion or waterborne), animals exposed to contaminated microplastics presented similar histopathological levels than those treated with uncontaminated microplastics, or even than control. The affection degree of each target organ revealed gills and liver as the most affected organs following by kidney, digestive tract and spleen. Lamellar lifting, fat vacuolation of hepatocytes and melanomacrophage centers were the most prevalent alterations noticed in gills, liver and whole-body of fish, respectively. These findings may suggest that other factors, even natural occurrence, could be the cause of these mild histopathological alterations. Moreover, the use of *Blennius* gobies in multi-organ histopathology showed to be a suitable organism and tool for assessing the potential adverse effects caused by microplastics and their potential role as contaminants entrance. Therefore, smaller microplastic particles, both contaminated and uncontaminated, should be tested in gobies in order to clarify and evaluate their potential adverse effects via feeding.

**Keywords:** microplastic, gobies, histopathology, ingestion, contaminant release, bioavailability, adverse biological effects.



## Resumo

Os microplásticos têm aumentado drasticamente no meio marinho, ampliando a preocupação sobre os potenciais efeitos adversos na biodiversidade. Estes poderão ser causadores de danos físicos, como abrasões, bloqueios internos, ou atuar como vetores de entrada de contaminantes nos organismos marinhos. Por conseguinte, o objetivo geral deste trabalho foi avaliar o estado de saúde de dois cabozes (*Blennius pholis* e *Blennius galerita*) expostos a microplásticos com e sem revestimento de pintura anti-incrustante (*i.e.* fonte comum de metais no meio marinho) através da alimentação, durante um mês. Com esse propósito, foi feita, em cabozes, a avaliação qualitativa e semi-quantitativa histopatológica de vários órgãos (*i.e.* brânquias, fígado, rim e trato digestivo). Os resultados, não mostraram sinais de ingestão de microplásticos, sugerindo os cabozes (*B. pholis* e *B. galerita*) como incapazes de ingerir microplásticos daquele tamanho. Consequentemente, não foram observadas quaisquer alterações significativas em ambas as espécies de peixes ao longo do ensaio laboratorial. Da mesma forma, independente emente da exposição aos contaminantes (*i.e.* por ingestão ou por via aquosa), os animais expostos a microplásticos contaminados apresentaram níveis histopatológicos similares aos expostos apenas a microplásticos ou mesmo aos do controlo. O grau de afetação de cada órgão, revelou brânquias e fígado como os órgãos mais afetados seguindo-se rim, trato digestivo e baço. Deslocamento epitelial da lamela secundária, vacuolização dos hepatócitos e centros de melanomacrófagos, foram as alterações mais prevalentes observadas nas brânquias, fígado e em todo o corpo do peixe, respetivamente. Em conclusão, as alterações histopatológicas registadas sugerem que a causa poderá ter sido resultado de outro fator ou de alterações naturais. No entanto, o uso de cabozes (*Blennius*) em histopatologia (avaliação de vários órgãos) revelou ser um método adequado para avaliar os potenciais efeitos adversos causados pelos microplásticos e seu papel como potencial entrada de contaminantes. Por esse motivo microplásticos de menores dimensões devem ser testados nos cabozes, a fim de esclarecer e avaliar os seus potenciais efeitos adversos através da alimentação.

**Palavras-chave:** microplásticos, cabozes, histopatologia, ingestão, libertação de contaminantes, biodisponibilidade, efeitos biológicos adversos.



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## **Abbreviation list**

<b>AF</b>	Antifouling paint
<b>Cu</b>	Copper
<b>Digestive tract HI</b>	Histopathological index of digestive tract
<b>DPX</b>	Mounting medium fast (toluene base)
<b>FCUL</b>	Faculty of Science at the University of Lisbon
<b>Fe</b>	Iron
<b>Gills HI</b>	Histopathological index of gills
<b>Hg</b>	Mercury
<b>Kidney HI</b>	Histopathological index of kidney
<b>KW</b>	Kruskal Wallis – Multiple Comparisons
<b>Liver HI</b>	Histopathological index of liver
<b>MSFD</b>	Marine Strategy Framework Directive
<b>PS</b>	Polystyrene
<b>PVC</b>	Polyvinyl chloride
<b>w/w</b>	Weight/weight
<b>Zn</b>	Zinc



## 1. Introduction

In modern society, plastic has achieved a pivotal status, with extensive commercial and industrial applications. In fact, the plastic has made a real contribution to meet targets for resource efficiency in many areas, including zero energy buildings, water saving, sustainable land use, extended shelf-life for products, diversified raw materials, greener mobility and renewable energies (PlasticsEurope, 2012). Hence, plastic production has dramatically increased from 1.5 million tonnes in the 1950 to approximately 322 million tonnes in 2015 (PlasticsEurope, 2016). This increasing plastic usage is provoking the uncontrollable accumulation of plastic wastes mainly in the terrestrial environment through accidental release and indiscriminate plastic discards (Barnes *et al.*, 2009). Nevertheless, these plastic wastes also reach the marine coast by, wind and river-driven transport, becoming a prevalent widespread element of marine litter due to its lightweight durable nature (Moore, 2008; Thompson *et al.*, 2009).

The first reports of plastic debris in the oceans were performed in early 1970s (Carpenter *et al.*, 1972). From the following decades to the present, owing to the deepening knowledge on the marine ecological consequences of plastic wastes, the environmental problem of plastic debris accumulation has become of great concern both within scientific community and society ( *e.g.* Cole *et al.*, 2011; Fowler, 1987). In fact, a recent study finds that minute fragments of plastic debris occur in aquatic environments worldwide, even in Antarctica (Barnes *et al.*, 2009; Zarfl and Matthies, 2010). Likewise, as human population continues to increase, it is believed that the production of plastics and their wastes will also probably enhance, which may turn this social dilemma in a long-term environmental problem (Browne *et al.*, 2011; Wright *et al.*, 2013).

In this context, there has recently been a remarkable emerging concern about inconspicuous microscopic plastic debris, namely microplastics, which are defined as any plastic particle smaller than 5 mm in size. In general, microplastics can be of primary (*i.e.* purposefully manufactured to be of microscopic size) or secondary (*i.e.* derived from the fragmentation of macroplastic items) origin. Since microplastics present the same size fraction as sediments and some planktonic organisms, they are potentially bioavailable to a wide range of marine organisms. Thus, microplastics can be ingested and bioaccumulated by low trophic suspension, filter and deposit feeders, detritivores and planktivores (Browne *et al.*, 2008; Graham and

Thompson, 2009; Murray and Cowie, 2011). Considering the high relevance of this environmental problem, more knowledge on microplastics inputs, spatial and temporal distributions, transport dynamics, interactions with biota and adverse potential threat to marine biota and ecosystems is required. The assessment of microplastics in the marine environment, in fact, has recently been recognized by its inclusion as a priority descriptor in the Marine Strategy Framework Directive (MSFD, 2008/56/EC) as Descriptor 10 “Marine Litter”. More specifically, this descriptor demands more information related to types, sizes and composition of marine litter. By this way, this ecological data may help in the decision-making of scientific community, managers, policy makers and stakeholders (Boerger *et al.*, 2010; De Stephanis *et al.*, 2013; Graham and Thompson, 2009; Lazar and Gračan, 2011; Murray and Cowie, 2011). Nevertheless, a few scientific researches have been focused on the environmental problem caused by microplastics, up till now, especially with regard to potential adverse effects in marine organisms (Cole *et al.*, 2011).

The microplastics bioaccumulation may result not only in physical harm such as internal abrasions and blockages, but also in a potential vehicle for the introduction of anthropogenic toxics by desorption processes (Andrady, 2011; Brennecke *et al.*, 2016; Fossi *et al.*, 2012, 2014; Koelmans *et al.*, 2013; Wright *et al.*, 2013). In fact, the capacity of microplastics to increase bioavailability of contaminants from marine environment has already stated (Brennecke *et al.*, 2016). Among others, metal pollution is categorized of great concern, since it is originated from multiple and common sources such as industrial wastes, fuel combustion and antifouling paints (Deheyn and Latz, 2006). The antifouling paints, in particular, are one of the major sources of heavy metals into the marine environment, through paint deterioration, metal release and subsequent dispersion (Berto *et al.*, 2012; Tuner, 2010). The most modern marine antifouling paints contain a copper based biocidal pigment, which are applied to ship hulls and several other fixed marine structures (*e.g.* pilings, pontoons and buoys) to prevent the growth of fouling organisms (Almeida *et al.*, 2007; Clode *et al.*, 2011; Kiil *et al.*, 2001; Valkirs *et al.*, 2003). The major environmental concern about the use of antifouling paints is currently related to the passive leaching of Cu, among others, into waters (Katranitsas *et al.*, 2003; Warnken *et al.*, 2004). Likewise, particles from antifouling paints may also be released into aquatic systems, which are mainly generated during repair, cleaning and painting procedures of vessel hulls (Parks *et al.*, 2010).

One of the advantages of using biological effect techniques is that they indicate links between contaminant exposure and ecological endpoints (Au, 2004; Stentiford *et al.*, 2003). Biomarkers are measurements of body fluids, cells or tissues at cellular, biochemical and molecular levels that indicate the presence of pollutants (exposure biomarkers) or the magnitude of the organism response (effects biomarkers) (NRC, 1987). Histopathology, in particular, is defined as the study of diseases and dysfunction of natural biological processes at tissue and cell levels (Chapman and Hollert, 2006) and thus measuring histopathological alterations in living organisms has been considered one of the most important approaches to assess pollution-driven adverse effects to organisms both in biomonitoring and laboratory studies (Costa *et al.*, 2013; Schultz *et al.*, 2013; Stentiford *et al.*, 2003). Moreover, histopathological techniques have already been employed in studies related to microplastics and their respective adverse biological effects (Andrady, 2011; Cole *et al.*, 2011; Fossi *et al.*, 2012, 2014; Koelmans *et al.*, 2013).

With the aim of addressing the assessment of adverse biological effects caused by microplastics, this study used two sympatric gobies, *Blennius pholis* and *Blennius galerita*, as potential test organisms. The *B. pholis* and *B. galerita* are common intertidal fishes living in exposed rocky shores in the marine littoral zones (Falcon *et al.*, 2003), more specifically in the eastern Atlantic Ocean, the Mediterranean Sea and the Black Sea (Falcon *et al.*, 2003; Zander, 1986). The *Blennius* fishes have a short generation time (12- 18 months) and can live in a variety of substratum types during its life cycle, inhabiting in shallow pools when they are juveniles and in exposed rocky shores under stones and other protected microhabitats when they become adults (Faria *et al.*, 2001). The *Blennius* fishes are believed to have limited mobility, hence organisms resilience to the inherently varying physico-chemical conditions is fundamental to their survival (McLusky and Elliott, 2007; Mitamura *et al.*, 2005). Both fish species spawns from spring to summer in the Atlantic coast, although the reproductive cycle varies with latitude (Almada *et al.*, 1996). In Portugal, in particular, the spawning season occurs in the cooler months, from October to May (Almada *et al.*, 1990a; Faria *et al.*, 2001). The main food items for this species consist of meiofauna and small macrofauna (Pihl, 1985; Salgado *et al.*, 2004). Owing to abovementioned characteristics, gobies have widely been used as suitable sentinel and test organisms for assessing adverse biological effects of exposure to both pollutants and microplastics (Cuevas *et al.*, 2016; Sá *et al.*, 2015).

## 2. Objectives

The primary goal of this thesis is to assess histopathological alterations and lesions in whole body of two fish species of *Blennius* (*i.e.* *B. pholis* and *B. galerita*) exposed via feeding to several concentrations of microplastics (uncontaminated) for a month. As a secondary objective, heavy metal release from microplastics covered with an antifouling paint (contaminated) was assessed through the histopathological analyses in same testing species in order to detect whether microplastics may play a role as vector for heavy metal contamination and levels of these toxic compounds found in microplastics may cause potential adverse biological effects. For abovementioned purposes, histopathological disturbances were firstly evaluated qualitatively and then through the development of histopathological condition indices in several target organs (*i.e.* gills, gills, liver, kidney, digestive tract and spleen), as well as histochemical evaluation.

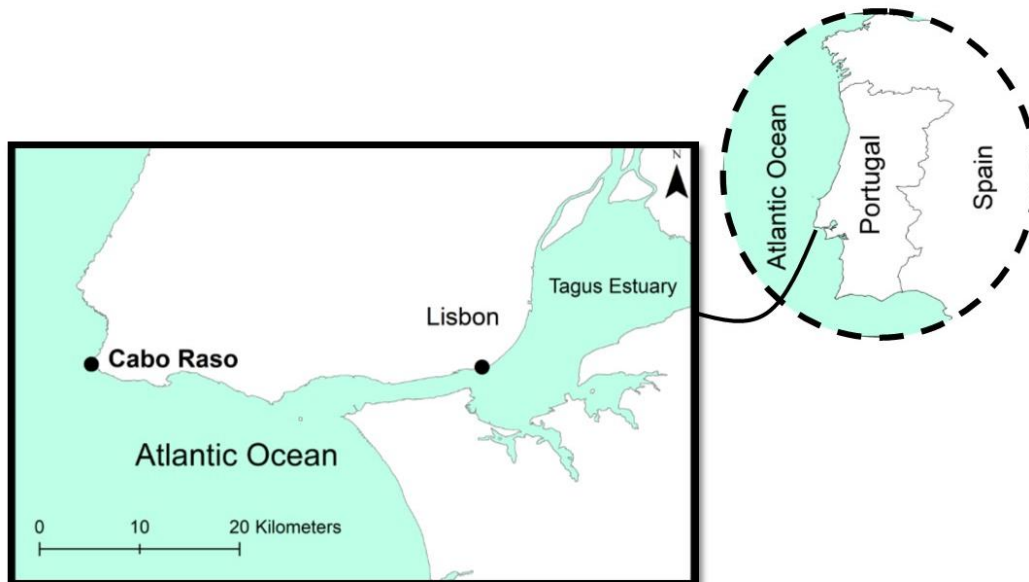
The present thesis attempts to address the following specific objectives:

- To identify histopathological alterations in multiple target organs (*i.e.* gills, liver, kidney, digestive tract and spleen) of two *Blennius* fishes after exposure (30 days) to uncontaminated microplastics via feeding.
- To determine and evaluate abovementioned histopathological effects in multiple target organs of gobies through qualitative and semi-quantitative histopathological assessment.
- To assess a potential role of microplastics covered with an antifouling paint (contaminated) as vector for entrance to heavy metal contamination in biota from marine environments.
- To investigate the potential adverse biological effects qualitatively and semi-quantitatively caused by heavy metal released contamination from microplastics in whole body of gobies.
- To compare histopathological alterations recorded in two sympatric *Blennius* fishes exposed to contaminated and uncontaminated microplastics via feeding for a month.
- To contribute to a histological description of *Blennius* fish species.

### 3. Materials and methods

#### 3.1. Experimental procedure

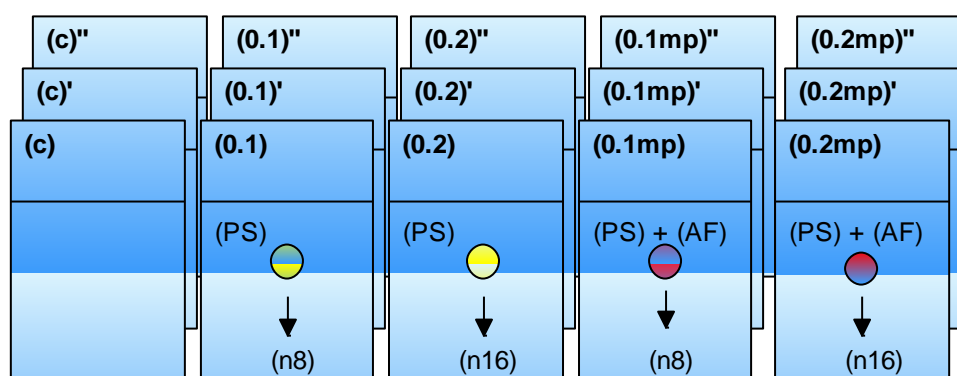
The laboratory experiment was designed and carried out by the Faculty of Science at the University of Lisbon (FCUL). Two fish species of *Blennius* (*B. pholis* and *B. galerita*) were collected in July 2016 at Cabo Raso (Portugal) (Fig. 1), a negligibly disturbed area. Fish were transported to the laboratory in aerated seawater containers and 25 individuals were randomly placed into each aquarium for 10 days of acclimation. The animals were fed daily with 2.19 g of frozen grinded shrimp and maintained in continuous artificial sea water flow ( $20 \pm 0.5^\circ\text{C}$ ; 34ppm).



**Fig. 1.** Location of sampling area (Cabo Raso, Portugal) where the animals were collected.

The two fish species, *B. pholis* and *B. galerita*, were afterwards exposed separately to two different concentrations of microplastics (0.1% and 0.2% w/w) via feeding for 30 days according to [Pedá et al. \(2016\)](#) (Fig. 2). Note that the microplastic spheres employed in two of the treatments were previously immersed in an antifouling paint (AF) (Micron Optima Base YBA953 and Micron Optima Activator YBA953, International Paint Ltd., Southampton, UK), containing copper oxide (25 - 50%) and zinc oxide (10 - 25%) (Fig. 2). Abovementioned concentrations of microplastics were mixed with daily feed. The microplastic spheres employed in this experimental assay were virgin Polystyrene (PS) (0.7 - 0.9 mm diameter; Styropor® P 326, BASF, Ludwigshafen, Germany), which is a common plastic type found in

marine and estuarine environments (Andrady, 2011). Specifically, 8 microplastic PS spheres were added to daily feed in 0.1% (w/w) experimental groups, while in 0.2 % (w/w) treatments 16 microplastic PS spheres were provided. To sum up, as Figure 2 illustrates, this experimental procedure presented five experimental groups, treatment C as control, treatments 0.1 and 0.2 with uncontaminated PS microplastics and, treatments 0.1mp and 0.2mp with microplastics covered with AF (contaminated). Three replicate tanks were assigned to each treatment diet and species.



**Fig. 2.** Experimental design showing five treatment diets [(C) control; (0.1) 0.1% w/w; (0.2) 0.2% w/w; (0.1mp) 0.1% w/w with AF; and (0.2mp) 0.2% w/w with AF] carried out for each testing species and their respective replicate tanks. PS: Polystyrene; AF: antifouling paint; n: number of microplastic spheres provided daily.

The number of the individuals sampled per treatment and species at the end of the exposure period (*i.e.* after 30 days) and processed for histological process is illustrated in Table 1. During the experiment, fish were monitored for any possible signs of impaired health status.

**Table 1.** Number of sampled individuals per treatment and species at the end of the exposure period (*i.e.* after 30 days). n.d.: no data.

	<b>C</b>	<b>0.1</b>	<b>0.2</b>	<b>0.1mp</b>	<b>0.2mp</b>
<b><i>B. pholis</i></b>	6	6	n.d.	6	2
<b><i>B. galerita</i></b>	6	6	6	6	5

### 3.2. Histological procedure

Collected fish samples were immersed in Bouin-Hollande's (10% v/v formaldehyde and 7% v/v acetic acid to which picric acid was added till saturation) or Davidson (formalin- ethanol- acetic acid) fixative for 24 - 36 h at 4°C. Afterwards, whole body samples were dehydrated in a graded progressive series of propanol (70, 96, 100%) followed by an embedding in paraffin. Embedded tissue samples of whole fish were cut (5 µm - 7 µm) using a rotary microtome (Jung

RM2035, Leica Microsystems). At least four slides from each sample were obtained, each containing 4 - 6 sections. Tissue samples were deparaffinated and rehydrated before staining. Diverse dyes were employed for histochemical and histopathological evaluation: (1) Haematoxylin and Eosin (H&E) for structural and morphological screening; (2) Rubanic Acid solution counterstained with Nuclear Fast Red in order to analyze and evaluate copper deposits and (3) tetrachrome stain based on Alcian Blue, Weigert's Hematoxylin and van Gieson's to the differentiation of distinct cell types, though enhanced chromatic segregation of tissues and cells within tissues (Costa and Costa, 2012 with few modifications). Afterwards, all slides were dried and mounted with glycerine solution (50 - 75%). Unlike common histopathological procedure, isopropanol and glycerine were employed instead ethanol and DPX, respectively, to avoid microplastics degradation (Gonçalves *et al.*, 2017).

### 3.3. Histopathological assessment

The histopathological analyses were made by ADMLB model microscope equipped with a DFC480 digital camera (Leica Microsystems). A preliminary qualitative histopathological screening was performed on the cellular structure of different target organs (*i.e.* gills, liver, kidney, digestive tract and spleen) in order to diagnose alterations such as inflammatory responses, regressive disturbances and progressive changes. The semi-quantitative histopathological approach was performed using already reported histopathological indices according to Bernet *et al.* (1999) and adapted by (Costa *et al.*, 2013). The histopathological indices considered the relative biological importance (weight) of each alteration and the degree of dissemination (score) of each lesion within the studied organ. The weights were 1 (slight), 2 (moderate) and 3 (severe); while the score ranged from 2 to 6 depending on the dissemination degree of the alteration. The histopathological alterations were classified into four reaction patterns: (1) circulatory disturbances, (2) regressive changes (functional loss), (3) progressive changes (altered function). The global histopathological indices ( $I_h$ ) were calculated for each individual and organ as detailed below:

$$I_h = \frac{\sum_1^j w_j a_{jh}}{\sum_1^j M_j}$$

Where  $I_h$  is the histopathological condition indices for the individual  $h$ ;  $w_j$  the weight of the  $j$ th histopathological alteration;  $a_{jh}$  the score attributed to the  $h$ th individual for the  $j$ th alteration and  $M_j$  is the maximum attributable value for the  $j$ th alteration, *i.e.*, weight  $\times$  maximum score. The equation's denominator normalizes  $I_h$  to a value between 0 and 1, thus permitting comparisons between distinct situations (such as different organs and species).

A list of all observed pathologies and respective condition weigh, considered for the estimation of histopathological indices are illustrated in Table 2. A blind review of slides was performed to ensure the accuracy of histopathological evaluation.

**Table 2.** Histopathological alterations recorded in liver, gills, kidney and stomach of *Blennius* and their respective biological significance ( $w$ ). MMCs: melanomacrophage centers; HP: hyperplasia.

	<b>Liver</b>	<b>w</b>	<b>Gills</b>	<b>w</b>	<b>Kidney</b>	<b>w</b>	<b>Stomach</b>	<b>w</b>
<b>Circulatory disturbances</b>	Hyperaemia	1						
	Haemorrhage	1						
<b>Inflammation response</b>	MMCs	1	MMCs	1	MMCs	1	MMCs	1
<b>Regressive changes</b>			Lamellar Lifting	2				
<b>Progressive changes</b>	Fat vacuolation of hepatocytes	1	HP Goblet	2				

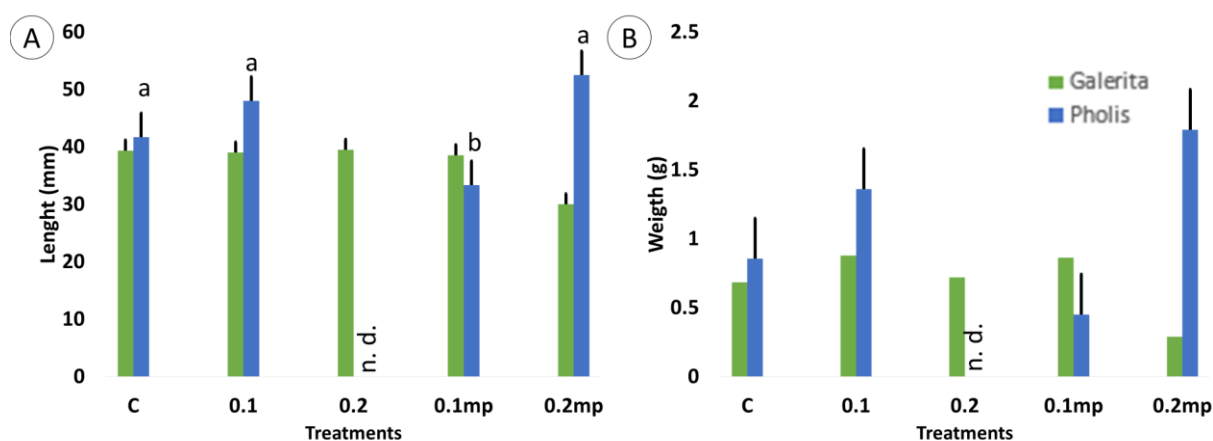
### 3.4. Statistical analyses

The non-parametric test Kruskal-Wallis Multiple Comparison test (KW) was employed in quantitative and semi-quantitative parameters after invalidation of at least one of the assumption of parametric tests (homocedasticity by Levene's test and normality by Kolmogoroff-Smirnoff's test). Spearman rank correlations were also performed in order to associate analyzed variables (*i.e.* biometric data and histopathological indices). A significance level  $\alpha$  was set at 0.05 for all analyses. The statistical analysis was conducted with the Statistica 8.0 software (Statsoft, USA).

## 4. Results

### 4.1. Biometric parameters

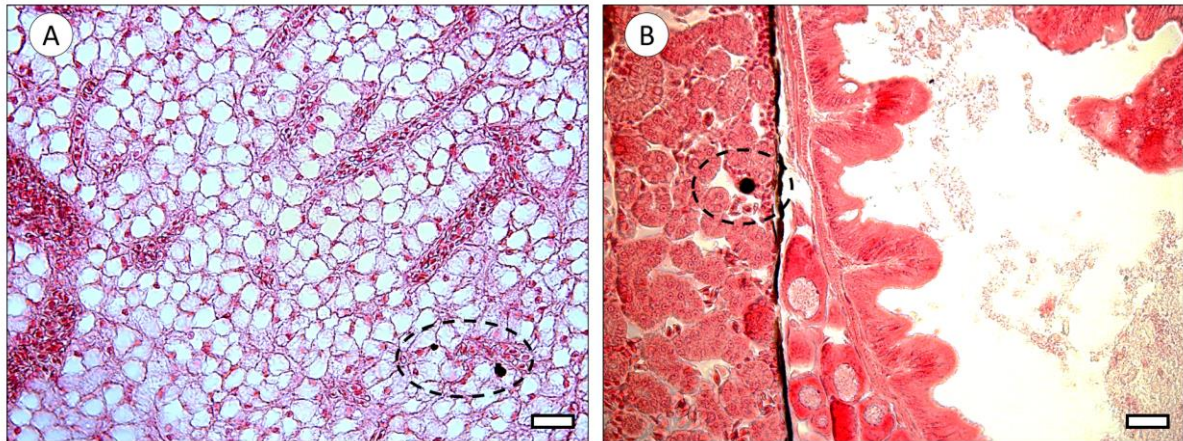
The length and weight of gobies ranged between 2.0 - 6.5 mm and 0.02 - 3.0 g, respectively. Mean values of both biometric variables were illustrated in Figure 3, grouped according to the fish species and experimental treatment. Significant differences in the length and weight were observed between individuals from the control and treatment 0.1mp for the *B. pholis* fish species (KW,  $p < 0.05$ ). On the contrary, no significant differences in biometric parameters were obtained among treatments for the *B. galerita* fish species (KW,  $p < 0.05$ ). Moreover, biometric parameters in both fish species were similar in all experimental treatments (KW,  $p < 0.05$ ).



**Fig. 3.** Means  $\pm$  SEM of biometric data (A: length; B: weight) of collected *Blennius* fishes. Statistical differences among experimental treatments in *B. pholis* fish species were reflected by letters. C: control; 0.1: 0.1% w/w; 0.2: 0.2% w/w; 0.1mp: 0.1% w/w with AF; 0.2mp: 0.2% w/w with AF; n.d.: no data.

### 4.2. Signs of microplastics ingestion and copper deposits

The microplastic particles were not observed histopathologically in any of the fish samples analysed for both fish species throughout the laboratory test. Similarly, differences were observed neither in the presence and density of copper deposits found in all analysed organs nor between species. Copper deposits frequently appeared in the analysed target organs as small and punctual centres, even in the control, as it is illustrated in Figure 4. Thus, the size and density of these copper deposits registered in both fish species were considered low, or even null, in all treatments. Therefore, no quantitative or semi-quantitative assessment of copper deposits and subsequent statistical analyses were performed.



**Fig. 4.** Liver and Kidney stained of *Blennius* fishes with Rubenic Acid solution and counterstained with Nuclear Fast Red for observation of copper deposits: (A) Liver section showing a blood vessel surrounded by small copper deposits (circle); (B) Kidney section presenting copper deposits into a renal tubule (circle). Scale bar: 35  $\mu$ m.

### 4.3. Histopathological alterations and indices

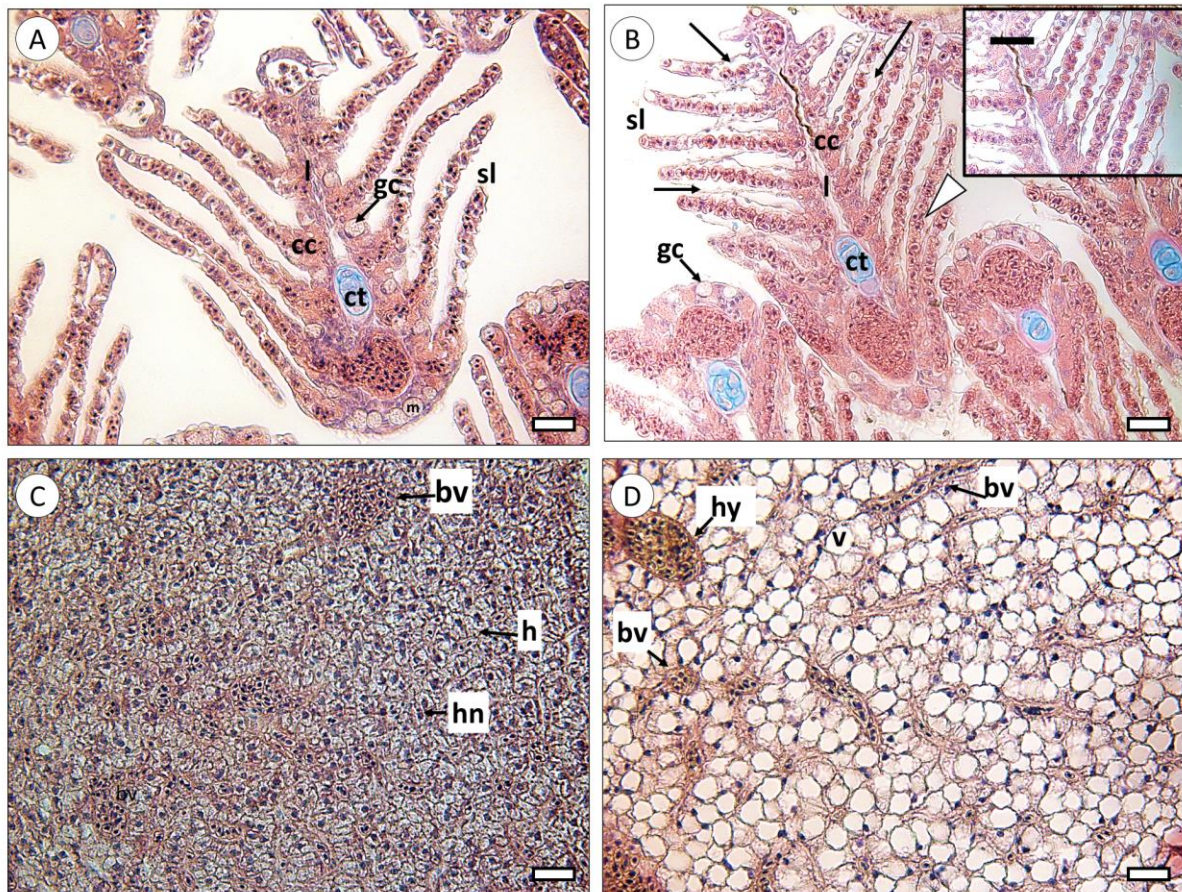
No gross histopathological lesions were observed in any of the organs analysed (*i.e.* gills, liver, kidney, digestive tract and spleen) for both species in all experimental groups. The main histopathological alterations registered in target organs were illustrated in abovementioned Table 2 (see material and methods section). The biological importance (weigh), according to the histopathological index applied, ranged between 1 and 3, although in the present work the maximum weigh registered was of 2, in the case of lamellar lifting and hyperplasia of goblet cells, both lesions in gills (Table 2). Likewise, the dissemination degree (score) of recorded histopathological alterations was relatively low, being values between 2 and 4 the most common within the multi-organ histopathological assessment in both fish species.

#### 4.3.1. Gills

Normal gills revealed the typical structure of lamellae (*i.e.* primary and secondary), in which the single-cell thick lamellar epithelia contained goblet and chloride cells supported by a complex system of blood vessels (Fig. 5A).

Regressive changes were the most relevant histopathological reaction pattern for both fish species, representing lamellar lifting (*i.e.* separation of epithelium from secondary lamellae) the most remarkable alteration (Fig. 5B). Animals of both *B. pholis* and *B. gallerita*, except for the control (67%) and 0,1mp (67%) respectively, revealed this latter alteration in a prevalence of 100% in all experimental groups. Inflammation responses, that include only the presence of melanomacrophage centers (MMCs), were the second reaction pattern of lesions most noticed

in the gills for both fish species. *B. pholis*, in particular, displayed a prevalence ranged between 0 - 33%, while *B. galerita* demonstrated less frequency (0 - 20%). This latter fish species also presented one case of progressive changes in gills, more specifically hyperplasia of goblet cells. No circulatory disturbances were observed in both fish species within all experimental groups.



**Fig.5** Gills and liver sections of *Blennius* fishes stained with Tetrachrome based on Alcian Blue, Weigert's Hematoxylin and van Gieson's: (A) Normal gills with well differentiated primary (l) and secondary lamellae (sl), gill filament support cartilage (ct), goblet cell (gc) and chloride cell (cc); (B) Gills affect by lamellar lifting (arrows and inset); (C) Normal liver presenting hepatocytes (h) with a homogenous cytoplasm and a central spherical and well defined nucleus (hn). (D) Liver tissue affected entirely by fat vacuolation (v) of hepatocytes and hyperaemia (hy). bv: blood vessel. Scale bar: 35  $\mu$ m, inset 20  $\mu$ m.

In gills, *B. galerita* presented similar histopathological indices (HI Gills) in all experimental treatments, being the highest indices in individuals from the control group. *B. pholis*, however, showed slightly higher HI Gills in animals from treated experimental groups than from the control, being the highest HI Gills in the treatment 0.2mp (Fig. 6). Moreover, *B. pholis* demonstrated higher HI Gills than *B. galerita* species in all treatments, except in individuals from the control.

No significant statistical differences were found among the treatments in each fish species. However, significant statistical differences (KW,  $p < 0.05$ ) were observed between species for the treatments 0.1, 0.1mp and 0.2mp (Fig. 7).

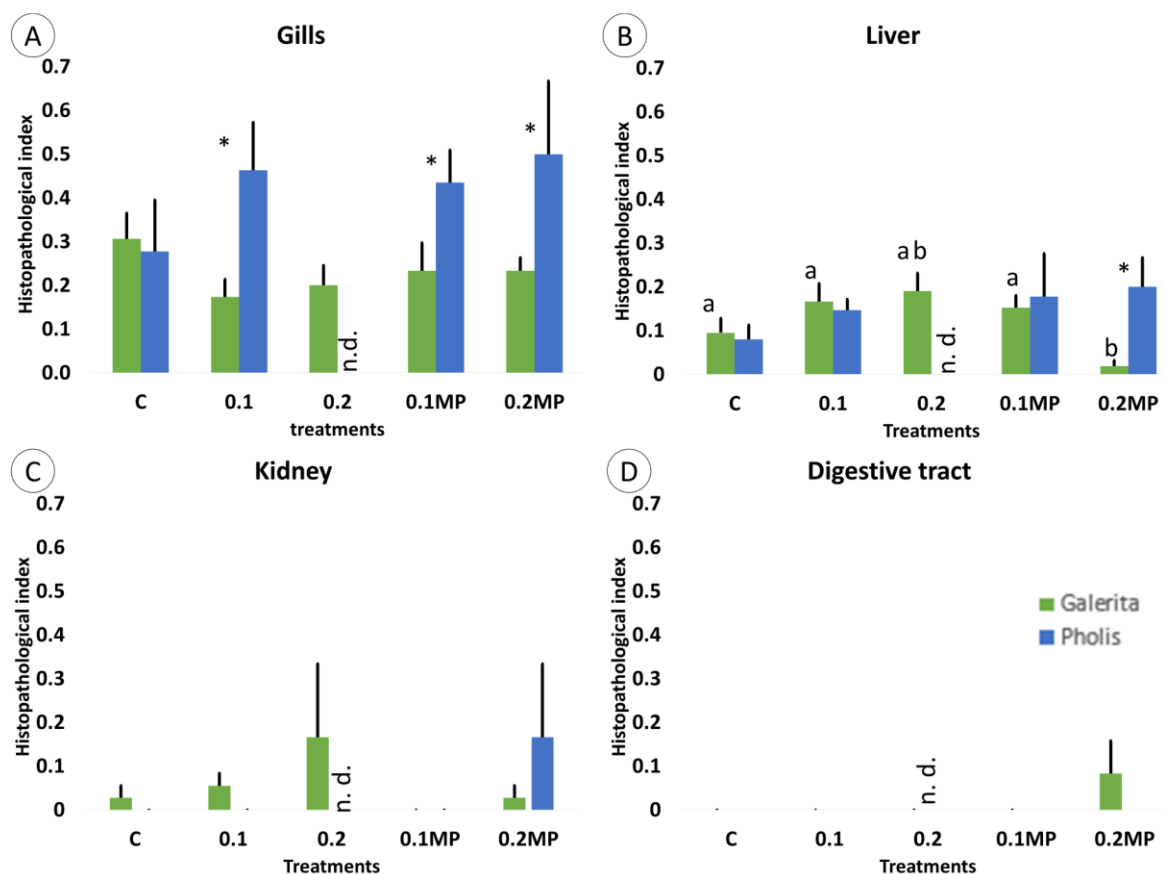
#### 4.3.2. Liver

The normal liver structure of gobies was characterized by hepatocytes presenting a homogenous cytoplasm and a central spherical nucleus, aligned with sinusoids (Fig. 5C). In this fish species, pancreatic tissue was comprised within the liver forming a single organ known as hepatopancreas.

The occurrence of lesions in the liver of collected fish was relatively low, being fat vacuolation of hepatocytes the most frequent alteration in both fish species. In those cases, nuclei of hepatocytes were remarkably displaced to the periphery of the cells becoming difficult to distinguish the normal hepatic lobular architecture (Fig. 5D). *B. pholis* presented prevalence ranged between 60 - 100%, showing the lowest value in animals from the control group. In the case of *B. galerita*, fat vacuolation was shown in frequencies of 0 - 80%. Noted that *B. galerita* fish collected from the treatment 0.2mp did not demonstrated any fat vacuolation of hepatocytes. Inflammatory responses, which include presence of melanomacrophage centers (MMCs), were the second most common reaction pattern, observing that *B. pholis* presented lower prevalence (0 - 50%) than *B. galerita* (40 - 100%). It should be emphasized that both species presented fat vacuolation as the second highest prevalence (40% *B. pholis* and 83% *B. galerita*) in individuals from the control group. Circulatory disturbances such as haemorrhage and hyperaemia in individuals of *B. pholis* presented prevalence ranged between 0 - 50% and 0 - 60%, respectively. Nevertheless, no signs of these circulatory alterations were shown in fish from the control for the *B. galerita* species, while in individuals from the rest of treatments presented prevalence between 0 - 20% for haemorrhage and 0 - 100% for hyperaemia. According to *B. pholis* results, hyperaemia was not shown in individuals from the control.

*B. galerita* presented the lowest histopathological index of liver (HI Liver) in the treatment 0.2mp, while the rest of experimental treatments presented very similar HI Liver. It has to note that animals from the control showed the second lowest HI liver for this specie (Fig. 6). On the other hand, *B. pholis* revealed similar HI Liver in all experimental treatments.

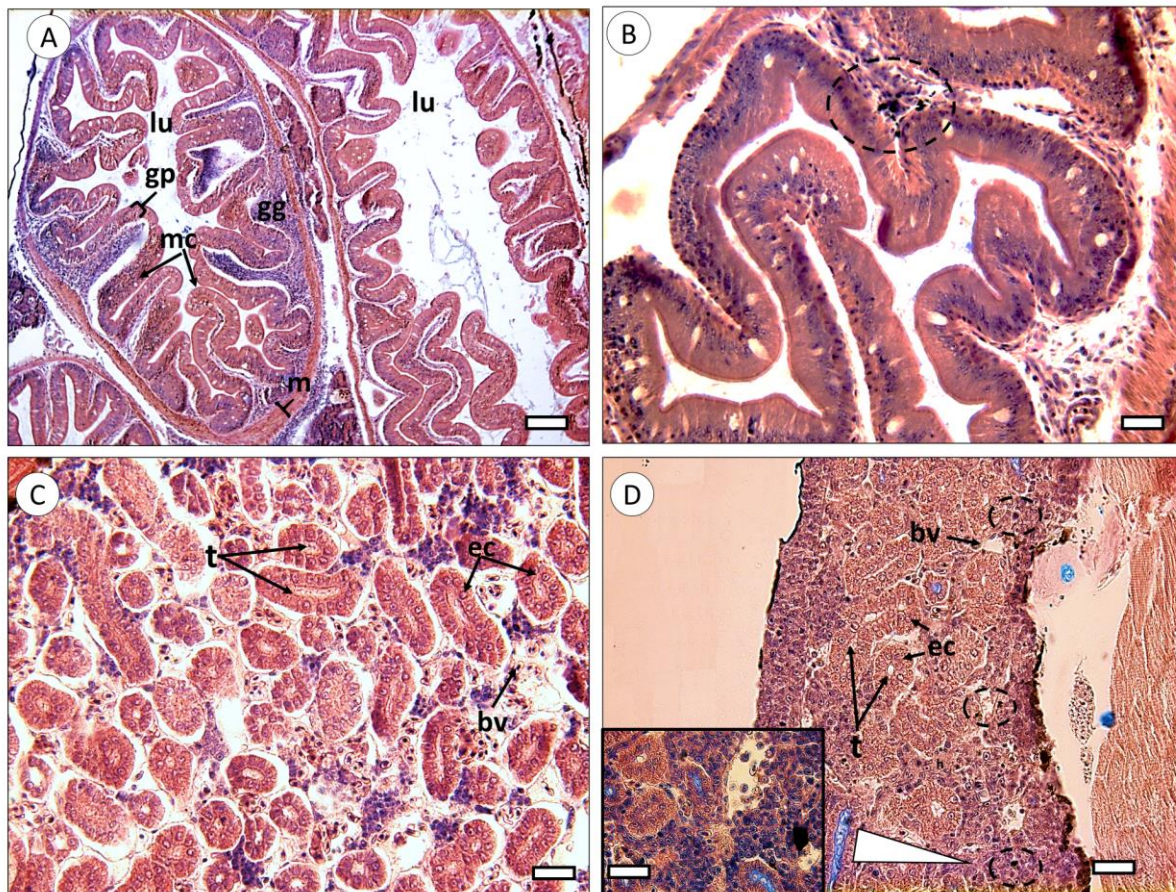
Significant statistical differences (KW,  $p < 0.05$ ) were found for the species *B. galerita* (Fig. 6) between control and the treatments 0.2 and 0.2mp. Differences were also observed between species for the treatment 0.2mp (KW,  $p < 0.05$ ) (Fig. 6).



**Fig. 6.** Mean histopathological indices of liver, gills, kidney and digestive tract of two fishes collected in the experimental assay. Letters indicate statistically significant differences among the treatments for the *B. galerita* fish species and (\*) between the species. Error bars indicate the standard error (SEM). C: control; 0.1: 0.1% w/w; 0.2: 0.2% w/w; 0.1mp: 0.1% w/w with AF; 0.2mp: 0.2% w/w with AF. n.d.: no data

#### 4.3.3. Digestive tract

The normal structure of the digestive tract, were composed by intestines mostly and stomach, are remained intact in animals of both species from all experimental treatments, without any remarkable alteration or lesion after the conduction of the experiment (Fig. 7A-B). As exception, one individual of *B. galerita* collected from the treatment 0.2mp presented an inflammation response of MMCs. Therefore, the histopathological index was only calculated for *B. galerita* species in the treatment 0.2mp (Fig. 6). Thus, the estimation of significant statistical differences was not feasible.



**Fig. 7.** Sections of digestive tract and kidney of *Blennius* fishes stained with Tetrachrome based on Alcian Blue, Weigert's Hematoxylin and van Gieson's. (A) Normal structure of digestive tract composed by: gastric gland (gg) and gastric pit (gp); mucous cell (mc); muscularis mucosae (m) and lumen (lu). (B) Digestive tract with MMCs (circle). (C) Normal structure of kidney composed with renal tubule (t) lined with a normal epithelial cell (ec); (D) Kidney tissue with MMCs (dashed circle and inset). Scale bar: 35  $\mu$ m; inset 20  $\mu$ m.

#### 4.3.4. Kidney

The kidney normal structure was composed of renal tubules surrounded by hematopoietic interstitial tissue containing mainly erythrocytes and blast (Fig 7C). Within this study, presence of MMCs was only observed, which is characterized as inflammatory change. This latter alteration was recorded in individuals of *B. pholis* from the treatment 0.2mp with a prevalence of 50%, while in *B. galerita* was registered in all treatments, except in the treatment 0.1mp with prevalence between 33 and 67% (Fig. 7D).

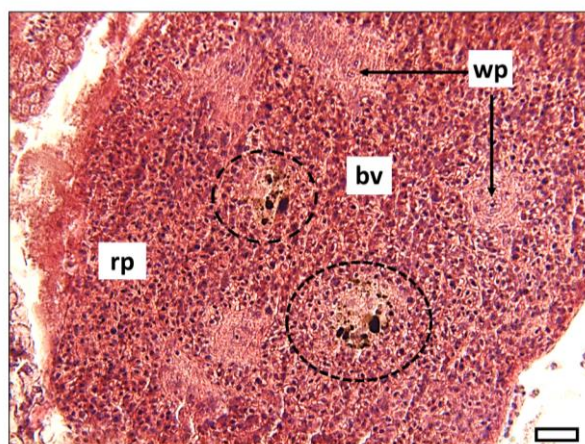
According to the estimation of histopathological indices, individuals of *B. galerita* presented a slightly higher affection degree than *B. pholis*. In fact, the histopathological indices of kidney (HI Kidney) were calculated in four of the five treatments including the control, while in

animals of *B. pholis* this alteration was only observed in the treatment 0.2mp (Fig. 6). Nevertheless, when the statistical evaluations were possible, no significant statistical differences were observed between the species and among the treatments (Fig. 6).

#### 4.3.5. Spleen

The spleen structure was composed mainly of blood vessels, red pulp and white pulp. The red pulp, which occupied the majority of the organ, consisted of a cellular reticulum with hematopoietic tissue and blood sinuses. The white pulp, on the contrary, was often scarce (Fig. 8).

In this organ, only inflammation responses were observed in both fish species from all experimental groups, more specifically the presence of MMCs (Fig. 8). *B. pholis* showed a prevalence of 100% in the control group and treatment 0.1. *B. galerita* presented prevalence ranging between 0 - 100%, being the control the most affected group, while the treatment 0.2 and 0.1mp did not showed any inflammation responses. Note that the sample size of this organ obtained in individuals from different experimental group was rather low and thus estimation of histopathological indices and subsequent statistical analyses were not performed and plotted.



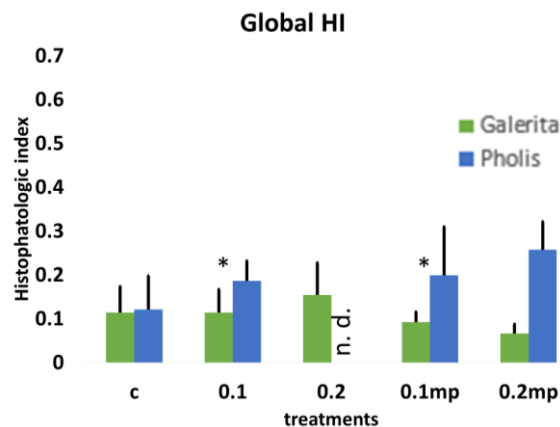
**Fig. 8.** Section of spleen of *Blennius* fish stained with Tetrachrome based on Alcian Blue, Weigert's Hematoxylin and van Gieson's. Spleen section affected by MMCs (circles). rp: red pulp; wp: white pulp; bv: blood vessels. Scale bar 35  $\mu$ m.

#### 4.3.6. Global histopathological indices

Global histopathological indices (Global HI) were calculated considering all alterations recorded in every target organ of each fish species, in order to assess the global health status

of tested individuals (Fig. 9). Considering the different affection degree in each target organ, gills and liver demonstrated to be the most affected organs in this laboratory experiment, while kidney and digestive tract, together with spleen, were less impacted (gills > liver > kidney > digestive tract). Thus, both liver and gills presented more influence in the estimation of global histopathological indices (Fig. 6, 9).

In general, *B. pholis* registered higher Global HI than *B. galerita*. There were no signs of tendencies among different treatments in the histopathological indices for both species (Fig. 9). Global HI, conversely, revealed significant statistical differences (KW,  $p < 0.05$ ) between species in the treatments 0.1 and 0.1mp (Fig. 9).



**Fig. 9.** Means of global histopathological indices, which are the sum of histopathological indices of the different target organs (*i.e.* liver, gills, kidney and digestive tract), registered in two *Blennius* fishes. (\*) indicates statistically significant differences between the species. Error bars indicate the standard error (SEM). C: control; 0.1: 0.1% w/w; 0.2: 0.2% w/w; 0.1mp: 0.1% w/w with AF; 0.2mp: 0.2% w/w with AF. n.d.: no data.

#### 4.3.7. Correlation analyses

According to spearman statistical test, there were found significant correlations among the treatments for each species. More specifically, in *B. galerita* significant correlations were observed between length and weight ( $R = 0.94$ ,  $n = 29$ ,  $\alpha = 0.05$ ). Additionally, there were correlations between HI Gills and the Global HI ( $R = 0.49$ ,  $n = 29$ ,  $\alpha = 0.05$ ) and between HI liver and Global HI ( $R = 0.64$ ,  $n = 29$ ,  $\alpha = 0.05$ ). Similarly, in *B. pholis*, the significant correlations were also found between weight and length, ( $R = 0.99$ ,  $n = 20$ ,  $\alpha = 0.05$ ), HI Gills and Global HI ( $R = 0.69$ ,  $n = 20$ ,  $\alpha = 0.05$ ), and HI Liver and Global HI ( $R = 0.54$ ,  $n = 20$ ,  $\alpha = 0.05$ ).

## 5. Discussion

In the present work, *Blennius* fishes, *B. pholis* and *B. galerita*, were exposed to two concentrations of microplastics (both contaminated and uncontaminated) during a month. Even so, the findings did not reveal signs of microplastics ingestion, which may suggest that these gobies were not able to ingest this kind of microplastic spheres. Therefore, negligible adverse biological effects were shown in the multi-organ histopathological assessment (*i.e.* liver, gills, kidney, digestive tract, and spleen) in gobies exposed to uncontaminated microplastics. Accordingly, regardless the pathway of contaminants (by ingestion or waterborne), exposure to contaminated microplastics did not cause gross histopathological adverse effects in gobies. Nevertheless, this study did not clarify whether microplastics may play a role as vector for entrance to heavy metal contamination in biota from marine environments.

According to the findings, the size of microplastics, selection of appropriate fish species (e.g. feeding behaviour) and its respective biometric parameters were relevant factors to be considered within experimental design, when the ingestion of these microplastic particles was expected. In comparison with the basis of this experimental design (Pedá *et al.*, 2016), the diameter of microplastic PS particles employed within this laboratory assay were almost three times larger (0.7 - 0.9 mm > 0.3 mm), while the size of fishes was considerably lower (3 g < 140 g). For instance, Oliveira *et al.*, (2013) reported the adverse effects of remarkably smaller microplastics (1 - 5µm diameter) ingestion in *Pomatoschistus microps* gobies, which feature similar size than *Blennius* species. Similarly, Mota (2017) presents a study with *Diplodus Sargus*, larger fish than *Blennius* fishes, based on the same bioassay conditions as in the present work, demonstrating histologically punctual ingestions of microplastic particles. Moreover, the feeding items of *Blennius* species mainly consist of meiofauna (Salgado *et al.*, 2004), whose size (50 and 500 µm) is considerably smaller than the dimensions of used microplastics. Likewise, Wright *et al.*, (2013) revealed that microplastics less than 0.5 mm of PVC were ingested 37 more times compared to other 17 higher size categories. Besides, gobies are characterized by a selective and highly flexible feeding strategy, allowing individuals to choose available food resources, even in food scarcity (Grabowska *et al.*, 2009; Lim *et al.*, 2017). On the other hand, the laboratory methodology applied in this work was carefully chosen to avoid the microplastic degradation, employing isopropanol and glycerin instead standard techniques as ethanol and DPX (Gonçalves *et al.*, 2017).

In view of that ingestion of microplastics may not occur, the potential role of these particles as entrance vector of metals from antifouling paints by ingestion pathway was not evidenced within the present study. The release of metals from antifouling paints may be held directly in seawater, being these contaminants diluted in the medium. Therefore, the levels of metals in waterborne and its bioavailability, specifically of copper, did not cause any relevant bioaccumulation and histopathological alterations in diverse target tissues of both fish species. The punctual copper deposits observed into the target organs may be caused from natural occurrence, since metals such Cu, Fe and Zn are essential metals and play important role in biological metabolic processes (Arulkumar *et al.*, 2017). Conversely, Sivaperumal *et al.*, (2007) and Velusamy *et al.* (2014) stated that essential metals can also produce toxic effects at high residual concentrations. Indeed, according to Brennecke *et al.*, (2016) and Soroldoni *et al.*, (2017), antifouling paints can be a source of metals to be desorbed through microplastics into the seawater and thus be bioavailable and bioaccumulated in marine living organisms.

Among the most obvious adverse effects caused by microplastics ingestion in fish species are the physical blockage of the digestive tract and its influence in feeding (Sá *et al.*, 2015; Jovanovic, 2017). Some authors, conversely, have reported more severe histopathological alterations caused by microplastics such as widening of the lamina propria and hypertrophy of goblet cells in diverse fish species after exposure of 30 days (Pedá *et al.*, 2016). Thus, contrary to the expectations, digestive tract was the less impacted target organ, showing only mild inflammatory responses. As reported in other studies, microplastics can pass through the digestive tract and be expelled from the body or be retained in the gastrointestinal tract, causing internal abrasion and inflammatory responses (Moos *et al.* 2012). Regarding other target organs, differences in histopathological affection degree were observed, being listed from most impacted to less as gills, liver, kidney and spleen. As it has been already reported, this different affection degree was probably owing to the specific biological function and sensitivity of each target organ (Bernet *et al.*, 1999; Costa *et al.*, 2009, 2013). Nevertheless, assessed organs within this work have already stated as suitable target organs to evaluate adverse biological effects in fish (Bernet *et al.*, 1999; Costa *et al.*, 2009; Cuevas *et al.* 2016).

The fish gills, in particular, are a multifunctional organ involved not only in respiration, but also in a variety of homeostatic activities such as osmoregulation, metabolism of circulating hormones and nitrogen excretion. Likewise, this organ is characterized by providing an extensive surface of contact with the aquatic environment and thus responds rapidly to various

contaminants resulting in adverse biological effects (Evans et al., 2005; Movahedinia et al., 2009). Unlike in other studies in which liver is reported as the most affected organ after contamination exposure (Costa et al., 2009; Cuevas et al., 2016; Karami et al., 2016), gills presented the highest histopathological indices in this work. This may be explained due to the abovementioned fairly slight waterborne metal release from microplastic particles, which may suggest gills as the only organ in direct contact with potential metal pollutants. On the other hand, it should be noted that animals collected from natural sites, often present a baseline level of non-specific gill lesions supporting the statement of the natural occurrence (Costa et al., 2009; Cuevas et al., 2015).

In the case of liver, kidney and spleen, they were employed within several works as target organs for the assessment of adverse biological effects (Bernet 1999; Costa et al., 2009, 2013; Cuevas et al., 2016). Liver, in particular, presents a relevant detoxification role, which regulates metabolism, allows the digestive system to extract more nutrients and remove wastes from blood, to aid in recycling old blood cells, any damage to the liver could impair circulatory lipid levels in the body (Begriche et al., 2011). Liver presented vacuolation of hepatocytes as the most common alteration in both fish species. Some authors defend that the condition of hepatocytes is highly dependent on the reproductive stage and the availability of an adequate supply (Davies and Vethaak, 2012). On the contrary, this histopathological disturbance has also been related to exposure of mixtures of metal and organic contaminants (Van Dyk et al., 2007; Triebkorn et al., 2008). Kidney, in turn, operates as an important organ related to electrolyte and water balance and the maintenance of a stable internal environment (Bernet et al., 1999; Costa et al., 2010). Melanomacrophage centers (MMCs) were the only alteration registered in this organ, although this alteration was observed in whole body of gobies. This frequent alteration in fish has specific functions such as deposition sites for intracellular bacteria, retention of iron, presentation to immune cells and collection of products of cellular degradation (Agius et al, 2003; Evans and Nowak, 2016; Wolke, 1992). However, according to some authors the number and dissemination of MMCs can be influenced by dietary supplementation, as well as exposure to infectious agents (Hur et al., 2006; Manrique et al., 2014; Pronina et al. 2014). Spleen, generally, is located close to the stomach and it mainly acts as filter for purifying the blood and producing the white blood cells for the immune system (Muiswinkel et al., 1991; Secombes & Manning, 1980, 1982). Nevertheless, the latter organ was not considered for the multi-organ histopathological assessment owing to the low number of assessed samples.

According to the results, *B. pholis* and *B. galerita* were not suitable species to evaluate potential adverse effects of the selected PS microplastic spheres owing to their large size for this fish species. However, gobies were reported as suitable test organisms for assessing adverse effect of both microplastic ingestion and potential pollutants (Sá *et al.*, 2015; Cuevas *et al.*, 2016; Oliveira *et al.*, 2013). Likewise, this work presented a setback related to the low sample size of treatments 0.2 and 0.2mp in *B. pholis* fish, which hindered the comparison between species. Nevertheless, these sympatric fish species presented similar biological adverse effect within this study, reflecting that both fish species may be used indistinctly, which may facilitate upcoming field and laboratory works.

This work did not show the expected results, thus it is important to rehash the experimental design as further research, to assess the biological effects of both microplastics and release of metals through waterborne and ingestion pathways. Furthermore, taking into account the work reported by Peda *et al.* (2016), it would be relevant to evaluate same objectives stated within this work, but extending the exposure period of microplastics (both contaminated and uncontaminated) to 60 and 90 days. By this way, more severe histopathological alterations may be expected. Additionally, the interaction between microplastics and other pollutants would be evaluated in gobies, especially those that are hazard in low contamination levels in marine environment (*i.e.* hg and pyrene).

## 6. Conclusions

Two *Blennius* fish species were exposed within this work to contaminated (by antifouling paints) and uncontaminated microplastic spheres via feeding during a month. Nevertheless, no signs of microplastics ingestion were recorded, revealing that both fish species were not able to ingest used microplastics, most probably due to the incompatibility between particles size, feeding behaviour and other characteristics of these fish species. Thus, mild adverse biological effects were observed in all individuals exposed to microplastics, even in fish tested with contaminated microplastics. This may suggest that microplastics and their contaminant release, regardless the pathway of contaminants exposure (by ingestion or waterborne), did not cause relevant adverse biological levels. Moreover, the target organs presented different affection degree (gills > liver > kidney > digestive tract > spleen), which is in accordance with their specific biological functions. Lamellar lifting, fat vacuolation of hepatocytes and melanomacrophage centers were the most prevalent alterations noticed in gills, liver, and whole-body fish, respectively. Moreover, the use of *Blennius* gobies in multi-organ histopathology showed to be a suitable organism and tool for assessing the potential adverse effects caused by microplastics and their potential role as contaminants entrance. However, the non-appearance of microplastic ingestion may suggest that other parameters, or even natural occurrence, may cause recorded negligible adverse biological effects observed in fish. Therefore, smaller microplastic particles should be assessed in gobies in order to verify the potential adverse biological effects that may cause microplastics within marine ecosystems.

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