UNIVERSIDAD DE LAS PALMAS DE GRAN CANARIA
Facultad de Veterinaria

IUSA Instituto Universitario de Sanidad Animal Seguridad Alimentaria

TESIS DOCTORAL

A PATHOLOGICAL STUDY OF THE POTENTIAL THREATS TO FISH HEALTH IN THE CANARY ISLANDS

Klee, 1925

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M. CAROLINA DE SALES RIBEIRO

DOCTORADO EN SANIDAD ANIMAL Y SEGURIDAD ALIMENTARIA

LAS PALMAS DE GRAN CANARIA

DICIEMBRE 2021

Para a minha família.

"Não largues esta mão no torvelinho
Pois falta sempre pouco para chegar
Eu não meti o barco ao mar
Para ficar pelo caminho"

José Mário Branco

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ABBREVIATIONS

Abbreviations

BOE	Boletín Oficial del Estado
CLSM	Confocal laser scanning microscopy
COVID-19	Coronavirus disease 2019
EGCs	Eosinophilic granular cells
EMODnet	European Marine Observation and Data Network
EEZ	Exclusive Economic Zone
EFSA	European Food Safety Authority
FAO	Food and Agriculture Organization of the United Nations
FTIR	Fourier Transform Infrared
GALT	Gut associated lymphoid tissue
GMS	Gomori's Methenamine Silver
HE	Haematoxylin and eosin
hpf	Hours post-feeding
IUSA	University Institute for Animal Health and Food Safety
MP	Microplastic
MPs	Microplastics
MSP	Maritime Spatial Planning
mm	millimetre
NPs	Nanoplastics
nm	nanometre
OIE	World Organisation for Animal Health
PAS	Periodic Acid–Schiff
PCR	Polymerase Chain Reaction
PE	Polyethylene
PEG	Polyethylene glycol
PS	Polystyrene
ULPGC	Universidad de Las Palmas de Gran Canaria
WHO	World Health Organization
ZN	Ziehl–Neelsen
µm	micrometre

Abbreviations

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INTRODUCTION AND OBJECTIVES

1.1. Introduction

The Canary Islands enjoy a privileged position in the Atlantic Ocean (Gobierno de Canarias - Consejería de Agricultura, 2021) and harbour a unique ecosystem of global importance (Popescu & Ortega Gras, 2013). Approximately 700 species of Osteichthyes and 85 species of Chondrichthyes inhabit the region (Espino et al., 2018).

Fishing activities are a fundamental part of the identity of the Canary Islands, as many coastal regions depend on fishing for their livelihoods (Popescu & Ortega Gras, 2013). In addition to providing multiple natural resources of economic value, coasts also provide invaluable opportunities for leisure and recreational activities (Espino et al., 2018). For example, fishing tourism, aquaculture tourism, and marine tourism have been developed over the past few years to protect, conserve, and regenerate marine resources and ecosystems while promoting responsible exploitation of existing marine resources (Ley 15/2019, de 2 de mayo, de modificación de la Ley 17/2003, de 10 de abril, de Pesca de Canarias. BOE, nº 141, de 13 de Junio, 2019) (Agencia Estatal Boletin Oficial del Estado, 2019). Because the tourism industry is built on advertising, it is essential to be aware of the challenges that limit its progress and to seek solutions that can maintain and strengthen the privileged position of the Canary Islands as a tourist destination. In this regard, a clean and healthy marine ecosystem and high-quality seafood are two keys to sustainable development.

Diverse fish species are important for the balance of marine ecosystems. In addition to being a significant part of the human diet, fish incorporate several trophic webs, granting the survival of many aquatic organisms (FAO, 2020). Fish are also acknowledged to be sentinels for multiple stressors that can impact biodiversity (Sebastian & Hering, 2018).

In the last fifty years, more fish was consumed globally than all other animal protein foods combined. Spain is one of the top 20 producers of marine capture fisheries (FAO, 2020). As markets become increasingly globalized, most fish products cross multiple borders to reach the consumer. Disease outbreaks can therefore severely impact supply distribution, price volatility, and public health. For that reason, criteria for food quality and safety have become increasingly relevant (FAO, 2020).

Wilson et al. (2010) invited scientists to submit questions about current knowledge gaps regarding how climate change impacts coral reef fish. The invited scientists came from 10 different countries and 23 institutions. More than 70% of the scientists identified reef fish habitat associations, community dynamics, diversity, and distribution patterns as key areas of research interest. In contrast, less than 20% of contributors listed reef fish physiology, productivity, and disease as research interests. Of the 53 questions presented in the final publication, only one addressed fish disease. However, this does not mean there are no knowledge gaps about fish diseases; rather, it highlights an apparent lack of scientific interest in the study of fish diseases.

The current COVID-19 outbreak, which is negatively impacting human health and the world economy, has reminded us that human health is closely connected to the health of animals and the shared environment. World Health Organization (WHO) Director-General Dr. Tedros Adhanom Ghebreyesus stated that "the pandemic is a reminder of the intimate and delicate relationship between people and planet. Any efforts to make our world safer are doomed to fail unless they address the critical interface between people and pathogens and the existential threat of climate change, that is making our Earth less habitable" (WHO, 2020). His comment implicitly raises the concept of '*One Health*,' which refers to the importance of interdisciplinarity in effectively detecting, responding to, and preventing outbreaks of zoonoses and food safety problems. Approximately 60% of the pathogens causing human disease have their source in domestic or wild animals, and 75% of emerging human pathogens are of animal origin (World Organisation for Animal Health, 2021). Active surveillance against pathogens is therefore crucial in the context of ongoing global climate changes. For example, increased water temperature, eutrophication, changes in ocean currents, and ocean acidification are expected to alter disease dynamics (Johnson et al., 2009; Lõhmus & Björklund, 2015; Marcogliese, 2008; Prowse et al., 2009). These global changes can lead to the redistribution, emergence, or re-emergence of several diseases (Marcogliese, 2008; Prowse et al., 2009).

In terrestrial environments, climate change has allowed vectors of Bluetongue virus (Jones et al., 2019; Purse et al., 2008; A. Wilson & Mellor, 2008) and Hantavirus (Clement et al., 2009) to spread to new locations. Global warming has similarly been linked to outbreaks of

the fungus *Batrachochytrium dendrobatidis*, which is responsible for declining amphibian populations (Pounds et al., 2006). In aquatic environments, the protistan oyster parasite *Perkinsus marinus* has extended its range northwards as water temperatures have increased (Cook et al., 1998).

Higher water temperatures have been associated with higher concentrations of viral DNA, the onset of clinical symptoms, and mortality in fish infected with Herpesvirus type 3 (Gilad et al., 2003, 2004; H. Soliman & El-Matbouli, 2020; St-Hilaire et al., 2005), and have contributed to the development of clinical signs in fish infected with *Aeromonas salmonicida* (Crumlish & Austin, 2020). Outbreaks of proliferative kidney disease, a disease caused by *Tetracapsuloides bryosalmonae*, have also been associated with increasing water temperature and eutrophication (Okamura et al., 2011; Sterud et al., 2007; Tops et al., 2009). Similar connections between climate change and disease prevalence or severity have been observed for other parasites including *Myxobolus cerebralis* (Hiner & Moffitt, 2001), *Ichthyophthirius multifilis* (Karvonen et al., 2010), and some trematodes and ectoparasites (Cairns et al., 2005; Hakalahti et al., 2006). Information regarding fungi-host dynamics in the context of climate change is limited. However, since adverse environmental conditions may compromise fish immune response, increased susceptibility to opportunistic pathogens such as fungi is likely to ensue (Dopazo, 2020; Roberts, 2012).

In addition to climate change, overfishing (Griffin et al., 2020), habitat destruction (Johnson et al., 2009; Marcogliese, 2008), invasive species (Lõhmus & Björklund, 2015), and contamination act as additional stressors in an already intricate suite of threats to fish and ecosystem health. For example, plastic contamination represents almost 90% of the total garbage floating in the sea (Espino et al., 2018). In the Canary Islands, the coastal areas and contiguous ocean are already contaminated with plastics (Álvarez-Hernández et al., 2019; Herrera et al., 2018; Rapp et al., 2020; Vega-Moreno et al., 2021). Plastic ingestion has also been documented in several species of stranded cetaceans (Puig-Lozano et al., 2018) and teleost fish (Herrera et al., 2019) from the Canary Islands. These observations underscore the need for detailed and accurate toxicity assessments for plastics and microplastics, as their impacts on marine ecosystems and public health are not fully understood.

Fish pathology has also been a neglected area of study among veterinary pathologists. Several pathologists (Baumann et al., 2016; Wolf et al., 2015) have highlighted the "broader pervasive problem of inaccurate histopathology data" in scientific publications. This situation is particularly problematic among ecotoxicological publications. Wolf & Maack (2017) found that only 54% of 189 studies containing fish histopathology data had either "highly credible" or "credible" data. In contrast, data were equivocal, dubious, or without credibility in the remaining 46% of those studies. Such inaccuracies unfortunately persist in the literature and serve as the basis for further misguided research (Baumann et al., 2016).

Studies on how microplastics affect fish are particularly prone to poor histopathology data. In several publications that address this question, reported histopathological changes were the result of misdiagnosis and misinterpretation. High-quality works that question and correct these inaccurate results are needed to prevent further inaccuracies.

At the same time, improved and more accurate diagnostic methods and surveillance efforts can mitigate the risks of future disease emergence (Walker & Winton, 2010).

1.2. Objectives

This study sheds new light on the current threats to wild fish health in the Canary Islands. The main objectives were to:

a. Determine the prevalence of various diseases in populations of wild fish from the Canary Islands.

b. Assess the pathological changes caused by infectious agents in these fish.

c. Assess whether fish can recognize microplastics as inedible.

d. Determine the extent to which microplastics are retained and translocated within fish following ingestion.

e. Identify the clinical and pathological effects of microplastic ingestion in fish.

1. Introduction and objectives

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STATE OF THE ART

2.1. **Scientific publication I:** First description of spontaneous granulomatous aerocystitis by *Phoma herbarum* in a wild greater amberjack (*Seriola dumerili Risso*, 1810)

2.1.1. Introduction

The incidence of diseases caused by fungal pathogens is increasing worldwide. However, the detection of these pathogens in aquatic ecosystems is hindered by challenges in directly observing their hosts (Gozlan et al., 2014).

In fish, fungal infections are most commonly secondary to other diseases or environmental stress (Yanong, 2003). In addition, most fungal infections are caused by opportunistic agents, such as plant pathogens (e.g., *Penicillium corylophilum* and *Phoma herbarum*) and soil fungi (e.g., *Paecilomyces lilacinus*), or fungi infecting immunosuppressed humans (e.g., *Exaphiala xenobiota*) (Gozlan et al., 2014; Yanong, 2003).

Reports of swim bladder inflammation (aerocystitis) due to fungal pathogens are rare in the literature. However, this lack of reports has been suggested to result from swim bladder examination often being overlooked during routine necropsies (Lumsden, 2006).

2.1.2. The anatomy and physiology of the swim bladder

The swim bladder (gas-bladder or air-bladder) is a gas-filled organ present in most bony fish (class Osteichthyes) (Helfman et al., 2009; Roberts, 2012). This organ locates ventral to the kidney and dorsal to the digestive tract (Helfman et al., 2009). The swim bladder works primarily as a hydrostatic organ controlling buoyancy (Helfman et al., 2009; Lumsden, 2006; Roberts, 2012). It may also play a role in respiration, sound production and reception and perception of pressure (Helfman et al., 2009; Lumsden, 2006; Roberts, 2012; Wildgoose, 2001).

Despite this, swim bladders are not essential for life as they are absent or reduced in size in many species, such as bottom-dwelling, deep-sea teleosts and fast-swimming pelagic species (Genten et al., 2009; Roberts, 2012).

Histologically, the swim bladder consists of a transitional epithelial layer, muscularis mucosa, submucosa, and serosa (Genten et al., 2009; Lumsden, 2006; Roberts, 2012). Cranially, the epithelial layer is modified, forming numerous folds comprising randomly arranged large polygonal cells with pale eosinophilic cytoplasm, in a structure known as the gas gland (Frasca et al., 2018). This gland is closely associated with a densely packed net of afferent and efferent capillaries, known as the rete mirabile (Bone & Moore, 2008; Genten et al., 2009). Together, these structures allow gas secretion into the swim bladder via the countercurrent capillary system (Genten et al., 2009). In addition, a layer of guanine crystals lines the swim bladder and makes it impermeable, such that gas is retained inside (Genten et al., 2009).

Embryonically, the swim bladder develops as a dorsal diverticulum of the foregut (Bone & Moore, 2008; Roberts, 2012).

The more primitive teleosts have maintained an embryonic connection between the foregut and the swim bladder, denoted the pneumatic duct (Roberts, 2012). Fish with a pneumatic duct are identified as physostomes (Roberts, 2012). This type of swim bladder is characteristic of sturgeons and primitive teleosts (Genten et al., 2009).

When the pneumatic duct is absent, fish are known as physoclists. This type of swim bladder occurs in advanced teleosts (Genten et al., 2009). However, during the larval stage, the pneumatic duct is present in many marine physoclists, thereby allowing larvae to swallow air at the surface and fill the swim bladder for the first time (Bone & Moore, 2008). The pneumatic duct is subsequently closed, and fish rely on intrinsic gas secretion and absorption mechanisms (Bone & Moore, 2008).

In physostomes (Fig. 2.1) with access to the water-air interface, inflation is achieved by swallowing air at the surface. The air enters the oesophagus, then passes through the pneumatic duct and reaches the swim bladder (Bone & Moore, 2008; Roberts, 2012). In physoclists (Fig. 2.2) and physostomes with no access to the water-air interface (Roberts,

2012), filling and emptying of the swim bladder occur via a secretory section (gas gland) and a resorbing section (the oval), respectively (Genten et al., 2009). Inflation results from gas release from the arterial blood to the gas gland. Gas reabsorption occurs when the oval's capillary plexus, emerging from the dorsal aorta, is exposed to gas from the swim bladder (Roberts, 2012). In addition, circular muscles contract and close off the oval, thus preventing the outflow of gases, whereas longitudinal muscles contract and expose the oval, thus allowing gas to escape (Helfman et al., 2009).

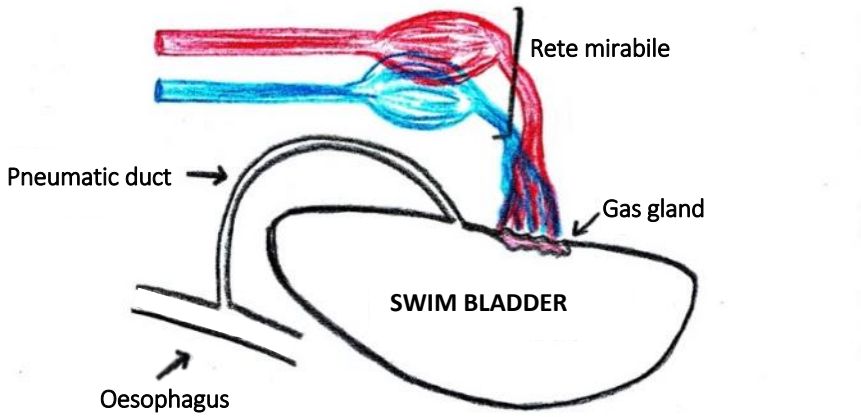

Figure 2.1. Illustration of a swim bladder from a physostomous fish. (Figures modified from Bone and Moore, 2008).

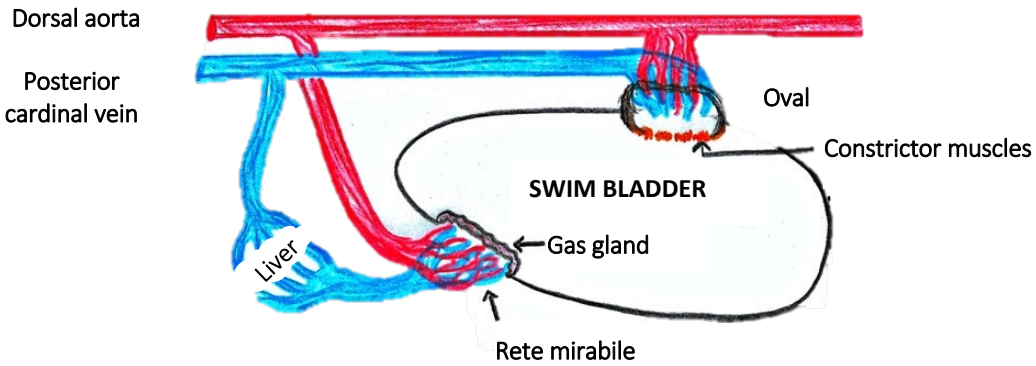

Figure 2.2. Illustration of a swim bladder from a physoclistous fish. (Figures modified from Bone and Moore, 2008).

Because of its anatomy, the swim bladder may be a common target for fungal infections.

In physostomous fish, the pneumatic duct acts as a bridge between the external environment and the swim bladder. This tubular structure enables pathogens, such as fungi, to enter the swim bladder. Primary infections may occur through direct invasion of the pneumatic duct after ingestion of contaminated food, ingestion of detritus, cannibalism of infected fish, airborne aspiration, or aspiration of contaminated water (Bruno, 1989; Lumsden, 2006; Newton, 2019).

In physoclistous fish and many physostomes, secondary aerocystitis occurs due to vascular invasion through the highly vascular gas gland (Camus et al., 2015). Vascular invasion facilitates the diffusion of fungi to the swim bladder and other organs, thereby causing systemic infection (Newton, 2019). Many fungal opportunists tend to cause vascular invasion and eventually thromboembolic events (Newton, 2019).

In addition, trauma due to intentional puncture of the swim bladder, a common practice to relieve swim bladder distention, may result in direct infection (Blaylock et al., 2001; Bowater et al., 2003; Newton, 2019). Swim bladder infections may also arise from traumatic lesions in the skin or gills, either through direct contact with adjacent tissues or hematogenous seeding (Blazer & Wolke, 1979; Camus et al., 2015; Nyaoke et al., 2009; Reuter et al., 2003).

2.1.3. Fungal aerocystitis

2.1.3.1. Phoma herbarum

Phoma herbarum (family Didymellaceae) is a saprophytic fungus and a known plant pathogen (Aveskamp et al., 2008; Bennett et al., 2018). *P. herbarum* has a ubiquitous distribution and has been isolated from vegetable debris, water sources, and inorganic material (Boerema, 1964; Boerema et al., 2004). In addition, it is an opportunistic pathogen in humans and other animals (Bennett et al., 2018)

P. herbarum is a facultative pathogen in fish, causing chronic progressive and often lethal visceral mycosis. Wood (1968) first described a swim bladder infection in juvenile Chinook

salmon *Oncorhynchus tshawytscha.* The mortality rate was 2%, and the etiological agent was identified as *P. herbarum*. Since then, this fungus has been associated with disease in fry and fingerlings of Coho salmon, *Oncorhynchus kisutch* (Ross et al., 1975); Chinook salmon (Faisal et al., 2007; Ross et al., 1975); and rainbow trout, *O. mykiss* (Řehulka et al., 2020; Ross et al., 1975). *P. herbarum* has also been isolated from Nile tilapia *Oreochromis niloticus* and detected at low prevalence in African catfish *Clarias gariepinus,* although the associated pathology has not been reported (Ali et al., 2011).

In affected fish, clinical signs include abnormal swimming behaviour and lethargy (Faisal et al., 2007; Ross et al., 1975). Physical examination indicates exophthalmia, areas of muscle softening, coelomic distention, and protruding and haemorrhagic vents (Faisal et al., 2007; Ross et al., 1975). Internally, the swim bladder is filled with a white, creamy, viscous mass (Faisal et al., 2007) Mycelia transmurally penetrate the swim bladder wall in severe cases, reaching the kidney and adjacent organs (Ross et al., 1975). The stomach contains a watery fluid in fingerlings, whereas the swim bladder is free of fluid (Faisal et al., 2007; Ross et al., 1975). In fry, opposite findings have been observed (Ross et al., 1975). Dark red areas have also been noted in the swim bladder, kidney, and contiguous muscles (Faisal et al., 2007).

Histologically, in the swim bladder, macrophages and lymphocytes are either distributed diffusely (Řehulka et al., 2020; Ross et al., 1975) or arranged in granulomas (Faisal et al., 2007). The epithelium is hyperplastic, and the lumen is filled with fungal mycelia (Faisal et al., 2007; Řehulka et al., 2020; Ross et al., 1975). In advanced cases of infection, hyphae invade the wall and lumen of the dorsal aorta, completely obliterating the swim bladder and infecting adjacent organs. Granulomatous inflammation with intralesional hyphae also occurs in the kidney, stomach and coelomic cavity (Faisal et al., 2007; Ross et al., 1975). The dorsal aorta and adjacent blood vessels are often congested with mycelia (Faisal et al., 2007; Ross et al., 1975).

Visceral mycosis caused by an unidentified species of the genus *Phoma* has been documented in farmed ayu *Plecoglossus altivelis* (Hatai et al., 1986). Grossly, the coelomic wall is opaque. Histopathological examination reveals hyphae in the swim bladder, kidney, intestine, liver, pancreas, and coelomic cavity. The swim bladder is the most affected organ, and the lumen is filled with hyphae, as well as necrotised and sloughed cells from the mucosa. In some cases, hyphae extend from the internal organs to the exterior, penetrating the skin.

The swim bladder is the primary target organ for *P. herbarum* infections (Faisal et al., 2007; Hatai et al., 1986; Ross et al., 1975). Therefore, transmission is suspected to occur during gas exchange by inhalation of conidia from infected water plants. Alternatively, ingestion of conidia from contaminated food has also been postulated (Burton et al., 2004; Faisal et al., 2007; Ochiai et al., 1977; Ross et al., 1975). *P. herbarum* may spread directly from the swim bladder into adjacent tissues (Faisal et al., 2007; Hatai et al., 1986), invade the blood vessels (Faisal et al., 2007; Ross et al., 1975), and spread systemically (Frasca et al., 2018). Nonetheless, these hypotheses are based on observations of *P. herbarum* infections from physostomous species, which have retained the pneumatic duct (Faisal et al., 2007; Ross et al., 1975).

In experimental infections, the highest mortality rates have been reported to occur after intraperitoneal injection (Ali et al., 2011; Burton et al., 2004; Easa et al., 1984; Řehulka et al., 2020), and oral (Easa et al., 1984; Ross et al., 1975) and airborne transmission (Ross et al., 1975). However, the results are not conclusive, owing to differences in experimental design.

Despite its ubiquity, *P. herbarum* is not easily transmissible under optimal environmental conditions (Burton et al., 2004; Faisal et al., 2007; Ross et al., 1975). Furthermore, the low incidence of *P. herbarum* in fish suggests that the outcomes of infection significantly depend on the host immune system (Faisal et al., 2007).

2.1.3.2. Differential diagnosis

2.1.3.2.1. Genus Exophiala

Fish from a public aquarium have been reported to develop systemic mycosis due to *E. pisciphila* infection (Blazer & Wolke, 1979). Grossly, the skin contiguous to the mandible and head presented dermal masses filled with a creamy, white material. In addition, round, raised, yellow to white, gritty areas were observed in the spleen, liver, heart, kidney, swim bladder, and brain. Histologically, the inflammatory response was characterised by necrosis surrounded by mononuclear and polymorphonuclear leukocytes (acute inflammation), or by granulomas with central areas of necrosis and calcification (chronic inflammation). In the first case, numerous hyphae were present, whereas these were rare in the chronic response.

In pretty tetra *Hemigrammus pulcher* from a public aquarium, a concurrent infection with *E. pisciphila* and *Phaeophleospora hymenocallidicola* has been reported (Řehulka et al., 2018). The fish showed anorexia, abnormal swimming motions, eroded scales, exophthalmia, and coelomic distension. The necropsy results indicated ascites, characterised by a light, amber fluid. The swim bladder was distended and thickened with a dark mass in the lumen. Microscopically, multifocal granulomas admixed with hyphae were observed in the swim bladder, liver, spleen, intestine, and kidney. An experimental study indicated that *E. pisciphila* was probably the primary pathogen in the reported cases.

E. xenobiotica has been isolated from a captive Queensland grouper *Epinephelus lanceolatus* with abnormal buoyancy and coelomic distension (Camus et al., 2015). Post-mortem examination revealed fight-induced skin lesions. In addition, the lumen of the swim bladder was filled with dry to pasty black nodules. Microscopically, the swim bladder wall was effaced by large granulomas and hyphae.

Exophiala sp. has been reported to infect wild-caught King George whiting *Sillaginodes punctatus* kept in indoor tanks (Reuter et al., 2003). Grossly, the skin showed white, necrotic foci and deep ulcers with necrosis of the underlying bones. Swim bladder distension with watery fluid and thickening of the wall were observed. The swim bladder mucosa was covered with soft, white plaques. In the kidney, multifocal areas of necrosis extended to the adjacent skeletal muscle. Histologically, the skin, bone, swim bladder, and kidney presented similar changes characterised by multifocal areas of necrosis, granulomas, and intralesional fungi.

Exophiala spp. are opportunistic pathogens occurring primarily in diseased, injured, or environmentally stressed fish (Camus et al., 2015).

Infections may result from traumatic injuries in the skin or gills (Blazer & Wolke, 1979; Camus et al., 2015; Nyaoke et al., 2009; Reuter et al., 2003), then spread to other organs after haematogenous seeding (Camus et al., 2015; Nyaoke et al., 2009). As with *Phoma* spp., infection via the oral route has also been suggested, either through dissemination from the intestinal mucosa to the visceral organs or via the pneumatic duct (Řehulka et al., 2018). In experimental studies, fish exposed to suspended hyphae have not been found to develop the disease, in contrast to fish intraperitoneally injected with spores (Blazer & Wolke, 1979; Řehulka et al., 2018).

2.1.3.2.2. Phialophora sp.

Phialophora sp. have been found to infect Atlantic salmon *Salmo salar* parr from a freshwater hatchery. Initially, the fish sank to the bottom and rapidly died (Ellis et al., 1983). Gross lesions included haemorrhages at the base of the fins, skin petechiae, and distended, hyperaemic vents. The coelomic cavity was filled with fluid and white to pink, firm masses adhered to the coelomic organs. Adhesions between the swim bladder and the visceral organs were also observed. The swim bladder wall was opaque, and the lumen was filled with white mucoid material. The kidney was grey and enlarged. Histologically, the mucosa of the swim bladder was ulcerated and haemorrhagic, with minimal inflammation. In the lumen, numerous hyphae and conidia were present. In addition, necrosis with intralesional hyphae was observed in the kidneys. Nonetheless, hyphae mainly invaded the blood vessels.

2.1.3.2.3. Isaria farinosa

Formerly known as *Paecilomyces farinosu*s, *I. farinosa* has been associated with aerocystitis and sporadic mortality in Atlantic salmon parr (Bruno, 1989; Lehmann et al., 1999). The affected fish presented reddening of the vent and distended celomic cavity. In addition, the swim bladder was thickened, distended, and filled with a white mass. Haemorrhaging around the swim bladder was also reported. Histological findings revealed hyphae infiltrating and effacing the swim bladder wall from the epithelial to the outer fibrous layer.

Since *I. farinosa* has been used as a biological control for insects, swim bladder infections in salmon may occur due to the consumption of infected insect larvae from the water surface (Bruno, 1989).

2.1.3.2.4. Penicillium corylophilum

P. corylophilum and *Cladosporium sphaerospermum* have been isolated from two marine, wild-caught, tank-held red snappers *Lutjanus campechanus.* Clinically, the fish displayed erratic swimming behaviour (Blaylock et al., 2001). Gross lesions included distension of the swim bladder with deposition of chalky white material and a discrete ulcer in the wall. Histologically, the swim bladder and kidneys presented foci of macrophages, lymphocytes, and polymorphonucleated leukocytes. Granulomas, haemorrhage, hyperplasia, necrosis, and hyphae were also observed. Despite the concurrent infection with *C. sphaerospermum,* only *P. corylophilum* was isolated from the swim bladder.

Introduction of contaminated material during puncture of the swim bladder to alleviate barotrauma was the suggested source of the infection. However, the infection has also been hypothesised to have been present before capture, and fungal expansion might have been potentiated by the stress of captivity or a compromised immune system.

2.1.3.2.5. Cladosporium sphaerospermum

An aerocystitis caused by *C. sphaerospermum* has been reported in a wild mullet *Mugil cephalus* (Sirri et al., 2016). Externally, a white, soft, exophytic mass protruded from an ulcerated area in the skin. This structure had multiple gas-filled cysts on the cut section and connected to the swim bladder through a funnel-shaped tissue. Histologically, the mass was characterised by multifocal cysts contiguous with the subepithelial rete mirabile and supported by abundant fibrous tissue. The skin adjacent to the exophytic mass (herniated swim bladder) was focally ulcerated and replaced by abundant granulation tissue and granulomas containing hyphae. Granulomas were also observed in the internal portion of the swim bladder, spleen, and pancreas. The source of the infection was probably a vegetal foreign body found in the herniated tissue.

Both *Cladosporium cladosporioides* and *Scopulariopsis brumptii* have been isolated from skin wounds in a wild-caught mature barramundi cod *Cromileptes altivelis* (Bowater et al.,

2003). While held in captivity, anorexia, lethargy, and difficulty in maintaining buoyancy were observed. Gross examination revealed a severely distended swim bladder with a large, olive-green to black, powdery mass filling almost the entire lumen. Histologically, multifocal granulomas with intralesional hyphae were present in the epithelium of the gas gland and extended through the swim bladder wall.

The source of the infection was not determined, but fungi have been suggested to have reached the swim bladder via traumatic injection with a contaminated hypodermic needle. Punction of the swim bladder is a common practice among fishers to prevent fish from floating on the tank surface after capture (Bowater et al., 2013).

2.1.3.2.6. Verticillum lecanii

A primary aerocystitis caused by *V. lecanii* has been documented in farmed Atlantic salmon parr (Aho et al., 1988). The clinically affected fish showed abnormal swimming motion, and infections were associated with low mortality. Grossly, the affected swim bladder had white to yellow, opaque, thickened walls. Haemorrhage was also observed. The lumen contained white fluid tinged with blood and in some cases a firm, white to yellow mass. Histological examination revealed erosion and ulceration of the epithelium where hyphae were present. In addition, necrotic debris, fibrin, fungal mycelia, and bacteria were present in the lumen. However, the presence of bacteria was considered a secondary event because the infection occurred at low temperatures, and primary bacterial infections are rarely seen under such circumstances (Aho et al., 1988).

2.1.3.2.7. Sporobolomyces salmonicolor

An extensive visceral mycosis with aerocystitis has been documented in Chinook salmon fry from a hatchery (Muench et al., 1996). Anorexia and increased mortality have been reported. During necropsy, coelomic distension was observed, owing to the presence of serosanguineous fluid. Histologically, the epidermis was eroded and ulcerated, thus allowing

water to enter the underlying connective tissue and muscle. Hyphae were observed in the epidermis, dermis, and hypodermis, with associated small aggregates of multinucleated cells. In the swim bladder, granulomas admixed with mats of fungal hyphae and pseudohyphae were present. These lesions extended into the kidneys, intestine, and muscles of the coelomic wall.

2.1.3.2.8. Ochroconis humicola

Ochroconis humicola has been isolated from the swim bladder of a dead mature barramundi cod after a force-feeding procedure (Bowater et al., 2003). Gross examination indicated that the swim bladder was primarily replaced by a large mass that extended to the adjacent kidney. Multiple abscesses were visible in the liver and spleen, together with fibrinous peritonitis with blood-tinged coelomic fluid. Histologically, numerous multifocal granulomas containing fungal hyphae were observed in the swim bladder, liver, kidney, and spleen. As in previous reports, puncture with a contaminated hypodermic needle was suggested to be the most likely source of infection (Bowater et al., 2003).

2.1.4. Specific objectives

The main objectives of this study were to:

a. Identify the external and internal gross and histological lesions produced by an opportunistic fungus in wild greater amberjack.

b. Determine the potential route of entry for fungi in physoclistous fish.

c. Identify the etiological agent.

2.2. **Scientific publication II:** A Study on the Pathological Effects of Trypanorhyncha Cestodes in Dusky Groupers *Epinephelus marginatus* from the Canary Islands

2.2.1. Introduction

For the past few decades, the marine environment has suffered from the effects of anthropogenic activities (H. W. Palm, 2011). Fishing pressure, habitat degradation, and pollution have altered fish population structures and the broader ecosystems (Brander, 2010; H. W. Palm, 2011). The effects of climate change, including rising sea levels, increasing water temperature, and salinity variations, have also affected parasite composition and distribution (H. W. Palm, 2011; Poulin, 2007).

Because the occurrence and abundance of parasites are strictly associated with the distribution, migration patterns, and population biology of their hosts, parasites may be valuable bioindicators for environmental changes (H. W. Palm, 2011). Studies of fish parasites are therefore essential for fully understanding how these parasites affect fish health, marine ecosystems, and public health.

2.2.2. Class Cestoda: order Trypanorhyncha

There are over 1400 species of cestodes described in marine habitats (Caira & Reyda, 2005). Current taxonomic classifications divide the order Trypanorhyncha into five superfamilies and 15 families (J. Y. Zhang et al., 2017), comprising some 350 documented species of which 95% infect marine species (Caira & Reyda, 2005).

Parasites of the class Cestoda are commonly known as tapeworms on account of their dorsoventrally flattened, tape-like body (Bowman, 2014; Mehlhorn, 2016). Because they are hermaphrodites with an acoelomate parenchymatous body, cestodes (Fig. 2.3a) resemble trematodes (Fig. 2.3b) (Bowman, 2014). However, unlike trematodes, cestodes lack a digestive tract and instead absorb nutrients through a specialised integument (Bowman, 2014; Mehlhorn, 2016).

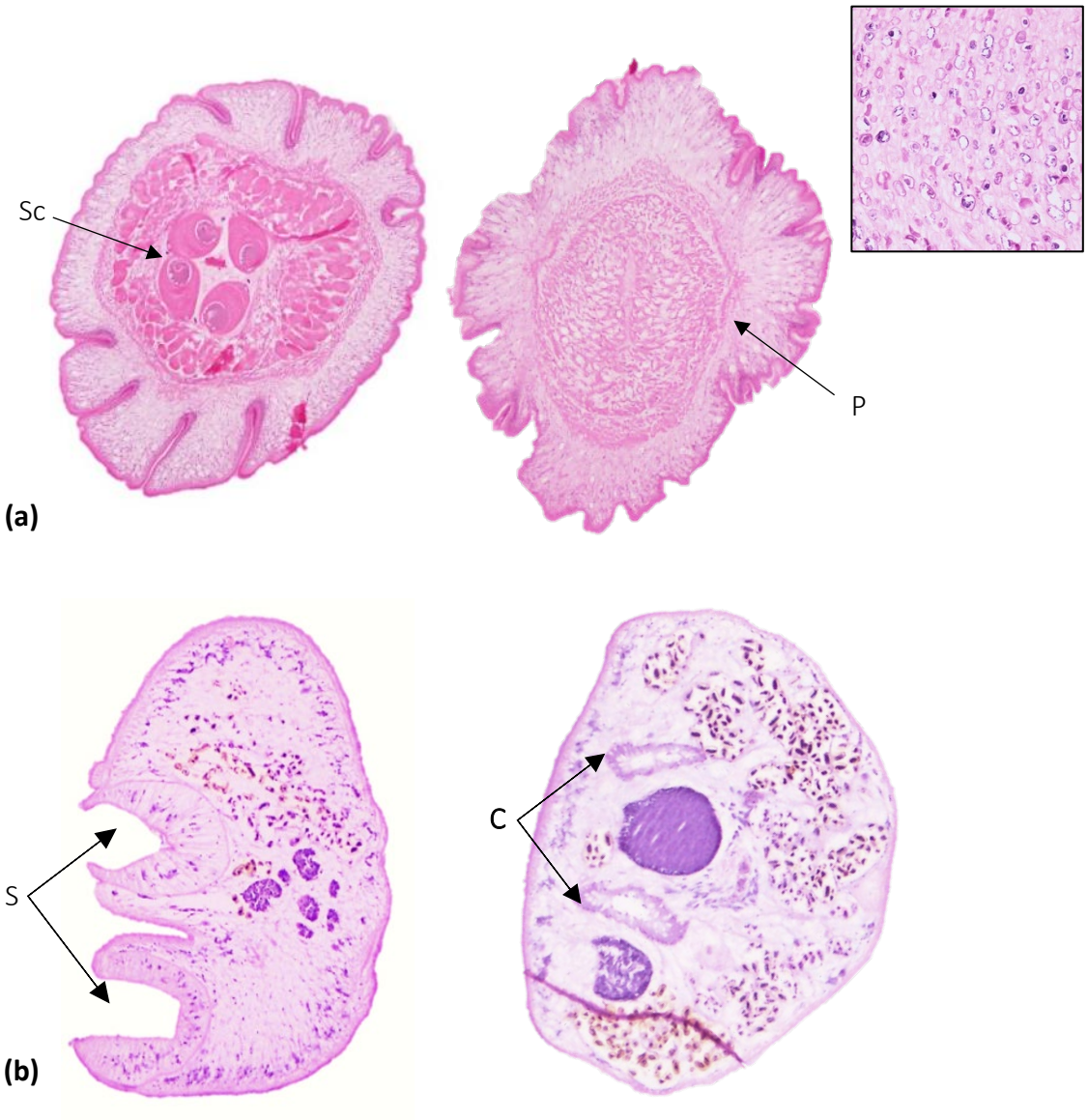

Figure 2.3. (a) Transversal sections of a cestode with a scolex (Sc), solid parenchyma (P) and embedded calcareous corpuscles (inset, 20x) (HE, 4x). **(b)** Transversal sections of a trematode with suckers (S) and paired intestinal caeca (C) in the parenchyma (HE, 4x). (Personal collection).

Trypanorhynchs are characterised by a scolex comprised of two or four bothria (Caira & Reyda, 2005; Grabda, 1991) and a tentacular apparatus (Mehlhorn, 2016) with four retractable tentacles (Fig. 2.4). Each tentacle has numerous hooks that are arranged in complex patterns and adapted to specific attachment sites inside the parasite's host (Campbell & Beveridge, 1994; Mehlhorn, 2016). The hook pattern along the tentacles is identical in both the plerocercus and adult stages of trypanorhynchs, thus enabling the easy identification of these parasites (Mehlhorn, 2016).

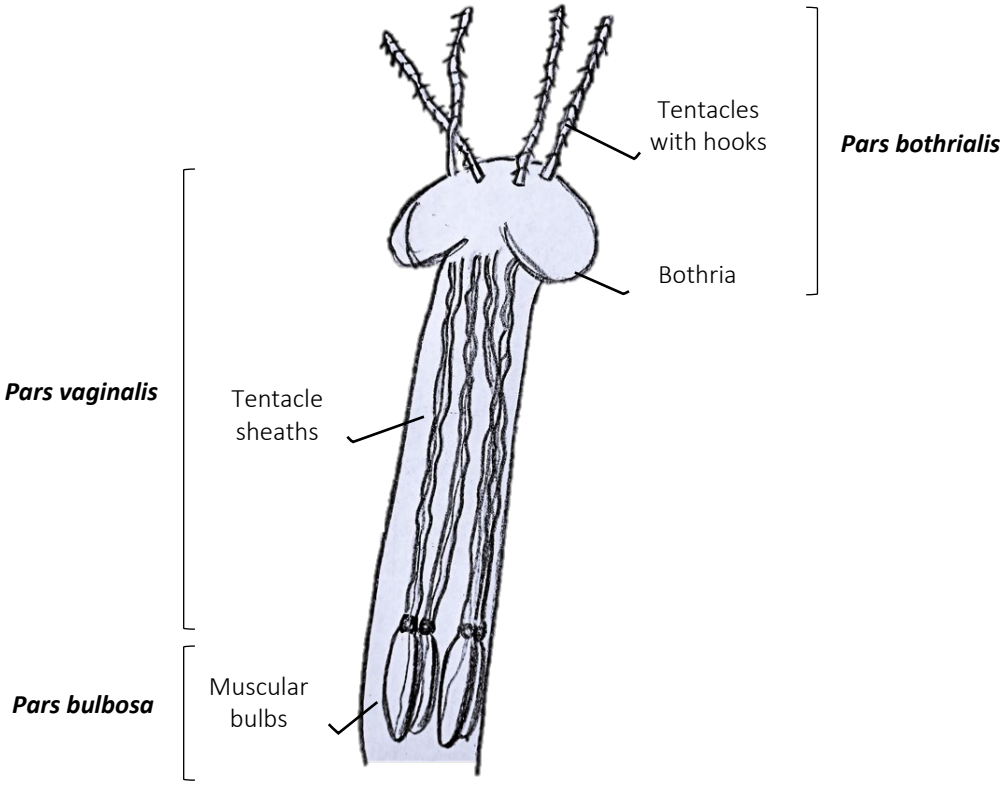

Figure 2.4. Scolex of a Trypanorhyncha. (Figure modified from Campbell & Beveridge, 1994).

Trypanorhyncha have complex life cycles (Fig. 2.5) (Mehlhorn, 2016). The adult parasite inhabits the gastrointestinal tract of elasmobranch fish, which are the parasite's definitive host (Tamaru et al., 2016). The body of the adult cestode is segmented into numerous proglottids, each containing a single set of reproductive organs (Roberts, 2012). Proglottids with eggs, are shed from the posterior end of the parasite (Bruno et al., 2013). Eggs can be either operculated or non-operculated.

Operculated eggs are not embryonated when laid and instead develop into coracidium in the water (Mehlhorn, 2016). When a coracidium is ingested by a first intermediate host, such as copepods and other small crustaceans, it migrates to the body cavity and matures into a procercoid (Caira & Reyda, 2005; Grabda, 1991).

In contrast, non-operculated eggs contain a fully developed embryo that is released from the eggshell when the egg is eaten by the first intermediate host. From there, the embryo develops into a procercoid (Caira & Reyda, 2005; Mehlhorn, 2016).

Once the procercoid is ingested by a suitable host at a higher trophic level, such as teleost fish or invertebrates, the procercoid penetrates through the stomach and intestinal wall and encysts in the coelomic cavity or skeletal muscle. The procercoid then develops into a plerocercus (or, in some species, a plerocercoid or merocercoid) (Moser et al., 1984; Palm & Caira, 2008; Roberts, 2012).

Teleost fish act as second intermediate or paratenic hosts. Specifically, small planktivorous teleost fish can serve as intermediate hosts, whereas larger piscivorous teleost fish act as paratenic hosts. Larger teleost fish function as a bridge between the smaller teleost fish and the final host (Caira & Reyda, 2005). Plerocerci do not undergo further development while in paratenic hosts.

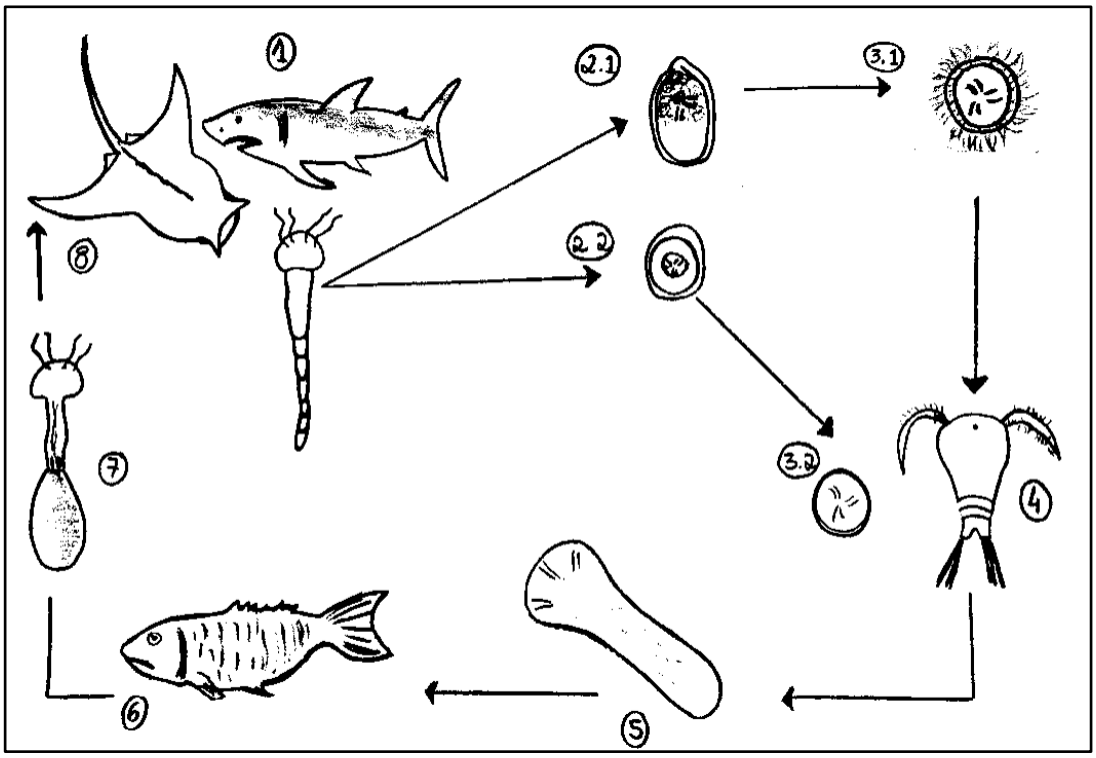

Figure 2.5. Postulated life cycles for Trypanorhyncha. (1) Adult stages inhabit the digestive tract of elasmobranchs. From there, they can lay two types of eggs – (2.1) operculated or (2.2) non-operculated. (3.1) Operculated eggs hatch in the water and develop into a coracidium, whereas (3.2) non-operculated eggs only hatch when ingested by a crustacean, shedding an embryo. (4) In the crustacean, both the coracidium and the embryo mature into (5) a procercoid. (6) Once the crustacean is ingested by a teleost, the procercoid migrates to the coelomic wall and muscle where it develops into (7) a plerocercus (plerocercoid or merocercoid in some species). (8) When the teleost is finally ingested by an elasmobranch, the plerocercus larva matures into the adult stage. (Diagram information obtained from Caira & Reyda, 2005; Grabda, 1991; Melhorne, 2016).

Pintneriella is a genus of Trypanorhyncha within the family Rhopalothylacidae (Palm et al., 2009; Palm & Caira, 2008). Four species of *Pintneriella* have been identified to date: *P. musculicola, P. gymnorhynchoides, P. pagelli*, and *P. maccallumi* (Palm, 2004). The adult stage of these cestodes inhabits the gastrointestinal tract of elasmobranchs (Tamaru et al., 2016), where it releases a free-swimming coracidium into the marine environment (Palm et al., 2017; Rohde, 2005). The coracidium then matures into a procercoid and a plerocercus (Tamaru et al., 2016), as described above.

2.2.3. Pathological changes in infections with Trypanorhyncha

Despite numerous studies detailing the biology of Trypanorhyncha, there are few reports on the pathological effects of Trypanorhyncha in fish.

In groupers, Trypanorhyncha larvae have been shown to infect the coelomic cavity, visceral organs, and skeletal muscle. In skeletal muscle, *P. musculicola* larvae (Hassan et al., 2002) and *Floriceps* sp. (Ibrahim, 2000) cause lymphocyte infiltration at the attachment site and focal oedema, degeneration, atrophy, and necrosis in the adjacent myofibers. In the coelomic cavity, encysted larvae are surrounded by concentric layers of dense connective tissue and lymphocytes (Ibrahim, 2000). Larvae have also been observed in the liver, which was decreased in size relative to healthy fish (Ibrahim, 2000). By contrast, other studies have not observed pathological changes in groupers infected with Trypanorhyncha (Rizgalla, 2016; Soliman et al., 2011).

For other fish species, previous studies have reported parasite encapsulation by dense connective tissue as well as macrophage and lymphocyte infiltration (Abdelsalam et al., 2016; Santoro et al., 2018; Sattari et al., 2014). Similar studies have noted serpiginous-shaped necrosis and fibrosis caused by larval migration (Santoro et al., 2018), as well as extensive fibrosis with peritoneal adhesions (Abdelsalam et al., 2016).

Trypanorhynch larvae are usually enclosed within cysts that are white-to-yellow (Beveridge et al., 2014; Hassan et al., 2002; Ibrahim, 2000). However, brown to black nodules have also been often reported. The latter type of nodule typically contains larval remnants (Beveridge et al., 2014; Ibrahim, 2000; Rizgalla, 2016). The dark colour results from the deposition of pigmented necrotic debris and calcification, similar to what has been observed in infections with other cestodes (Arme & Owen, 1968, 1970; McAdam et al., 2015; Rizgalla, 2016). In some species of serranids, fibrotic encapsulation has been associated with the deposition of ceroid, lipofuscin, and melanin pigments (Overstreet & Thulin, 1989).

This type of response allows the parasite to be separated from the host tissues, preventing further tissue damage. Darker nodules are likely the result of a host-initiated immune response mounted to isolate and kill the parasites (Rigby & Dufour, 1996). However, in severe infections, extensive fibrosis may cause compression and atrophy of adjacent organs (Lumsden, 2006), eventually leading to organ malfunction and death (Ackermann, 2012).

2.2.4. Differential diagnosis

Similar tissue responses occur in fish infected with the cestodes *Diphyllobothrium* spp., *Ligula* spp. (order Diphyllobothriidea), or *Paradilepis scolecina* (order Cyclophyllidea).

Previous studies have observed a short acute phase characterised by congestion, oedema, and haemorrhage in the areas adjacent to cysts of *Diphyllobothrium* spp. (Otto & Heckmann, 1984). Migration tunnels have also been reported in the stomach and intestine (Halvorsen, 1970; Sharp et al., 1992).

In the first phases of *Diphyllobothrium* spp infection, lymphocytes, macrophages, and eosinophilic granular cells are mobilised to the infection site (Sharp et al., 1989, 1992). However, neutrophil infiltration is typically short-lived and diminishes as chronic inflammation develops (Sharp et al., 1989). The surrounding tissues completely encapsulate the larvae and undergo angiogenesis (Halvorsen, 1970; O'Neill et al., 1988; Sharp et al., 1992). The walls of the parasite cysts are characterised by the deposition of fibroblasts with an extracellular collagenous matrix and of mononuclear inflammatory cells, especially activated macrophages with abundant cytoplasm (i.e., epithelioid cells) (Sharp et al., 1992). In contrast with mammals, multinucleated giant cells (Ackermann, 2012; McAdam et al., 2015) are not commonly observed in fish. Larvae progressively separate from the cyst wall, which slowly becomes thicker (Halvorsen, 1970).

Peritoneal fibrosis occurs in areas adjacent to degenerated parasites, whereas cysts with intact larvae do not cause significant reactions (Otto & Heckmann, 1984). Tissues adjacent to the degenerated parasites are often infiltrated by macrophages and lymphocytes (O'Neill et al., 1988; Otto & Heckmann, 1984). In mammals infected with cysticerci, cysts similarly evoke little host inflammatory response when intact. However, once the cysts degenerate, the surrounding tissues demonstrate inflammation followed by focal scarring and calcification (McAdam et al., 2015). Fibrous peritonitis (van Kruiningen et al., 1987), with viscera adhering to the body wall, has also been reported in heavily infected fish (Sharp et al., 1989).

The first stages of *Ligula intestinalis* infections are characterised by massive deposition of fibroblasts, macrophages, and "polymorphonuclear leukocytes" (Arme & Owen, 1968, 1970). As the infection progresses, the number of inflammatory cells decreases and the

deposition of connective tissue around the larvae increases (Arme & Owen, 1968, 1970; Sweeting, 1977).

Paradilepis scolecina produces a similar response, with parasite encapsulation and a mild inflammatory reaction evidenced by lymphocyte activity. Degenerated parasites with hook fragments have often been observed surrounded by pigmented macrophages (Williams et al., 2012).

Nematodes such as *Philonema spp.* (Lumsden, 2006; Molnár et al., 2006) and *Philometra* spp. (Fig. 2.6) can also be found encapsulated in the coelomic cavity and visceral organs of fish (Molnár et al., 2006). Both parasites have been associated with severe peritonitis with visceral adhesions (Densmore, 2019; Roberts, 2012).

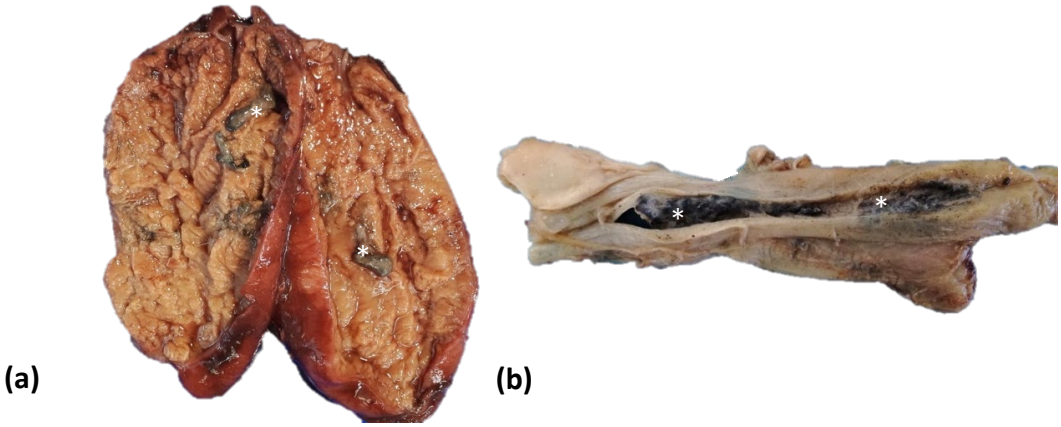

Figure 2.6. **(a)** Ovary of greater amberjack and **(b)** testis of dusky grouper with dark brown to black, firm, and round to irregular nodules (*) containing degenerated nematodes, likely *Philometra* spp. (Photographs courtesy of Miguel Rivero, IUSA, ULPGC).

Overall, migrating parasites may trigger minimal inflammation, but they can also activate peritoneal macrophages, promote migration of neutrophils and eosinophilic granular cells, and eventually produce extensive adhesions between abdominal organs (Bruno et al., 2013; Lumsden, 2006; Noga, 2010).

Even though microsporidia are currently classified as fungi, *Glugea* sp. and *Pleistophora* sp. produce similar brown or black nodules of various sizes in the coelomic cavity and visceral

organs of groupers (Fig. 2.7) (*Database of Parasites in Fish and Shellfish*, n.d.; Jithendran et al., 2011; W. S. Soliman et al., 2011; J. Y. Zhang et al., 2005). However, these nodules are filled with spores that can be easily identified in wet mounts or histological sections (Cruz-Lacierda & Erazo-Pagador, 2004; Noga, 2010).

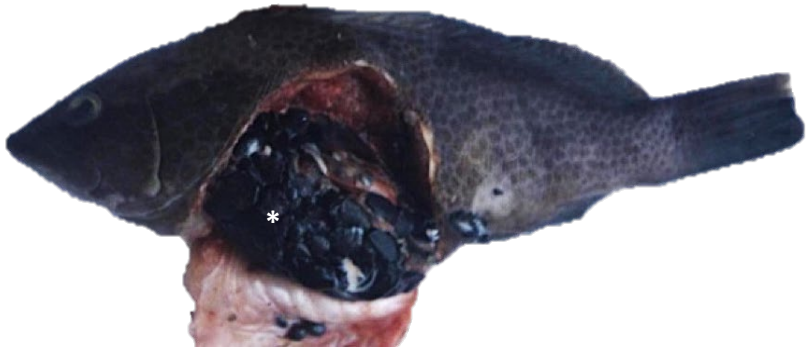

Figure 2.7. Black nodules of microsporidia *Glugea* sp. (*) in the coelomic cavity of Hong Kong grouper *E. akaara*. (Photograph edited from the original by Jin-Yong Zhang via Database of Parasites in Fish and Shellfish, n.d., and Zhang et al., 2005).

2.2.5. Trypanorhyncha in groupers

Dusky groupers *Epinephelus marginatus* are keystone species necessary for the preservation of several marine ecosystems (Condini et al., 2018). Determining the pathological effects caused by Trypanorhyncha in dusky groupers is especially vital because dusky groupers are considered vulnerable and several efforts have been made to culture them (Condini et al., 2018; Pollard et al., 2018).

Several Trypanoryncha species have been documented in groupers (subfamily Epinephelinae, family Serranidae) from numerous regions of the world. Some of the most commonly reported species include *Callitetrarhynchus gracilis* (Abdou & Palm, 2008; Al-Zubaidy & Mhaisen, 2011; Beveridge et al., 2014; Justine et al., 2010; Kleinertz et al., 2014; Kleinertz & Palm, 2015; Neubert et al., 2016), *Floriceps minacanthus* (Abdou & Palm, 2008; Beveridge et al., 2014; Justine et al., 2010), *Pseudotobothrium dipsacum* (Beveridge et al.,

2014; Haseli et al., 2011; Justine et al., 2010) and *Pintneriella musculicola* (Beveridge & Campbell, 2000; Haseli et al., 2011).

Despite their global prevalence, Trypanorhyncha species have not yet been documented in groupers in the Canary Islands. Nevertheless, one study reported a severe *Gymnorhynchus gigas* infection in the skeletal muscle of the Atlantic pomfret *Brama brama* (Grabda, 1991). In the same region, *Floriceps saccatus* infected the coelomic cavity and visceral organs of the common dolphinfish *Coryphaena hippurus* (Carbonell et al., 1998). *Pseudogrillotia epinepheli* was found attached to the visceral organs of a blacktail comber *Serranus atricauda* (family Serranidae) from the Madeira Islands, near the Canary Islands (Costa et al., 2013). Larval stages of *Tentacularia coryphaenae*, *Sphyriocephalus tergestinus*, *Nybelinia lingualis*, and *Grillotia* sp. have also been reported in other fish species (Costa et al., 1996, 2003, 2016).

Pintneriella gymnorhynchoides has been reported in the Portuguese dogfish (*Centroscymnus Coelolepi*) from the Azores (Beveridge & Campbell, 2003), and *P. maccallumi* has been found in smooth-hounds (*Mustelus* spp.) from the east Atlantic Ocean (Palm, 2004). Given that both elasmobranch species are present in the region, they may act as final hosts for *Pintneriella* sp.

2.2.6. Specific objectives

Assessment of the health condition of wild populations is vital, not just for wild stocks, but also for cultured fish that are also susceptible to infectious agents transmitted by broodstock that naturally live in or in the surroundings of the net cages (Rückert et al., 2009). Even though these infections do not appear to represent a threat to human health, recent research has shown that ingestion of fish with Trypanorhyncha may cause allergic disorders (Gòmez-Morales et al., 2008; Mattos et al., 2015; Rodero & Cuéllar, 1999). In addition, severe cestode infections may reduce the fish market value by making them unappealing to consumers (Tamaru et al., 2016). In line with this, the main objectives of this study were to:

a. Determine the prevalence of Trypanorhyncha in dusky groupers caught in the Canary Islands.

b. Assess the pathological changes produced by these parasites in dusky groupers.

2.3. **Scientific publication III:** An end to the controversy over the microscopic detection and effects of pristine microplastics in fish organs

2.3.1. Plastics and microplastics

Along with climate change (Provencher et al., 2017), the pervasive presence of plastic in the aquatic and terrestrial environments has become one of the most significant environmental challenges (Cole et al., 2011).

The ever-growing plastic production, copious use of single-use plastic products, indiscriminate waste disposal, and accidental releases have contributed to the accumulation of plastic in the natural environments (Cole et al., 2011; Wright et al., 2013). In addition, plastics' low degradation rates greatly contribute to their persistence in the environment for long periods, constituting a long-standing issue (Cole et al., 2011; Duis & Coors, 2016).

Plastic debris enters the aquatic environment from land-based sources, such as industrial and urban effluents, landfills, runoff, and neighbour fields (Auta et al., 2017; Avio et al., 2017; Barboza, Dick Vethaak, et al., 2018; Murphy et al., 2016). Extreme weather events, such as flooding or hurricanes, can intensify the transfer of plastic debris to the aquatic environment (Cole et al., 2011). Plastic can also enter the aquatic environment directly from offshore industrial activities, recreational and commercial fishing, and litter released during sea activities, such as aquatic and coastal tourism (Barboza, Dick Vethaak, et al., 2018; Cole et al., 2011).

Microplastics (MPs) are small plastic particles measuring up to 5 mm in diameter (Auta et al., 2017). They can be manufactured specifically to be small, for domestic or industrial purposes, such as in exfoliating scrubs or the ones used to clean machinery and boat hulls (Auta et al., 2017; Browne et al., 2007; Cole et al., 2011). Even more widespread in the aquatic environment are the secondary MPs. These typically result from the fragmentation of larger plastics. Fragmentation occurs during use or after disposal when exposed to photolytic, mechanical, and biological degradation (Andrady, 2017; Auta et al., 2017; Browne et al., 2007).

Despite their ubiquitous prevalence in the aquatic environment, most approaches to determine the number of MPs are underestimated (Jovanović, 2017). The use of different mesh sizes to sample MPs predictably leads to different cut-off sizes for the analysed MPs and

is a recurring limitation (Andrady, 2017; Jovanović, 2017). In most reports, particles collected have a diameter above 300 µm (Andrady, 2017; Crawford & Quinn, 2017; Hidalgo-Ruz et al., 2012; Li et al., 2018) excluding smaller particles in the range of 1 µm to 300 µm.

2.3.2. MPs in the aquatic environment

In 1972, Carpenter & Smith (1972) reported the presence of tiny plastic particles in the Northwest Atlantic Ocean. In the same year, microbeads were found in the digestive tracts of several fish species from the coastal waters of southern New England (Carpenter et al., 1972). Since then, MPs have been detected in the oceans and seas (Barboza et al., 2020; Colton et al., 1974; Cózar et al., 2015; Desforges et al., 2014; Faure et al., 2012; Herrera et al., 2020; Reisser et al., 2013), estuaries (Lima et al., 2014; Pazos et al., 2017; Yonkos et al., 2014), rivers (Castañeda et al., 2014; Dris et al., 2015; Horton et al., 2017; Klein et al., 2015; Mani et al., 2015; Moore et al., 2011), lakes (Baldwin et al., 2016; Biginagwa et al., 2016; Corcoran et al., 2015; Eriksen et al., 2013; Faure et al., 2012, 2015; Fischer et al., 2016; Free et al., 2014; Sruthy & Ramasamy, 2017; Su et al., 2016; K. Zhang et al., 2016), beaches and coastal areas (Browne et al., 2011; Claessens et al., 2011; Dekiff et al., 2014; Herrera et al., 2018; Imhof et al., 2013; Rapp et al., 2020) and even in the remote polar waters (Lusher, Tirelli, et al., 2015).

MPs are of particular concern among plastic litter due to their small size. Small plastics are a potential prey even for small organisms that may accumulate and transfer MPs through the food web (Batel et al., 2016, 2020; Setälä et al., 2014; Wright et al., 2013). In addition, the high prevalence of MPs in the aquatic environment makes aquatic organisms prone to ingest them inadvertently or willingly. The ingestion of MPs has been documented in several wild aquatic species worldwide. From crustaceans (Desforges et al., 2015; Devriese et al., 2015) to fish (Bellas et al., 2016; Bessa et al., 2018; Foekema et al., 2013; Herrera et al., 2019; Jabeen et al., 2017; Tanaka & Takada, 2016), marine turtles (Duncan et al., 2019), and marine mammals (Besseling et al., 2015; Lusher, Hernandez-Milian, et al., 2015; Montoto et al., 2021). Even farmed aquatic animals have been shown to ingest MPs (Cheung et al., 2018; Feng et al., 2019; Reinold et al., 2021).

Dietary habits, concentration of MPs, availability of food in the environment (Collard et al., 2019; Kim et al., 2019), confusion with prey (Barboza, Dick Vethaak, et al., 2018) as well as the size of the particles may influence the ingestion of MPs (Collard et al., 2019). Furthermore, it seems that MPs are easily ingested when mixed with food (Ory et al., 2018).

2.3.3. Retention time and uptake

Retention time is an essential factor to consider when assessing the potential for MP accumulation in organisms. A high retention time means prolonged exposure to exogenous material with toxic potential. Furthermore, prolonged exposure may increase both pathological effects over time and the probability of trophic transfer (Bour et al., 2020).

In studies exposing fish to MP particles of various sizes, it was observed that microbeads ranging from 6 to 200 µm were excreted predominantly after 24–96 hours (Bour et al., 2020; Cong et al., 2019; Elizalde-Velázquez et al., 2020; Grigorakis et al., 2017; Jovanović et al., 2018; Mazurais et al., 2015). In contrast, larger microbeads (2–5 mm) and micro-fragments (up to 1.2 ± 0.2 mm) took longer, with a half-life of up to 13 days (dos Santos & Jobling, 1991; Ory et al., 2018). Elimination of microfibres (up to 500 µm) was achieved after 33.4 to 48 hours (Bour et al., 2020; Grigorakis et al., 2017). Therefore, retention time appears to be directly proportional to particle size. However, retention time is also likely to increase after multiple intakes (dos Santos & Jobling, 1991).

From a biological perspective, the attachment of particles to external surfaces (e.g., gills) or their transfer into the lumen of the gastrointestinal tract must be regarded as external to the body (Triebskorn et al., 2019). Nonetheless, some researchers (Lu et al., 2016) have used the term ‘uptake’ when describing MPs adhering to soft tissues like the intestinal epithelium or gills. The terms ‘uptake’ and ‘translocation’ should only apply when particles have penetrated either cells or tissues beyond the epithelial surface (Triebskorn et al., 2019).

Uptake of 10–20 µm MPs into the epithelial cells of the intestine (enterocytes) has been reported in zebrafish *Danio rerio* (Batel et al., 2016). However, complementary analyses were not undertaken on the specimens to confirm the internalisation of these particles.

The routes through which MPs cross the epithelial layer in fish are not clear. In mammals, paracellular and transcellular pathways have been suggested as possibilities. In a paracellular pathway, uptake would occur between cells through intercellular junctions and spaces; in a transcellular uptake, absorption would occur through the microvillous border (Carr et al., 2012).

The paracellular route is the most likely for the passage of larger particles in mammals (Volkheimer, 1977). Severe tissue injuries may also facilitate microparticle uptake (Triebskorn et al., 2019). In fish, intestinal inflammation (enteritis) and erosion may increase the permeability of the mucosal epithelium, thus facilitating the passage of particles through damaged tissue. Responses to acute and subacute injuries are often expressed as epithelial hyperplasia. In chronic cases, enterocyte detachment from the basement membrane, loss of enterocytes, goblet cell hyperplasia and epithelial metaplasia may be present (Handy et al., 2008; Peterson, 2015; Volkheimer, 1975).

Transcellular uptake occurs mainly via M cells in Peyer's patches and gut-associated lymphoid tissue (GALT) (Behrens et al., 2002; Hussain et al., 2001). Fish do not have organised GALT, but instead have lymphoid cells and occasional macrophages scattered throughout the epithelium and lamina propria. Until recently, it was believed that fish lacked M cells; however, recent studies with salmonids (Fuglem et al., 2010) and zebrafish (Løvmo et al., 2017) identified specialised enterocytes with M cell-like activity in the distal region of the middle intestine. In fish, this region is the primary site for the uptake and transfer of macromolecules to closely associated intra-epithelial macrophages (Løvmo et al., 2017). In zebrafish, these specialised enterocytes have large supra-nuclear vacuoles (Løvmo et al., 2017) and may deliver luminal contents to scattered immune cells underneath the epithelial layer (Brugman, 2016). Nanoparticles (NPs) have been detected in the phagocytes of the lamina propria and supranuclear vacuoles of specialised enterocytes (Rességuier et al., 2017). However, phagocytic activity was not limited to these cells and was also observed in regular enterocytes (Løvmo et al., 2017).

The role of goblet cells in the uptake of microparticles in mammals has also been discussed. These cells may have looser tight junctions and hence be more permeable to small particles (Carr et al., 2012).

The transcellular route has been suggested for the uptake of NPs in fish. Since fish enterocytes can take up larger materials by endocytosis than those of mammals (Handy et al., 2008; Jovanović & Palić, 2012), the uptake of small MPs can also be speculated.

2.3.4. Particle translocation

After uptake, plastic particles need to be translocated into tissues before any plastic-associated adverse effects are seen (Triebskorn et al., 2019).

The translocation of MPs into the liver has been reported to occur in different fish species over the years. Avio et al. (2015) observed polyethylene (PE) and polystyrene (PS) MPs ranging from 200 µm to 600 µm in the liver of mullets. Similarly, MPs ranging from 100 µm to 567 µm were also claimed to have been found in the livers of other fish species (Abbasi et al., 2018; Collard et al., 2017, 2018).

The translocation of such large particles is difficult to explain with current knowledge on translocation pathways for MPs in fish. The transfer of exogenous particles sized 100–600 µm from circulation in blood to tissues would likely cause an inflammatory response in the surrounding areas (Batel et al., 2016). However, this has not been documented in any of the abovementioned studies.

Studies investigating smaller particles (0.5–5 µm) have also reported translocation of MPs to the liver. For example, a study exposing red tilapia *Oreochromis niloticus* to 0.5 µm MPs found that translocation of MPs to the liver did occur (Ding et al., 2018). However, examination of the data used to support these observations revealed an absence of fluorescence in the liver. Fluorescence was diffusely spread in the tissues of the remaining organs, likely due to leaching of the fluorescent dye (Schür et al., 2019). In another study with zebrafish and 5 µm MPs (Lu et al., 2016), the evidence presented was either inaccurate or poorly presented, as detailed in a published comment (Baumann et al., 2016).

With the increasing interest in research on MPs in the past decade, it has become necessary to find adequate and accurate tools to assess the uptake and translocation routes as well as determine the exact location of MPs in tissues.

Confocal laser scanning microscopy (CLSM) is a valuable tool for confirming the cellular uptake and depth of penetration of small fluorescent particles (Rossetti et al., 2013) that offers the possibility of making reporting on MP translocation more accurate. CLSM produces high-resolution images (Chirayil et al., 2017) taken from different depths and can reconstruct them as three-dimensional structures (Chirayil et al., 2017; Lamprecht et al., 2000), which provides an advantage when attempting to locate fluorescent particles.

In fish, the hepatic portal system drains blood from the intestines through a short hepatic portal vein then and discharges it to the sinusoids in the liver (Gamperl & Shiels, 2014). MPs can therefore enter the circulatory system and reach the hepatocytes through the endothelial fenestrae and the space of Disse in the liver. Thus, MP uptake could occur across the basal membrane of hepatocytes (Hinton et al., 2001). The fenestrae vary in size depending on physiological and pathological conditions (Cheng, 2018). Latex beads sized 1 µm (i.e., the size of MPs) and 100 nm (the size of NPs) were observed within the hepatic sinusoids of juvenile and adult zebrafish (Cheng, 2018). NPs have been detected in blood vessels and internalised in endothelial cells and the liver, suggesting that these particles are transported to the liver by the bloodstream (Rességuier et al., 2017).

The properties of particles also play a significant role in mediating cellular uptake. Therefore, insights gained for one type of polymer, with specific properties, are not transferable to other polymers. Polymer size and surface charge are essential factors that affect for uptake (Triebskorn et al., 2019).

2.3.5. Effects of MPs in fish tissues

Although the direct consequences of plastic ingestion have been detailed in the literature (i.e., damage and physical blockage of the digestive system and limitation of food intake) (Jovanović, 2017), our understanding of the impacts of MPs is still limited and often ambiguous.

Significant histopathological changes were not observed in previous experimental studies that exposed fish to different types of MPs either directly (Ašmonaite et al., 2018; Batel

et al., 2016; Jovanović et al., 2018; Rainieri et al., 2018; Romano et al., 2018) or through the trophic chain (Batel et al., 2020).

In contrast, numerous studies have reported histopathological changes in the intestine of fish after exposure to pristine[1] or contaminated MPs. Reported histopathological changes include "cilia defects" (Qiao, Deng, et al., 2019), "collapsed brush border", "beheading of villi" (Pedà et al., 2016), "deformation of columnar cells" (Hamed et al., 2021), "breakage of the epithelium" (Jabeen et al., 2018), "detachment of epithelium from lamina propria" (Pedà et al., 2016), "cracking of villi and splitting of the enterocytes" (Lei et al., 2018), "villi damage and epithelial damage" (Qiao, Sheng, et al., 2019), "epithelial detachment" (Limonta et al., 2019), "disepithelization" (Pedà et al., 2016), "destructed intestinal mucosa structure" (Yang et al., 2020), "crypt cell proliferation", "degeneration of crypt cells" (Hamed et al., 2021), enlarged intestine lumen (Yin et al., 2019), "larger enteric cavity", "separated intestinal adventitia and muscular layer, loose submucosal structure" (Yang et al., 2020), "atrophy of submucosa" and "atrophy of subserosa" (Hamed et al., 2021), "degenerated basement membrane" (Hamed et al., 2021), "infiltration of leukocytes" (Jabeen et al., 2018), higher density of "mucosal neutrophils" (Limonta et al., 2019), "higher lymphocytic focus scores in the submucosa" (Montero et al., 2022), infiltration of "inflammatory cells" in the lamina propria (Hamed et al., 2021), increase in rodlet cells (Pedà et al., 2016), "congestive inflammation" (Qiao, Sheng, et al., 2019), presence of one or two eosinophilic granule cells or "mast cells" (Qiao, Deng, et al., 2019), "atrophy of goblet cells" (Hamed et al., 2021), increases in the volume of mucus (Jin et al., 2018; Limonta et al., 2019), "necrosis of the enterocytes and mucosal epithelium" (Hamed et al., 2021), pyknotic nucleus in the mucosa (Hamed et al., 2021), haemorrhage (Hamed et al., 2021), oedema of the serosa, muscularis mucosa, submucosa and mucosa (Pedà et al., 2016).

Similarly, in the liver, several histopathological changes were reported, such as necrosis (Espinosa et al., 2019; Hamed et al., 2021; Iheanacho & Odo, 2020a; Karami et al., 2016; Lu et al., 2016) and infiltration of the hepatocytes (Espinosa et al., 2019; Iheanacho & Odo, 2020a; Jabeen et al., 2018; Lu et al., 2016), hemocyte infiltration (Yang et al., 2020), haemorrhage, "passive hyperaemia" (Jabeen et al., 2018), dilated blood sinusoids (Hamed et al., 2021), oedema (Karami et al., 2016), "increased hepatocyte interstitial space" (Yang et al., 2020),

1 Pristine or virgin plastics are the ones that have not been used previously nor mixed with waste.

"increased number of Kupffer cells" (Hamed et al., 2021), "severe deformation of hepatocytes", pyknotic nuclei, mitotic nuclei (Hamed et al., 2021), "severe parenchymal dystrophy" (Hodkovicova et al., 2021).

Different outcomes are expected in experimental sets (Wolf & Wheeler, 2018) that may result from variations in fish species, age, sex, or reproductive status; environmental conditions; tested concentrations; size, type, surface chemistry or hydrophobicity of MPs; feeding routine, exposure route, exposure time, and number of animals or replicates per treatment group; specimen collection; and preparation methods.

However, the above-mentioned histopathological changes allegedly found in fish exposed to MPs result from inaccurate interpretations of the histopathological data.

2.3.6. The (lack of) accuracy in MPs studies

Within the fish pathologist community, there have been increasing concerns about the overall quality of histopathological findings presented in peer-reviewed literature (Baumann et al., 2016). This situation is especially alarming in cases in which the conclusions of a study depend heavily on histopathological results. Furthermore, such observations will persist in the literature and result in further misguided research. This is particularly problematic for students and researchers working in fish pathology who search for reliable sources of information in peer-reviewed publications (Baumann et al., 2016).

One example is the study by Lu et al. (2016), who allegedly showed that MPs accumulated in fish gills, liver, and intestine, causing necrosis and infiltration. Despite the evident inaccuracies and poorly presented histopathological data already highlighted in a published comment (Baumann et al., 2016), this work has over 700 citations and is still considered reliable. It has become one of the most influential sources in the January 2019 proposal from the European Chemicals Agency on the restriction of intentionally added MPs (Batel et al., 2020).

Other studies have reported defects in ciliated cells in the intestines of adult zebrafish (Qiao, Deng, et al., 2019). However, ciliated cells are only found in the intestinal epithelia of

lampreys, chondrosteans, and dipnoids and are not present in the intestinal mucosa of most fish species (e.g., *D. rerio)* (Wilson & Castro, 2011). Most fish have brush border microvilli instead. Likewise, hyperplasia of Kupffer cells was also reported in teleosts (Hamed et al., 2021), but this group of fish lack these cells (Roberts, 2012).

Since the publication of our study (de Sales-Ribeiro et al., 2020), which extensively detailed the inaccuracies in histopathological findings in several MP studies, 26 publications have cited our work. Some studies, however, are citing our work incorrectly and thereby contradicting our results.

An example is a recent study by Abarghouei et al. (2021) that exposed *Carassius auratus* to PS microparticles of different sizes (0.25 µm and 8 µm) and concentrations (0.05–5 mg/L). The authors reported observing dose-dependent histological lesions. Despite the importance of such findings, photographic evidence of these lesions was not presented. Moreover, the authors cited our work to support their unsubstantiated pathological findings when it clearly states the opposite.

Likewise, Cera & Scalici (2021) cited our work when stating that MP microbeads did not translocate. However, our results showed the opposite.

Also, a recent publication (Solomando et al., 2021) citing our work mentioned that "diverse studies have…observed few particles of more than 250 µm in the liver of various species of fish, such as *Mugil cephalus*, *Saurida tumbil*, and zebrafish, suggesting a paracellular route of entry". Again, these contradicted the results presented in our study as we only confirmed the translocation of plastic microbead sized up to 1.6 µm to the liver.

Recent studies (Abarghouei et al., 2021; Guerrera et al., 2021; Montero et al., 2022; Solomando et al., 2021) are continuing to cite publications that present inaccurate histopathological findings. Hence, as previously highlighted (Batel et al., 2020), it is of the highest importance that unsubstantiated methods and uncertain results presented in scientific publications are questioned and corrected with additional high-quality work.

2.3.7. Further studies

Although pristine MPs do not appear to produce imminent damage, they may act as vehicles for chemicals, including those incorporated into them during manufacturing (e.g., endogenous chemical additives) and those adsorbed during their residence in the environment (e.g., persistent organic pollutants and metals) (Barboza, Vieira, et al., 2018; Rochman, 2015; Rochman et al., 2013). Therefore, further research is needed to identify the translocation pathways and effects that MPs and associated contaminants may have on animal and human public health.

In conclusion, despite their small size, MPs are a significant environmental issue requiring high-quality studies to correctly determine their toxicology and the potential threats they pose to the environment and public health.

2.3.8. Specific objectives

a. Assess fish feeding behaviour after exposure to different types of pristine MPs either free or mixed with a control diet.

b. Determine the intestinal retention time, uptake, and elimination of MPs in fish.

c. Evaluate the potential for accumulation of different types of pristine MPs after a sub-chronic exposure and depuration period.

d. Assess the translocation of MPs in fish organs using confocal microscopy.

e. Determine the pathological effects in fish from the sub-chronic exposure to different sizes, shapes, and polymers of pristine MPs.

3

MATERIAL AND METHODS

3.1. Scientific publications I and II

3.1.1. Area of study

The Canary Islands (Fig. 3.1) are an autonomous community of Spain in the Eastern Central Atlantic Ocean (FAO major fishing area 34, subarea 34.1 and division 34.1.2) (FAO, 2021). Within the Spanish established Exclusive Economic Zone (EEZ), the extension of the EEZ surrounding the Canary Islands is over 400 000 km^2 (European Marine Observation and Data Network (EMODnet), 2020; European MSP Platform, 2021). The archipelago comprises seven main islands: Gran Canaria, Fuerteventura, Lanzarote, Tenerife, El Hierro, La Palma, and La Gomera.

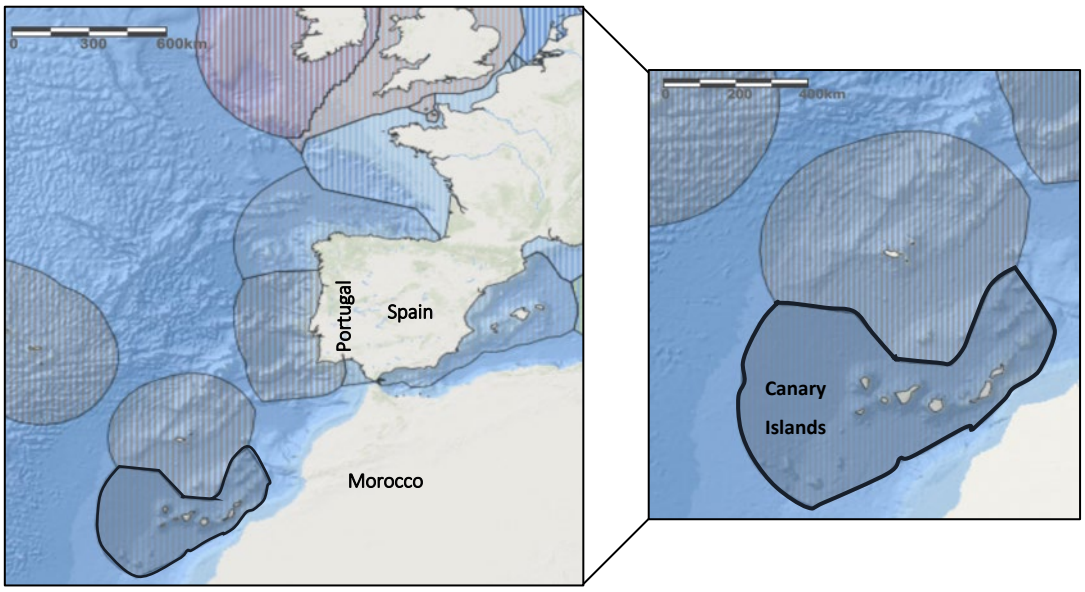

Figure 3.1. Map of some European Exclusive Economic Zones. The EEZ surrounding the Canary Islands is highlighted (see inset). (Maps modified from EMODnet, 2020).

3.1.2. Fish

Since 2011, professional fishers have submitted wild-caught fish species listed as high-risk for Ciguatera poisoning to the Laboratorio Oficial de Control de Ciguatera y Enfermedades de Peces at the University Institute for Animal Health and Food Safety (IUSA), Universidad de Las Palmas de Gran Canarias (ULPGC). This program is developed within the Official Control Program for Ciguatera Fish Poisoning established by the Directorate-General for Fisheries of the Canary Islands Government and supported by the European Food Safety Authority (EFSA) through the EuroCigua project (GP/EFSA/AFSCO/2015/03).

As a surveillance tool to monitor fish health status, post-mortem examination (necropsy) was performed in 30 adult dusky groupers *Epinephelus marginatus* and 65 greater amberjacks *Seriola dumerili* captured between 2017 and 2020.

3.1.3. Post-mortem examination

The necropsies were performed through a systemic approach and observation of external and internal structures, organs, and tissues was assisted by sample collection for further and complementary analysis (Bruno et al., 2013; Meyers, 2009; Roberts, 2012).

Fish were placed on the right flank with the head to the left side so that the internal organs could be immediately observed upon the section of the coelomic wall. In addition, weight, length, and overall body condition were assessed.

Externally, the body surface, fins, gills, and eyes were thoroughly observed. As fish skin contacts directly with the external environment, it tends to be more vulnerable to damage from various sources, such as primary and opportunistic pathogens (Bruno et al., 2013). Skin can also act as the portal of entry for numerous infectious agents that may spread to other tissues, originating systemic infections.

An incision was made along the mid-ventral line from the urogenital papilla to the pectoral girdle, caudal to the operculum for internal examination. Then, a second incision was made upwards from the pelvic girdle to the base of the lateral line. From there, it continued horizontally just below the lateral line and then towards the urogenital papilla. Finally, the obtained flap was removed to expose the internal organs. Once the coelomic cavity was opened, internal organs were examined in situ to assess the anatomic position, colour changes, the presence of fluid or other significant findings.

Gastrointestinal organs were removed as a unit by cutting through the cranial aspect of the oesophagus and the rectum. Once detached from the body, the organs were separated and individually assessed.

Series of transversal cuts were performed in the liver and spleen to inspect the parenchyma and associated vasculature. The gall bladder was opened to analyse the content. Finally, a longitudinal cut was made from the oesophagus to the rectum to expose the lumen and allow content and mucosa examination.

The pericardial cavity was evaluated in situ, and the whole heart was removed. Then, to observe the main heart structures, a longitudinal section was performed.

Gonads and the swim bladder were also separated and examined individually. For both organs, longitudinal sections were made. Gonads were additionally analysed to determine the sex and the stage of reproductive development. In the swim bladder, the lumen and the wall were examined with detail in the cranial region where the gas gland and rete mirabile are present. After detachment of the swim bladder, two parallel incisions were made from the cranial to the caudal end of the kidney to separate it from the vertebral column. Then, a longitudinal cut was made through the whole kidney to assess changes.

To expose the brain, the overlying bone and cartilage were detached. Finally, the whole brain was removed together with the eyes and evaluated.

3.1.4. Sample processing

a) Histological analysis

During the post-mortem examination, representative samples of the skin, muscle, gills, eyes, heart, liver and gall bladder, spleen, stomach, intestine, swim bladder, kidney, gonads, and brain were fixed in 10% neutral buffered formalin. After fixation, the tissue samples were placed into cassettes and processed. Processing of the tissues included dehydration through ascending grades of alcohols, clearing in xylene and finally impregnation with paraffin wax. The resultant block was refrigerated in ice and sectioned at 4 µm. The obtained samples were stained with haematoxylin and eosin (HE). The slides were mounted and examined with a light microscope (Olympus BX51TF, Japan).

Selected tissue sections were stained with complementary histochemical techniques to determine the etiological agent. Gram and Ziehl–Neelsen stains were employed to identify bacteria in tissue sections, and periodic acid–Schiff (PAS) and Gomori's methenamine silver (GMS) were used to highlight fungal structures. All the stains were performed following the protocols detailed in Bancroft et al. (2013). To assess the deposition of connective tissue and highlight the morphology of the trypanorhynch larvae, tissue, and parasite sections, respectively, were stained with Masson trichrome as follows:

1. Deparaffinization of the sections until distilled water.
2. Stain with Harris haematoxylin (8 minutes).
3. Rinse in distilled water.
4. Picric acid (15 minutes).
5. Rinse in distilled water.
6. Stain with a solution of Biebrich scarlet and acid fuchsin (5 minutes).
7. Rinse in distilled water.
8. Treat with a solution of phosphomolybdic acid and phosphotungstic acid (6 minutes).
9. Stain with aniline blue (6 minutes).
10. Rinse in distilled water.
11. Treat with 1% acetic acid (10 minutes).
12. Rinse in distilled water.
13. Dehydrate through ascending grades of alcohol.
14. Clear in xylene, mount in a permanent mounting medium.

b) Parasitological analysis

During the post-mortem examination, parasites were detached from the tissues, and their capsules were removed under a stereomicroscope (Motic SMZ-171 TL). They were washed with a saline solution and fixed in 70% ethanol. These samples were examined unstained under a stereomicroscope and a light microscope.

c) Molecular analysis

For molecular analysis, formalin-fixed paraffin-embedded samples were submitted to Instituto Valenciano de Microbiología for molecular identification of the infectious agent. DNA was extracted using DNeasy® Blood, and Tissue Kit (Qiagen) and a PCR was performed using MyTaqTM HS Mix (Bioline, UK). Fungal DNA detection was carried out using the initiator oligonucleotides of the gene 18S-28S rRNA and its subsequent sequencing. The percentage of similarity with sequences of the same species in GenBank was 99%.

3.2. Scientific publication III

3.2.1. Fish

Zebrafish (family Cyprinidae) were used as animal models for the experiments with microplastics. Zebrafish are teleost fish extensively used in animal and human health research (Bailone et al., 2020). Several reasons justify the popularity of this species as an animal model. First, they are small fish, requiring relatively small and inexpensive housing, as well as a smaller amount of testing agents (Chakraborty et al., 2016; Goodwin et al., 2016; Haque & Ward, 2018). In addition, critical organ systems, such as the digestive, nervous and cardiovascular systems, are very similar to mammals (Chakraborty et al., 2016; Haque & Ward, 2018). This underpins the extensive equivalence in response to testing agents between zebrafish and other mammalian species (Haque & Ward, 2018).

In recent years, zebrafish have been commonly used as sentinels for assessing environmental hazards, such as aquatic pollutants (C. Zhang et al., 2003), becoming an established animal model for toxicity assays with nanoparticles (Chakraborty et al., 2016; Haque & Ward, 2018) and microparticles (Batel et al., 2016, 2020; Hering et al., 2021; Kurchaba et al., 2020).

3.2.2. Microplastics characterisation

Green, fluorescent polymer microbeads with diameters of 1–5 µm were purchased from Cospheric LLC, USA. These fluorescent plastic microbeads were used to identify the distribution of small MPs in fish tissues after ingestion.

Polyethylene (PE) plastic microfragments with an irregular surface were extracted from a cosmetic body cleanser. The content of the cleanser was washed with distilled water and sieved. The obtained particles had a size range of 120–220 µm (mean: 175±42 µm).

White microfibres from a synthetic textile were cut under a stereomicroscope. The mean size of the resulting fibres was 1500 µm x 13.67 µm.

Fourier transform infrared (FTIR) spectroscopy was performed to determine the composition of the plastic polymers. A Bruker IFS 66/S spectrometer (Bruker, Spain) equipped with a deuterated triglycine sulphate detector and a diamond crystal attenuated total reflection module was used. FTIR spectra were acquired from an average of 64 scans with an 8 cm^{-1} resolution. The reflectance ratio (R/R_0) was calculated, where R and R_0 were the reflectance measured at the sample and the clean crystal, respectively. Positive bands represented the loss, while negative bands represented the gain of species at sampling. The microfragments from the cosmetic body cleanser were confirmed as PE. Regarding the fluorescent microbead, the obtained spectrum showed a mild similarity to that of polyethylene glycol (PEG) (<60%) but was insufficient to identify the composition of the polymer.

3.2.3. Experimental design

Three Petri dishes with ultrapure water were placed next to each work area and analysed as procedural blanks during the experiments. The procedural blanks were present at every step of the MPs evaluation process to assess sample contamination.

3.2.3.1. Acute experiment

Seventy adults of zebrafish of similar weight were kept in acclimation tanks for four weeks in the experimental animal facility (EGC00616436), ULPGC. Fish were placed in a semi-static system with tap water at a stocking density of~0.8 fish/dm^3 and under a photoperiod of 12:12 hours light: dark cycle and fed a control diet three times a day.

A preliminary study was performed to assess the ability of zebrafish to recognise plastic particles as indigestible material. Free fluorescent microbeads were added to an aquarium with five zebrafish. Likewise, cosmetic microfragments were added to another aquarium with the same number of fish. Two hours post-feeding (hpf), fish from both groups were sampled. Necropsies were performed under a stereomicroscope. For histology, the whole intestine was extracted and fixed in 10% neutral buffered formalin.

For the acute experiment, two sets of diets were used. Diet F_A was obtained by adding fluorescent microbeads to the control diet. Diet C_A was obtained by mixing cosmetic PE microfragments and textile microfibres with fish oil-aromatised gelatine. Following acclimation, the fish were starved for 24 hours, randomly collected, and separated into two groups (80 dm^3 per aquaria, n = 30). Fish from group 1 were fed a single intake of diet F_A, and group 2 was fed a single intake of diet C_A. Following this period, fish from both groups were separated into five aquaria in groups of six to prevent food contamination. Each aquarium represented a different sampling time (2, 6, 10, 12 and 24 hpf). During these procedures, fish were closely monitored. Water from each aquarium was filtered, and the faeces were collected and mounted on a slide. Fish from group 1 and group 2 were sampled 2, 6, 10, 12 and 24 hpf.

3.2.3.2. Sub-chronic experiment

Seventy-two zebrafish adults of similar weight and length were kept in acclimation tanks under the same conditions as those described for the acute experiment.

Two sets of experimental diets were used. First, each test diet was spiked with different types of MPs. Diet F_{SC} was obtained by adding fluorescent microbeads to the control diet. Similarly, diet C_{SC} was obtained by mixing cosmetic PE microfragments and textile microfibres with the control diet. Finally, a control group, held under identical conditions, was fed a control diet.

After the acclimation period, the zebrafish were weighed, and their general body shape and urogenital papilla were inspected to determine their sex. The fish were distributed into two test groups. Fish from group 1 were fed the F_{SC} diet, and fish from group 2 were fed the C_{SC} diet. All the treatments were carried out in triplicate, and each aquarium comprised an equal number of males (n=4) and females (n=4). Each batch (n=8) was placed in a 20 dm^3 aquarium. A control group (n=24) was added. Fish from all the groups were fed a control diet three times a day on a fixed schedule. Every two days, the first intake of the control diet was replaced with the F_{SC} diet and C_{SC} diet in the test groups.

The feeding experiment lasted 45 days. Mortalities and observable abnormalities regarding both appearance and behaviour were recorded. All the aquaria contained tap water

under constant aeration. After 30 and 45 days of feeding, two fish from each replicate aquarium were euthanised. The examination was performed under a stereomicroscope. A cut was made along the ventral line, and a celomic flap was cut open and removed to allow the fixative to penetrate the internal tissues. The whole fish was preserved for histological examination in 10% neutral buffered formalin for 24 hours. After that period, the entire intestine, liver, and muscle samples were extracted. Fish from the control group were similarly dissected.

A depuration period was designed to determine the potential for the accumulation of these plastic particles. At the end of the feeding period, the remaining fish were transferred to clean aquaria to avoid contamination with the test substance. Then, for 15 days, all the fish were fed only the control diet. After that, two fish per triplicate were euthanised, weighed, and similarly dissected.

3.2.4. Clinical examination

In the acute and sub-chronic studies, clinical examination of all fish was performed daily. The assessment was made before, during and after feeding to monitor behavioural changes, such as inappetence, lethargy or changes in the swimming patterns and position in the water column. Interactions with other fish from the same aquarium were also evaluated. External signs of pathology, such as colour changes or other noticeable lesions or physical abnormalities, were likewise monitored.

3.2.5. Histological assessment

The methodologies proposed by Saraiva et al. (2015) and Bernet et al. (1999) were used to assess the histological findings in the intestine and liver, respectively. In addition, an individual description, termed *degree of vacuolation,* was added for the liver. The degree of vacuolation was scored for all the fish using a semiquantitative scale: minimal (1), mild (2), mild to moderate (3), moderate (4), marked (5) and severe (6).

3.2.6. Confocal microscopy

Confocal microscopy was used to assess the presence and uptake of fluorescent MPs by the different tissues. Fluorescence images were obtained with a confocal microscope (Zeiss Confocal LSM800, Germany) at an excitation wavelength of 519 nm and emission wavelength of 543 nm for green and an excitation wavelength of 543 nm and an emitting wavelength of 567 nm for orange.

Panoramic images of the whole intestine were created. A series of two-dimensional images over the depth range of interest (Z-stacks) were performed to obtain a three-dimensional image. This allowed confirmation of the internalisation of the MP particles in the tissues. The diameter of the microparticles was measured using Zen Blue v2.3 software.

3.2.7. Statistical analysis

Wilcoxon's tests or Student's t-tests were used to compare groups, and the results are presented as the mean (standard deviation) or median [range]. Differences were considered significant when the two-tailed P value was below 0.05. The statistical analyses were performed by a commercial statistical software package (IBM SPSS Statistics Version 18, SPSS Inc., Chicago, IL).

4

SCIENTIFIC PUBLICATIONS

I. First description of spontaneous granulomatous aerocystitis by *Phoma herbarum* in a wild greater amberjack (*Seriola dumerili* Risso, 1810)

de Sales-Ribeiro, C., Sanchez-Henao, A., García-Álvarez, N., Real, F., Rivero, M. A., Fernández, A., & Caballero, M. J. (2019). First description of spontaneous granulomatous aerocystitis by Phoma herbarum in a wild greater amberjack (Seriola dumerili Risso, 1810). *Journal of fish diseases*, *42*(9), 1321–1325. https://doi.org/10.1111/jfd.13045

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SHORT COMMUNICATION

Journal of Fish Diseases WILEY

First description of spontaneous granulomatous aerocystitis by *Phoma herbarum* in a wild greater amberjack (*Seriola dumerili* Risso, 1810)

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KEYWORDS: aerocystitis, fish pathology, greater amberjack, *Phoma herbarum*, *Seriola dumerili*, swim bladder

Phoma herbarum Westend (family Didymellaceae) is a saprophytic fungus mostly recognized as a plant pathogen (Aveskamp, Gruyter, & Crous, 2008; Bennett, Ponder, & Garcia-Diaz, 2018; Kumla, Suwannarach, & Lumyong, 2016; Neumann Brebaum & Boland, 1999). With a ubiquitous distribution, *P. herbarum* can be isolated from vegetable debris, inorganic material, water sources, humans and animals (Boerema, 1964; Boerema, Gruyter, Noordeloos, & Hamers, 2004).

In fish, *P. herbarum* has been reported to act as a facultative pathogen, causing a chronic progressive and lethal visceral mycosis. Previous reports detailed the infection in coho salmon (*Oncorhynchus kisutch* Walbaum), chinook salmon (*O. tshawytscha* Walbaum) and rainbow trout (*O. mykiss* Walbaum) in hatcheries in the Northwest, Midwest United States and Alaska (Boerema et al., 2004; Burton, Meyers, Starkey, & Follett, 2004; Faisal, Elsayed, Fitzgerald, Silva, & Mendoza, 2007; Ross, Yasutake, & Leek, 1975). Moreover, a visceral mycosis with similar features was also found in farmed ayu (*Plecoglossus altivelis altivelis* Temminck & Schlegel) in Japan, caused by an unidentified species of the genus *Phoma* (Hatai, Fujimaki, Egusa, & Jo, 1986).

Infections by *P. hebrarum* in fish were characterized by lethargy and erratic swimming, for example swimming on their sides or in a vertical position, and resting on their sides or on the bottom, as infection progresses (Faisal et al., 2007; Ross et al., 1975). Physical changes included swollen and haemorrhagic vents, areas of muscle softening, petechiae along the lateral and ventral body surfaces and exophthalmia (Buller, 2014; Faisal et al., 2007; Ross et al., 1975). Evaluation of the internal organs revealed the presence of white caseous necrotic masses and congestive walls in the swim bladder. Adhesions between the swim bladder and gastrointestinal tract, stomach distension by yellowish fluids and haemorrhagic areas in the kidneys and adjacent musculature were also reported (Faisal et al., 2007; Ross et al., 1975). Histopathology showed abundant septate hyphae in the lumen of the swim bladder, extensive inflammatory infiltrate and degenerated necrotized and sloughed cells from the organ walls. Based on the anatomopathological observations, the swim bladder appears to be the most affected organ by *P. herbarum* (Burton et al., 2004; Faisal et al., 2007; Hatai et al., 1986; Roberts, 2012; Ross et al., 1975).

The exact portal of entry for *P. herbarum* in fish is still unclear. An often suggested theory is that ingested conidia, from feed or surface water, could pass through the pneumatic duct from the digestive tract to the swim bladder (Burton et al., 2004; Faisal et al., 2007; Ochiai, Kodera, Kon, Miyazaki, & Kubota, 1977). Nonetheless, the proposed theory was based on the reports of *P. herbarum* spontaneous infections exclusively in species that maintain the pneumatic duct—physostomous species (Burton et al., 2004; Faisal et al., 2007; Ochiai et al., 1977; Ross et al., 1975).

Greater Amberjack (*Seriola dumerili* Risso, 1810; family Carangidae) is a marine fish commonly found over offshore chasms, drop-offs or rocky outcrops (Harris, Wyanski, White, Mikell, & Eyo, 2007; Smith-Vaniz, Pina Amargos, Brown, Curtis, & Williams, 2015)

in the Atlantic and Indo-Pacific Oceans (Andaloro & Pipitone, 1997; Harris et al., 2007; Sley, Taieb, Jarboui, Ghorbel, & Bouain, 2016). As an opportunistic predator, it preys both on benthic and on pelagic species, either close to the surface or close to the bottom (Andaloro & Pipitone, 1997; Jerez & Vassalo-Agius, 2016; Smith-Vaniz et al., 2015). Whilst being a popular species in commercial and recreational fisheries, greater amberjack has also been recently introduced for aquaculture diversification (Jerez et al., 2018; Zupa et al., 2017). Hence, knowledge on pathologies affecting this species is of extreme importance in order to avoid the transmission of diseases, a major bottleneck in fish production and performance, and economic losses.

To the best of our knowledge, we present in this paper, the first description of a spontaneous granulomatous aerocystitis caused by *P. herbarum* in greater amberjack, a wild marine physoclist.

A female adult greater amberjack, 27 kg weight and 112 and 132 cm, furcal and total length, respectively, was captured alive by local fishermen in La Santa, the north-west Lanzarote, Canary Islands (Spain 29°06′40″N 13°40′00″W), in early autumn. It was submitted in October 2016 to the Division of Infectious Diseases and Ichthyopathology, Institute for Animal Health and Food Safety (IUSA), University of Las Palmas de Gran Canaria, Canary Islands, Spain.

A standard necropsy for finfish, according to Meyers (2009), was performed, and tissue samples were collected for histopathological analysis.

Tissue samples from the swim bladder, anterior and posterior kidney, liver, stomach, intestine, heart, gills and gonads were fixed in 10% phosphate-buffered formalin solution, embedded in paraffin wax, sectioned at four-μm sections and stained with haematoxylin and eosin (H&E), Gram, Ziehl–Neelsen, Gomori's Methenamine Silver (GMS) and Periodic Acid–Schiff (PAS), according to the protocols detailed in Bancroft, Layton, and Suvarna (2013).

Formalin-fixed paraffin-embedded samples from the swim bladder were submitted to Instituto Valenciano de Microbiología for fungal identification by PCR. DNA was extracted using DNeasy® Blood and Tissue Kit (Qiagen). PCR was performed using MyTaq™ HS Mix (Bioline, UK). Primers used for fungal amplification were the ones described by White, Bruns, Lee, and Taylor (1990). The initial sequence obtained from 264 base pairs after the Basic Local Alignment Search Tool (BLAST) analysis provided 99%–100% identities for several species of *Phoma* spp., *Leptosphaeria* spp., *Epicoccus* spp. and *Dothideales* spp. To confirm the identification, an analysis was performed with specific primers of *Phoma herbarum* for the MAT gene (accession: AY748945.1).

External examination showed no abnormalities or lesions. Gross findings in the internal organs were confined to the swim bladder. It presented a complete loss of morphology and generalized thickening and opacity of the wall with markedly increased size (36 mm × 16 mm) and diminished to obliterated lumen. Multiple, often coalescing, translucent to whitish variable-sized (20–80 mm) fluid-filled cystic lesions replaced the normal structure (Figure 1a,b). Irregular tan to dark brown, gritty and raised nodules, ranging from 7 to 20 mm, were also observed amidst the cystic lesions (Figure 1b). Both cystic and gritty nodules were well-demarcated and separated by smooth, thick bands of fibrous connective tissue. Multifocal haemorrhagic foci were noticed, with particular emphasis on the caudal aspect of the organ.

Histological examination of the swim bladder revealed multiple variable-sized cysts, lined by one up to twelve layers' thick of attenuated to cuboidal epithelial cells and distended by clear spaces or filled with eosinophilic homogenous proteinaceous fluid (Figure 2a). The remaining tissue was replaced by numerous often coalescent, variable-sized granulomas with a core of necrotic tissue, often pigmented and mineralized, surrounded by epithelioid macrophages, foreign body-type multinucleated giant cells and lymphocytes. Granulomas and cystic lesions were separated by prominent variable thick bands of vascularized loose connective tissue containing scant and disperse small aggregates of lymphocytes and macrophages (Figure 2b). Frequently, within the granulomas, large numbers of filamentous irregular branching septate hyphae, 3–6 μm width, with rare bulbous swellings, were observed (Figure 2c) and highlighted with GMS (Figure 2d) and PAS.

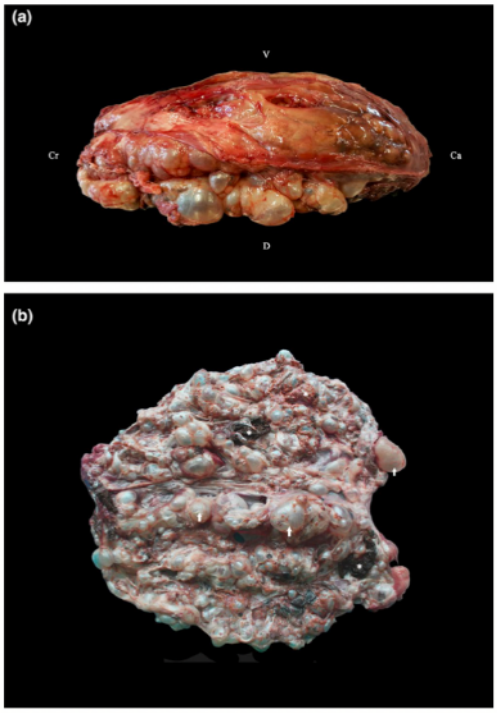

FIGURE 1 (a) Lateral view of the swim bladder with multiple cystic lesions and haemorrhagic foci (Cr—cranial, Ca—caudal, V—ventral, D—dorsal). (b) Inner aspect of the swim bladder. Numerous cystic structures (arrow) admixed with multiple variable-sized tan to dark brown solid nodules (.)

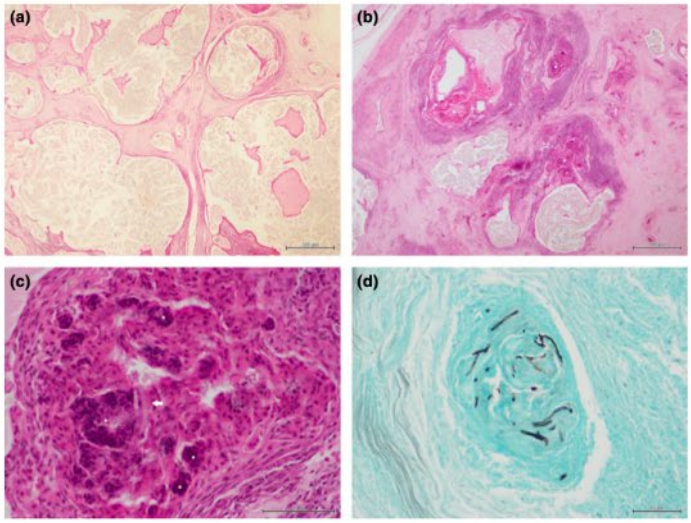

FIGURE 2 (a) Swim bladder. Variable-sized cysts, separated by thick bands of vascularized loose connective tissue (H&E). (b) Swim bladder. Multiple variable-sized granulomas with a core of necrotic tissue (,), surrounded by epithelioid macrophages, foreign body-type multinucleated giant cells and lymphocytes, admixed with variable-sized cysts, separated by thick bands of vascularized loose connective tissue with small aggregates of lymphocytes and macrophages (H&E). (c) Septate hyphae (arrow) surrounded by multinucleated giant cells (,) within a granuloma (H&E). (d) Branched septate fungal hyphae within a granuloma (GMS)

Tissue samples from other organs did not reveal the presence of fungal infection and no other pathological changes were observed, apart from a mild infection with nematodes (Anisakidae) in the stomach lumen, without associated lesions.

The detection and sequencing of fungal DNA allowed the identification of the species as *Phoma herbarum* in the swim bladder samples of the present study.

Phoma herbarum is a saprophytic fungus with a ubiquitous distribution (Boerema et al., 2004). In fish, it has been reported to act as a facultative pathogen, causing a chronic progressive and lethal visceral mycosis, being the swim bladder the most affected organ (Burton et al., 2004; Faisal et al., 2007; Roberts, 2012; Ross et al., 1975).

The swim bladder is a hydrostatic organ whose primary function is to regulate buoyancy (Bruno, Noguera, & Poppe, 2013; Genten, Terwinghe, & Danguy, 2009; Helfman, Collette, Facey, & Bowen, 2009). Two primary types of swim bladder are currently recognized. In physostomous species (e.g., Salmonidae), the swim bladder is connected to the oesophagus by the pneumatic duct. The pneumatic duct favours the entrance of gas to the swim bladder by swallowing air at the surface and passing it down the gut (Bone & Moore, 2008; Bruno et al., 2013). In contrast, in physoclists (e.g., Carangidae), the connection between the swim bladder and the digestive tract ceases to exist during the embryonic development and the filling and emptying mechanisms of the swim bladder are done by diffusion with the bloodstream (Bruno et al., 2013; Genten et al., 2009; Hughes, Rowland, Stewart, & Gill, 2016).

Prior reports of visceral mycosis in fish by *P. herbarum* appear to be restricted to hatchery-reared fish of the family Salmonidae in freshwater (Faisal et al., 2007; Ross et al., 1975; Wood et al., 1968). Because swim bladder was the most affected organ, it was hypothesized that infection could have resulted from the passage of the fungus from the digestive tract, after ingestion of food or detritus, to the swim bladder, through the pneumatic duct (Burton et al., 2004; Faisal et al., 2007; Hatai et al., 1986). In physoclistous species, this theory would not apply due to the absence of the pneumatic duct (Bruno et al., 2013). Transmission by inhalation, enabled during the swim-up stage, was another postulated route of infection. It was suggested that aerosolized conidia from plants with *P. herbarum* deposited in the water surface could be inhaled during gas exchange and pass to the swim bladder (Wood et al., 1968; Burton et al., 2004). Again, the absence of the pneumatic duct undermines this theory in physoclists. However, transmission may occur during gas exchange. When oxygen dissolved in the surrounding water enters the bloodstream via the efferent arterioles in gills, it goes through the dorsal aorta, reaching the swim bladder. Ross et al. (1975) and Faisal et al. (2007) reported vasculitis and fungal invasion of the dorsal aorta. In addition, it was suggested by Camus, Berliner, Hyatt, Hatcher, and Clauss (2014), in a case of aerocystitis by *Exophiala xenobiotica*, that the swim bladder infection could be the result of a vascular invasion at an undetermined primary site, through haematogenous seeding of the gas gland capillary rete. The fact that greater amberjack often preys close to the surface, where aerosolized conidia from plants with *P. herbarum* deposit (Andaloro & Pipitone, 1997), would make it particularly susceptible to an infection by this route. However, we cannot further hypothesize on this portal of entry, as dorsal aorta was not sampled in the present case. Recently, it was reported that, in some species of Carangidae (e.g., *S. dumerili*, *S. lalandi*), the swim bladder may not be completely sealed (Hughes et al., 2016). Hughes et al. (2016) detailed a specialized anatomical structure, consisting of a membranous opening, dorsal in the swim bladder, which led to a flattened tube that bifurcated around the vertebral

columns and exited via a small hole in the pharyngocleithral membrane underneath each operculum. This connection could be considered as an important portal of entry of *P. herbarum* in species with this feature.

In experimental infections with *P. herbarum*, other portals of entry apart from oral (Burton et al., 2004; Easa, Hatem, Sakr, & Refai, 1984; Ross et al., 1975) and airborne transmission (Burton et al., 2004; Ross et al., 1975) were considered, such as bath immersion (Ali, Hashem, & Al-Salahy, 2011), intraperitoneal injection (Burton et al., 2004; Easa et al., 1984), subcutaneous injection (Ali et al., 2011; Easa et al., 1984) and swabbing on scarified gills (Easa et al., 1984). Overall, the highest mortalities were obtained by intraperitoneal and subcutaneous injection (Ali et al., 2011; Burton et al., 2004; Easa et al., 1984) and in a less extent, by oral transmission (Easa et al., 1984; Ross et al., 1975), airborne transmission (Ross et al., 1975) and bath exposure (Ali et al., 2011). However, results were not conclusive due to differences between studies with regard to dosage, exposition time, water conditions and environment.

Despite being a ubiquitous fungus, few spontaneous infections by *P. herbarum* in fish have been reported. This allegedly low incidence supports the idea that the pathogenic effect of *P. herbarum* depends greatly on the immune status of its host (Faisal et al., 2007). In an experimental transmission of *P. herbarum* to chinook salmon, Burton et al. (2004) reported that survival rates tended to be higher under optimal environmental conditions. As already acknowledged, intrinsic factors such as species, genetic background, age, reproductive status and nutritional condition may have a negative impact in the immune system. Similarly, extrinsic factors, such as seasonality and temperature, handling, density and pollution, could also affect negatively the activity of the immune system (Magnadóttir, 2006; Plumb & Hanson, 2011; Voronin, 2014).

In previously reported infections with *P. herbarum*, fry and fingerlings were the most affected fish. These observations are in line with the idea that young fish are inherently more susceptible to diseases as they have not yet acquired natural resistance (Noga, 2010; Plumb & Hanson, 2011). Also, being poikilothermic animals, temperature significantly affects their metabolism, especially concerning immunity (Noga, 2010). A decrease in water temperature is reported to suppress the immune response. Fish from temperate and colder climates are particularly susceptible to infectious diseases during spring and autumn, when changes in water temperature are more abrupt (Noga, 2010; Plumb & Hanson, 2011). Stressful events, such as water temperature (Ross et al., 1975) and salinity changes (Hatai et al., 1986) or handling (Easa et al., 1984; Faisal et al., 2007; Hatai et al., 1986), were also described prior to the development of the infections by *P. herbarum*. However, the aforementioned cases of spontaneous infections by *P. herbarum* occurred in hatchery-reared species, under a controlled environment.

The present report concerns a wild adult greater amberjack, captured alive. In a wild environment, the number of stressors that may result in increased risk of infections is vast. Several factors could have prompted the infection. Reproductive status is known to modulate immunity, especially during the spawning season, when natural resistance of adult fish is reduced, as their energy is diverted into reproductive activities (Plumb & Hanson, 2011). Latent or opportunistic pathogens may also have the potential to cause immunosuppression, enabling the development of a secondary infection. In our case, the mild gastric nematode infection observed may have played a role in the suppression of the immune system, thus prompting the infection by *P. herbarum*. Also, a sudden change in water temperature could negatively affect the immune system. It is important to remark that *P. herbarum* optimal temperature for growth is between 20°C and 25°C (Boerema, 1964) and that temperatures in the eastern Atlantic Ocean usually hover around 20°C and 23°C from June to September. Nevertheless, it is important to bear in mind that *P. herbarum* is characterized by causing a chronic progressive disease and it is not possible to determine the definite role played by these stressors at the time of onset of the infection.

In summary, previous reports of spontaneous visceral mycosis by *P. herbarum* were limited to freshwater farmed species with a physostomous swim bladder configuration. To the best of our knowledge, this is the first report of a granulomatous aerocystitis by *P. herbarum* in a wild greater amberjack, a marine fish with a physoclistous swim bladder.

The importance of this finding lies mostly on the efforts that have been made for the past years to introduce greater amberjack in aquaculture. As *P. herbarum* has been associated with great losses in hatcheries (Faisal et al., 2007; Ross et al., 1975), it is essential to better identify potential diseases that may represent a bottleneck to fish production and performance, in order to be able to prevent or treat them and avoid economic losses.

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CONFLICT OF INTEREST

The authors declare that they have no conflict of interests.

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II. A Study on the Pathological Effects of Trypanorhyncha Cestodes in Dusky Groupers *Epinephelus marginatus* from the Canary Islands

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A Study on the Pathological Effects of Trypanorhyncha Cestodes in Dusky Groupers *Epinephelus marginatus* from the Canary Islands

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Simple Summary: Trypanorhyncha are common parasites of marine fish. Despite numerous studies detailing their biology, knowledge on the effects caused by these parasites in fish tissues is still limited. Dusky groupers are keystone species, necessary for the preservation of several marine ecosystems. Considering their vulnerable state of conservation and the efforts being made to culture them, identification of the effects caused by Trypanorhyncha is vital. Here, we have assessed the prevalence of Trypanorhyncha in dusky groupers from the Canary Islands and the associated pathological changes. Of the 28 fish examined, 27 presented trypanorhynch larvae. Macroscopically, in the abdominal cavity, there were numerous larvae-filled cysts and nodules embedded in abundant fibrosis, hindering the separation of the organs. Microscopically, in the peritoneum, stomach and intestine, there were numerous degenerated parasitic cysts and extensive deposition of fibrous connective tissue with minimal inflammatory responses. This study shows that Trypanorhyncha are common parasites of adult dusky groupers from the Canary Islands. Even though the immune system appears to isolate and eliminate the parasites, extensive fibrosis may have a detrimental impact on fish health when adjacent organs are compressed and their functions impaired.

Abstract: Trypanorhyncha are cestodes commonly infecting marine fish. Numerous studies have detailed the biology of Trypanorhyncha species, but information on the pathological changes produced by these parasites is limited. Dusky groupers are keystone species necessary for the preservation of several marine ecosystems. Considering their vulnerable state of conservation and the efforts being made to culture them, identification of the effects caused by Trypanorhyncha is vital. Here, we aimed to determine the prevalence and pathological changes produced by Trypanorhyncha in dusky groupers from the Canary Islands. The prevalence of trypanorhynch plerocerci was 96%. Grossly, in the abdominal cavity, there were numerous larvae-filled cysts and nodules. These were embedded in abundant fibrosis, producing visceral adhesions. Histologically, affecting the peritoneum, stomach, and intestine there were numerous degenerated encysted plerocerci and extensive deposition of mature connective tissue. These findings indicate that Trypanorhyncha is highly prevalent in adult dusky groupers from the Canary Islands, producing a progressive and chronic response. Furthermore, fish immune system appears to attempt to eliminate the parasites through fibrous encapsulation. Nonetheless, extensive fibrosis may have a detrimental impact on fish health when adjacent cells or tissues are compressed and their functions impaired.

Keywords: fish parasites; Pintneriella; Trypanorhyncha; Cestoda; *Epinephelus marginatus*; dusky grouper; fish pathology

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1. Introduction

Cestodes of the order Trypanorhyncha have been found in marine fish all over the world [1–9]. They are characterized by a scolex with two or four bothria [10] and a tentacular apparatus comprised of four retractable tentacles [11]. Tentacles are armed with numerous hooks arranged in complex patterns to adapt to the attachment site in the final host [12]. With a complex life cycle [12], most Trypanorhyncha species require a definitive host (elasmobranch fish) and a first (small crustacean) and a second intermediate host (teleost fish or invertebrate) [8,13], although in some cases, only a single intermediate host is necessary [14]. Alternatively, in some species, paratenic hosts may harbor the plerocerci until a final host is available [10]. In this case, larger teleosts may serve as paratenic hosts to bridge gaps in the food chain between smaller teleosts and the elasmobranchs [10].

Pintneriella (Yamaguti, 1934) is a genus of Trypanorhyncha within the family Rhopalothylacidae [11]. Four species of *Pintneriella* have been identified, *P. musculicola, P. gymnorhynchoides, P. pagelli* and *P. maccallumi* [15]. The adult stage of *Pintneriella* spp. inhabits the gastrointestinal tract of the definitive host [16] from where it releases the free-swimming coracidium larva into the marine environment [10,17]. Once free in the environment, these are ingested by a first intermediate host and converted into procercoids [16]. In turn, when the procercoid is ingested by a second intermediate host, it penetrates through the gut wall and encysts in the viscera or musculature, maturing into a plerocercus [18].

Dusky groupers, *Epinephelus marginatus*, are large predatory fish and keystone species in the rocky bottom's ecosystems, occurring at depths of up to 250 m [19]. Being a high-priced species [20] highly appreciated for the quality of their flesh [21], dusky groupers became a popular species among commercial and recreational fisheries [19]. As a result, their populations have suffered a major decline over the past decades [19] due to overharvesting, habitat destruction and juvenile extraction [22]. With the aim of replenishing wild fish stocks, some efforts have been made to develop aquaculture for this species [23]. To attain efficient and sustainable fisheries management, aimed at conservation of endangered species, it is essential to expand the knowledge of the diseases affecting fish populations and their ecosystems [19].

Over the years, several studies have documented infections by Trypanorhyncha in *Epinephelus* spp. [3,4,7,9,24]. Despite considerable progresses in the morphological and taxonomical aspects of Trypanorhyncha species, information regarding the pathological changes caused by these parasites is still limited. A previous study documented the presence of *Pintneriella musculicola* in *Epinephelus* spp. from the Arabian Gulf [25]. The authors [25] detailed the presence of long, whitish plerocercoids of *P. musculicola* in the muscle, with associated muscle fiber atrophy and oedema. Inflammation, necrosis and fibrosis of the skeletal muscle, abdominal cavity, mesentery and liver have also been reported in Areolate groupers *Epinephelus aerolatus* infected with *Floriceps* sp. [26]. The presence and gross presentation of trypanorhynch larvae in *Epinephelus* spp. were briefly referred to in a few other studies [3,27] but further details were not provided. Interestingly, in another work reporting an infection by trypanorhynch larvae in *E. marginatus*, pathological changes were not observed [28].

In elasmobranch fish from the region, *P. gymnorhynchoides* was reported in Portuguese dogfish, *Centroscymnus Coelolepis*, from Azores [29], and *P. maccallumi* was found in smooth-hounds *Mustelus* spp. from the east Atlantic Ocean [15]. Given the presence of *Mustelus* spp. in the region, these may act as final hosts for *Pintneriella* sp. To the best of our knowledge, trypanorhynch infections have not yet been reported in *Epinephelus* spp. from the Macaronesia.

Considering the large number and diversity of species of the order Trypanorhyncha, their high prevalence in a wide array of marine fish, the impact they might have on fisheries and the threat they may represent for mariculture species [30], knowledge on the pathological effects of Trypanorhyncha in fish is paramount. Based on this premise, the aim

of the present study was to assess and determine the prevalence and pathological changes caused by trypanorhynch larvae in wild dusky groupers from the Canary Islands, Spain.

2. Materials and Methods

2.1. Sample Collection

Between 2016 and 2018, a total of 28 specimens of adult dusky grouper were collected from the Eastern Central Atlantic from the Canary Islands. All fish were wild caught by professional fisherman and submitted dead to the Institute of Animal Health and Food Safety (IUSA), University of Las Palmas de Gran Canaria (ULPGC) for sanitary control within the Official Control Program of Ciguatera.

Though the exact catch location of the fish could not be determined, the date and approximate location of the capture were supplied. Dusky groupers were brought from Tenerife (n = 5), Gran Canaria (n = 1), Fuerteventura (n = 3) and Lanzarote (n = 19) (Figure 1).

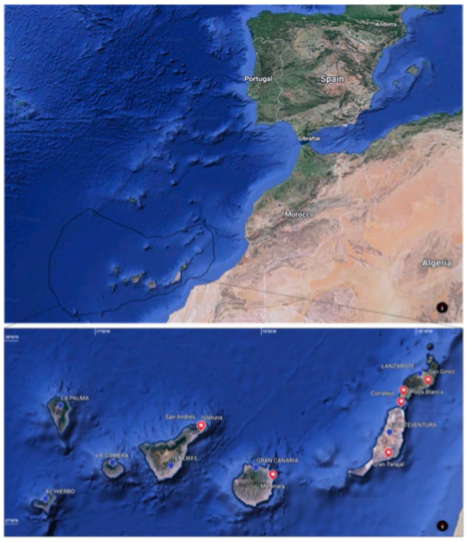

Figure 1. Spain established Exclusive Economic Zone in the Eastern Central Atlantic Ocean [31]. Inset: Capture zones in the Canary Islands (FAO fishing areas 34.1.2) [32] (maps obtained by Google Earth).

Total length (cm) and weight (kg) were measured for each specimen. Fish were necropsied, and samples of skeletal muscle, peritoneum, liver, spleen, stomach, intestine, gonads, swimbladder, kidney and heart were collected. Sex identification was determined by gross morphology of the gonads and confirmed by histology.

2.2. Histology and Parasitology

Representative segments of the collected tissues were stored in 10% neutral buffered formalin for histopathological examination. Encapsulated larvae were detached from the tissues, and the capsule was removed under a stereoscope (Motic SMZ-171 TL). Larvae were washed with saline solution and fixed in both 10% buffered formalin and 70% ethanol. The formalin-fixed tissues were then embedded in paraffin, sectioned at 4 µm and stained with hematoxylin and eosin (HE) and Masson's trichrome (MT) for histopathological assessment. Larvae samples stored in 70% ethanol were examined unstained under a stereoscope and an optical microscope (Olympus BX51TF). Stereoscope images (Moticam 1080) and photomicrographs of the larvae of the Trypanorhynch were taken (Olympus DP21).

3. Results

3.1. General Observations

Dusky groupers had an average body length and weight of 103.6 ± 6.2 cm and 22.4 ± 3.5 kg, respectively. Twenty-five were males, and three were females.

3.2. Gross Findings

External gross examination often showed distension of the abdominal cavity (Figure 2a). Internally, 27 out of 28 animals sampled presented numerous multifocal to coalescing parasitic cysts, attached or deeply embedded within the peritoneum and adhering to the serosal surfaces of the abdominal organs, often extending into the muscularis and submucosa of the stomach and intestine. These cysts were irregular, circular to oval with a narrowed end, variably sized (2 mm × 1.5 mm–6 mm × 3.2 mm), yellow to tan, larvae-filled, with small amounts of a serous fluid (Figure 3 left). Concurrently, there were numerous multifocal to coalescing dark nodules embedded within the fibrous tissue and adhering to the serosal surfaces of the celomic organs, occasionally extending into the muscularis and submucosa of the stomach and intestine and the hepatic parenchyma. These nodules were irregular, variably sized (10 mm × 25 mm–60 mm × 96 mm), dark brown to black, firm to hard, with a light brown and gritty core (Figure 3 right).

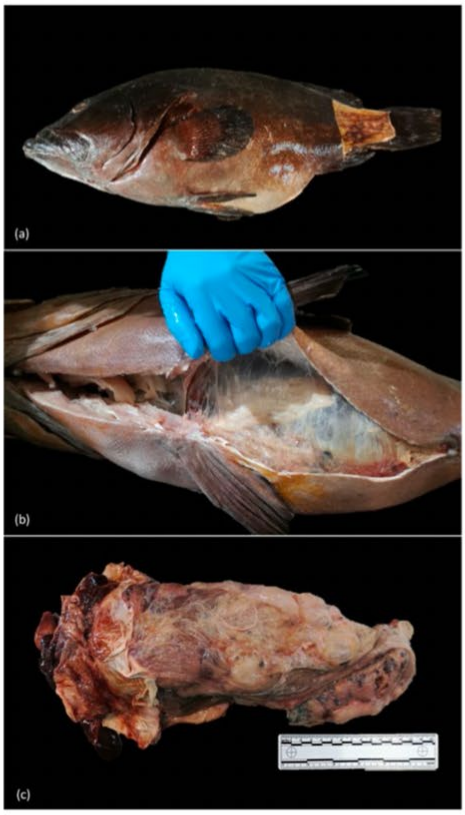

Figure 2. (**a**) Abdominal distension. (**b**) Abundant deposition of fibrous connective tissue in the abdominal cavity, (**c**) with diffuse intraperitoneal adhesions, hindering the separation of the individual organs, with numerous tan cysts and dark brown to black irregular nodules embedded.

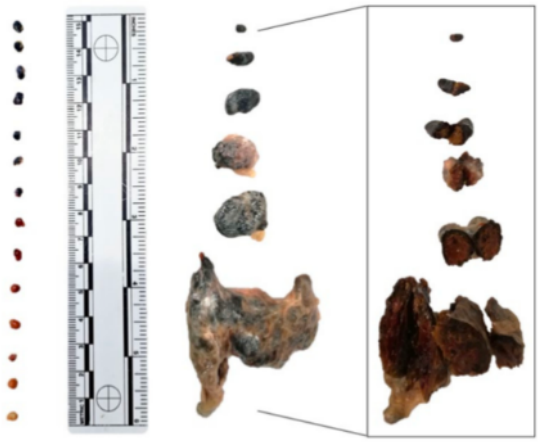

Figure 3. Size and color of the parasitic structures. Circular to oval, occasionally with a narrowed end, yellow to tan, larvae-filled cysts (**left**) and irregular, dark brown to black, firm to hard nodules (**center**) with a lighter gritty core at the cut surface (**right**).

In the abdominal cavity, surrounding and adhering to the peritoneum, mesenteric fat and abdominal organs (i.e., stomach, intestine, liver, spleen, kidney, gonads), both of the above-mentioned structures were embedded in abundant bands of fibrous connective tissue with diffuse intraperitoneal adhesions, hindering the separation of the individual organs (Figure 2b,c).

Larvae-filled cysts and nodules were mostly observed in the peritoneum and mesenteric fat, particularly surrounding the pyloric caeca (Figure 4a), and in the submucosa, muscularis and serosa of the stomach (Figure 4b) and intestine (Figure 4c). In a few cases, these structures were also present in the hepatic parenchyma (Figure 4d), adjacent to the gonads and adhering to the external wall of the bulbus arteriosus of the heart.

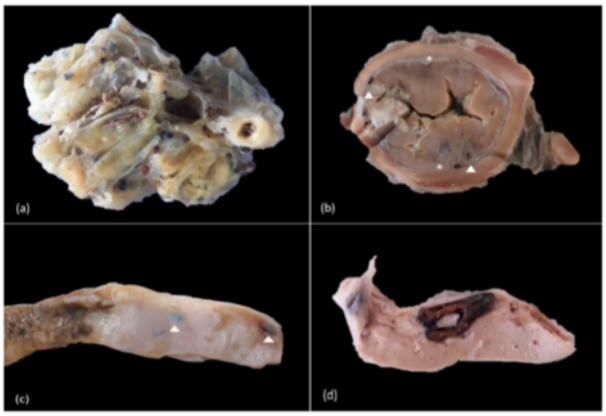

Figure 4. Location of the parasitic cysts and nodules. (**a**) Mesenteric fat and peritoneum, numerous cysts embedded in fibrous connective tissue. (**b**) Transversal section of the stomach, numerous cysts (arrowhead) in the submucosa with profuse deposition of mature fibrous connective tissue (*). (**c**) Intestine, multifocal, transmural larvae-filled cysts (arrowhead). (**d**) Liver, large dark brown nodule invading and displacing the hepatic parenchyma.

Individualized parasites appeared as round to elongated, yellow to tan brown, soft, with an average size of 2 mm × 1.5 mm.

3.3. Histopathology

Within the peritoneum (Figure 5a), adhered to the serosal surfaces (Figure 5b) and markedly expanding and compressing the muscularis and the submucosa of the stomach (Figure 5d) and intestine (Figure 5c), there were multifocal, variable-sized (up to 5.7 mm × 3.9 mm diameter), parasitic cysts containing a cross-section of a larval cestode (plerocercus). These were surrounded by an inner strongly basophilic, thin layer; a middle golden brown, thin cellular layer and an outer eosinophilic, thin layer of collagen with few fibroblasts.

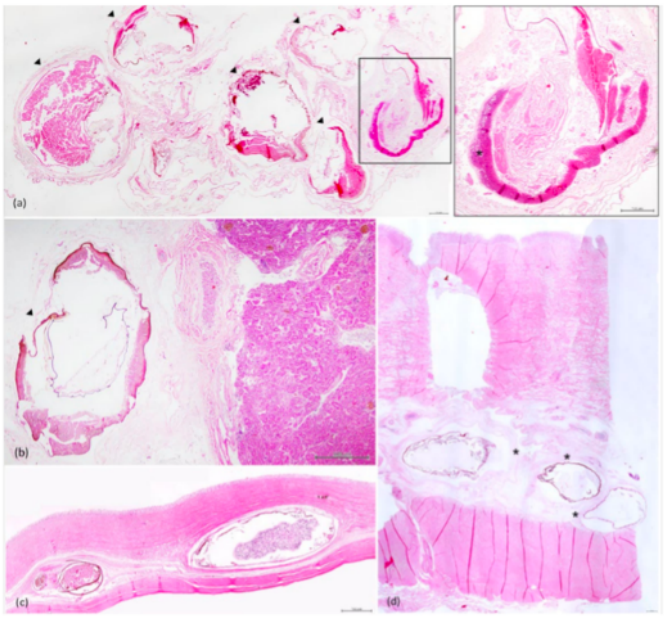

Figure 5. Histology, HE stain. **(a)** Peritoneum, cross-section of degenerated plerocerci (arrowheads) encapsulated in concentric layers of fibrous connective tissue (scale bar = 500 µm). Inset: severe inflammatory infiltrate and cellular debris (*) surrounding a ruptured cyst (scale bar = 500 µm). **(b)** Peritoneum, connective tissue adjacent to the kidney with a degenerated cyst (arrowhead) (scale bar = 500 µm). **(c)** Intestine, viable (**right**) and degenerated plerocerci (**left**) admixed with golden-brown macrophage aggregates (MA) (scale bar = 500 µm). **(d)** Stomach, numerous degenerated plerocerci embedded in a thick band of mature fibrous connective tissue (*) (scale bar = 500 µm).

Admixed and enclosed by a fibrous capsule, there were multifocally numerous, degenerated larvae, characterized by remnants of the plerocerci, on occasions with fragments of the hooked tentacles, and finely stippled to coarse, strongly eosinophilic, golden-brown and basophilic debris (mineralization) (Figure 6a,b). Surrounding the degenerated larvae cysts, there were often multifocal aggregates of golden-brown pigment-laden macrophages and, occasionally, scattered lymphocytes (Figure 6c). On occasions, in the peritoneum, when cysts were ruptured, there were variably sized aggregates of lymphocytes, and macrophages were observed surrounding and infiltrating the remnants of the parasite (Figure 5a).

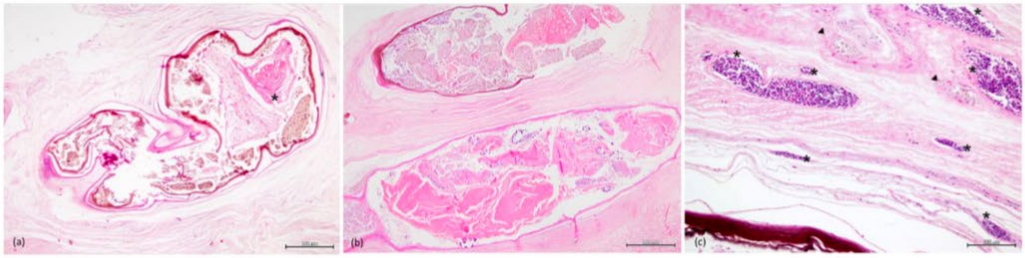

Figure 6. Histology, HE stain. (**a**,**b**) Encysted degenerated plerocerci, on occasions with fragments of the hooked tentacles (*), and finely stippled to coarse, strongly eosinophilic, golden-brown and basophilic debris (mineralization), enclosed by a fibrous capsule (scale bars = 500 µm). (**c**) Stomach submucosa, proliferation of small-caliber blood vessels (*) and MA surrounding (arrowheads) the degenerated plerocerci (scale bar = 100 µm).

Surrounding the cysts, there was profuse deposition of mature fibrous connective tissue, characterized by densely packed collagen fibers, as highlighted by the MT stain (Figure 7), and minimal inflammation, with occasional interspersed small aggregates of lymphocytes and golden-brown macrophages and oedema. Diffusely, in the fibrotic areas of the submucosa of the stomach, there was proliferation of small-caliber blood vessels (Figure 6c). Furthermore, small and medium caliber arteries within the fibrous connective tissue had markedly thickened walls, expanded by dense layers of connective tissue with numerous small caliber blood vessels.

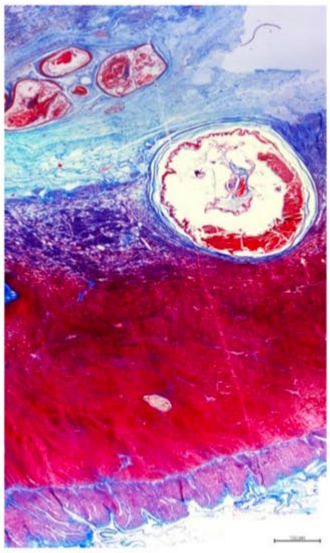

Figure 7. Histology, MT stain. Stomach, submucosa and muscularis adjacent to the cysts effaced and replaced by extensive areas of fibrous connective tissue (scale bar = 500 µm).

Occasionally, in the mucosa of the stomach, there were multifocal areas of mild necrosis and fibrosis.

The liver showed no significant pathological changes, but occasionally, hepatocytes were interspersed with small aggregates of lymphocytes and multifocal proliferation of

macrophage aggregates (MA) with a golden-brown pigment. This proliferation of MA was also observed in the spleen, kidney, intestine and stomach.

Sections of the individualized plerocerci showed an eosinophilic tegument and lacy, fibrillar, eosinophilic parenchyma with numerous, basophilic to clear, round calcareous corpuscles embedded and a scolex with four muscular bulbs and armed tentacles. Calcareous corpuscles are often dissolved during the fixation or histological processing, in which cases, the shape of the corpuscles remains, but they present as clear oval structures [33]. In some sections, the anterior end of the plerocerci was observed with the scolex, characterized by muscular fibers concentrically arranged (muscular bulbs) (Figure 8a–d), stained red by MT and the armed tentacles lined by series of refractile hooks (Figure 8e–g).

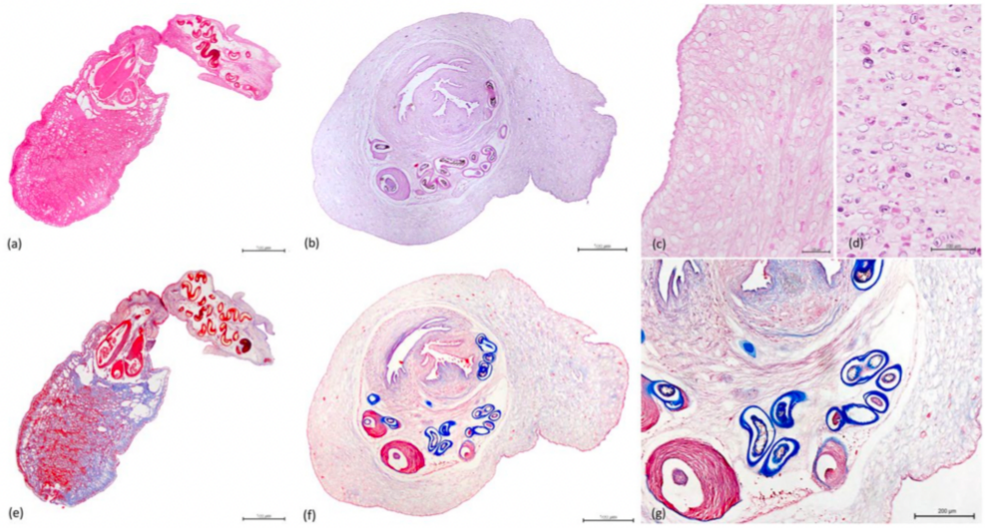

Figure 8. Histology, HE stain. Trypanorhyncha plerocerci. (**a**) Longitudinal section, eosinophilic tegument, lacy, fibrillar, eosinophilic parenchyma and scolex with muscular bulbs and armed tentacles (scale bar = 500 µm). (**b**) Transversal section, muscular bulbs and armed tentacles (scale bar = 500 µm). (**c**) Eosinophilic tegument (scale bar = 100 µm). (**d**) Parenchyma, numerous, basophilic to clear, round calcareous corpuscles (scale bar = 100 µm). Histology, MT stain. (**e**) Longitudinal section, tentacle sheaths and muscular bulbs staining red with MT (scale bar = 500 µm). (**f**) Transversal section, muscular bulbs and armed tentacles (scale bar = 500 µm). (**g**) Detail of the muscular bulbs and hooks (scale bar = 200 µm).

Severity and extension of the lesions was directly proportional to the intensity of the infection. Fish with a higher number of parasites ($n > 30$) presented extensive fibrosis of the peritoneum as well as the muscular and submucosa of the stomach and intestine. On the other hand, fish with a lower number of parasites ($n < 30$) showed less extensive fibrosis and the areas of the stomach and intestine affected were smaller. Differences were not observed between fish captured from different regions. However, the number of fish specimens received was not similar for all the islands; hence, it was not possible to establish any correlation.

3.4. Parasitology

The overall prevalence of *Pintneriella* sp. larvae was 96.4% (27/28) with 3/3 specimens from Fuerteventura, 1/1 from Gran Canaria, 18/19 from Lanzarote and 5/5 from Tenerife islands.

Observation of the parasites preserved in 70% alcohol under a stereoscope and optic microscope showed two presentation forms of the plerocercus: a plerocercus enclosed within

an oval, amber, blastocyst of 2 mm × 0.9 mm (Figure 9a) and a plerocercus with a blastocyst and a protruding scolex, amber, up to 10 mm × 2.1 mm (Figure 9b). The plerocercus showed a scolex peduncle (Figure 10a) with tentacles armed with hooks (Figure 10a,b), two bothridia (Figure 10b), pars vaginalis with the tentacle sheaths (Figure 10c) and pars bulbosa with the muscular bulbs (Figure 10d). Within the scolex peduncle, there was a characteristic rhyncheal apparatus with a metabasal and basal armature, tentacle sheaths and muscular bulbs.

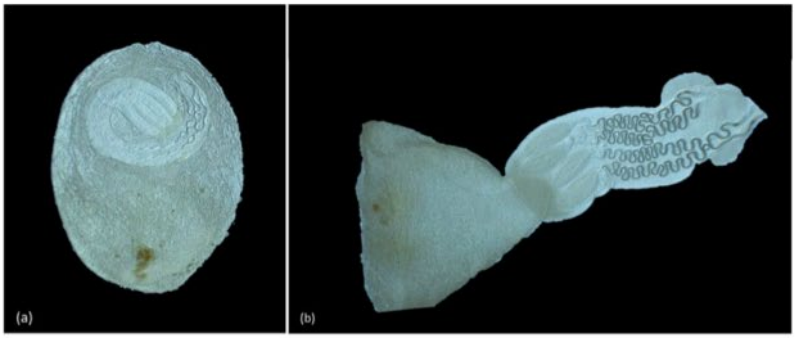

Figure 9. Plerocercus under stereoscope (0.75×). (**a**) Plerocercus within blastocyst. (**b**) Scolex of the plerocercus emerging from the blastocyst.

Figure 10. Plerocercus under a light microscope. (**a**) Scolex of the plerocercus emerging from the blastocyst (Bo—bothridium; Tb—tentacle bulb; Te—tentacle) (scale bar = 500 µm). (**b**) Detail of the hooked tentacles. (**c**) Detail of the bothridium. (**d**) Detail of the tentacle bulb.

4. Discussion

Numerous studies have detailed the presence and morphological features of Trypanorhyncha species infecting groupers [3,4,7,9,24–26,28,34–36]. Notwithstanding, information on the pathological effects produced by these parasites remains limited. Therefore, the main goal of this study was to assess and determine the prevalence and pathological changes produced by Trypanorhyncha in dusky groupers caught in the Canary Islands, Eastern Central Atlantic Ocean.

The prevalence of *Pintneriella* sp. in our study has been estimated as 96.4%. This result was considerably higher compared to those reported in previous studies. In *Epinephelus* spp. from the Arabian Gulf, the prevalence of *P. musculicola* and *Floriceps* sp. was around 15% [25] and 24.2% [26], respectively. Similarly, in white groupers, *Epinephelus aeneus,* and dusky groupers from the Turkish Mediterranean coast, the prevalence of *Grillotia* sp. was 17.7% and 18.4%, respectively [28]. In groupers from the Red Sea, the overall prevalence of *Callitetrarhynchus gracilis* was 9.2% [35]. Furthermore, in areolate groupers [37] and the orange-spotted grouper, *Epinephelus coioides* [36], from Bali, the prevalence ranged from 3.3% to 8.6%. These variations may be due to differences in the availability of the first intermediate host as prey [7,38], ontogenetic diet shifts [19,39,40] and the amount of prey usually ingested [41]. Larger fish typically feed on macrocrustaceans, smaller fishes and cephalopods [19,20] and need greater amounts of food, hence being more likely to ingest first intermediate hosts [41]. Fish from our study had a larger average length when compared to groupers from previous studies. Similarly, it has been observed in previous studies that trypanorhynch larvae occurred primarily in larger fish [25,26,42].

Grossly, fish presented with a distended abdominal cavity with diffuse and extensive fibrosis in the peritoneum, mesenteric fat and serosal surfaces of the abdominal organs. Embedded within this fibrous tissue, there were numerous, round with a narrowed end, yellow to tan-brown, larvae-filled cysts and irregular, dark brown to black, firm to hard nodules. These structures were also observed in the muscularis and submucosa of the stomach and intestine and, on a few occasions, invading the hepatic parenchyma and the pericardial cavity. In *Epinephelus* spp. from the Arabian Gulf [25], long, whitish plerocercoids of *P. musculicola* were observed in the muscle and were associated with muscle fiber atrophy and oedema [25]. Here, plerocercoids of *Pintneriella* sp. were not observed in the muscle, despite the similarities shared regarding the parasite genus and the host. In areolate groupers infected with *Floriceps* sp., Ibrahim [26] did not observe external lesions, but the liver showed atrophy with focal firm and necrotic areas. Fibrosis and adhesion of the abdominal organs were also observed [26]. These results differ from the findings presented here. In our study, the liver was only occasionally affected by dark brown nodules and scattered lymphocytes and macrophage aggregates, but atrophy was not observed. The presence and gross features of trypanorhynch larvae in *Epinephelus* spp. was also briefly mentioned in a few other studies. In dusky groupers from the Libyan coastal waters, larvae-filled cysts were mostly present in the head kidney, followed by the external surface of the stomach, ovary, and testis [27]. In our study, no evidence of plerocerci was detected in the head kidney. Plerocerci enclosed within both white and brown to black capsules were also recovered from the body cavity attached to the mesentery of *Epinephelus* spp. from Australia and New Caledonia [3]. Contrary to our results, pathological changes were not observed in dusky groupers from the Turkish Mediterranean coast infected with trypanorhynch plerocerci [28].

Histologically, we observed in the peritoneum and mesenteric fat, extensive areas of fibrosis with embedded plerocerci cysts adhering to the serosal surfaces of the abdominal organs and invading the muscularis and the submucosa of the stomach and intestine. Areas of the muscularis and submucosa adjacent to the cysts were often effaced and replaced by fibrous connective tissue with proliferation of macrophage aggregates with a golden-brown pigment and small caliber blood vessels. Occasionally, in the mucosa of the stomach, there were multifocal areas of mild necrosis and fibrosis. Similarly, fibrosis of the mesentery with adhesion of the internal organs and encapsulation of the plerocerci with fibrous connective

tissue were also reported in areolate groupers infected with *Floriceps* sp. [26]. However, contrasting with our findings, those lesions were also accompanied by severe degenerative and necrotic changes in the liver and marked tissue destruction with intense inflammatory reaction in the skeletal muscle, caused by the entrance of the motile larvae [26]. By contrast, other studies reporting trypanorhynchs in fish tissues did not detail associated pathological changes [28], and some have suggested that these parasites were not likely to cause significant pathological changes [27,43].

Extensive proliferation of connective tissue with encapsulation of the plerocerci appears to be a common response in long-standing infections with cestodes [44–48]. Containment in this way serves to separate the parasite from the tissues to prevent further damage to the host [45,46]. Another reported trait in long-standing cestode infections is a marked decrease in the number of cells participating in tissue reaction and the predominance of fibrous connective tissue [44,49]. In most vertebrates, there is a particular immune response implicated in the production of collagen [50], a major component of mature connective tissue and an important agent of tissue repair [51] that is believed to play a role in both parasite encapsulation and tissue reconstruction [50]. Proliferation of small caliber blood vessels admixed with the connective tissue has been associated with an attempt to repair the injured tissues to get the necessary nutrients and oxygen [51]. In heavily infected fish, fibrosis tends to extend throughout the body cavity, causing adhesion of the viscera to each other and to the body wall [45]. When tissue injury is severe or recurring or if the wound-healing response is not appropriately regulated, it will result in overzealous or persistent wound-healing responses, becoming detrimental and contributing to the development of fibrotic pathology [50,52]. In fish, cestode infections have caused extensive fibrosis, producing compression and atrophy of the adjacent abdominal organs [53]. In severe cases, fibrosis may eventually lead to organ malfunction and death [52,54].

Macrophage aggregates (MA) are commonly found in the spleen, kidney, and liver in some fish species [55]. However, mobilization and proliferation of MA [55] may also be features of a chronic inflammatory response [55,56]. It has been shown that these pigment-laden aggregates may be found surrounding and within the encapsulating response of parasites [47,55,57], as observed in our study. Thus, MA are likely to act as collections of scavenging cells stimulated by excessive degenerating tissue [58].

Color variations grossly observed in the yellow to tan-brown larvae-filled cysts and dark brown to black nodules may be due to the deposition of different types of pigments. Histologically, in the larvae-filled cysts, the plerocerci remained intact. On the other hand, dark brown to black nodules represented the degenerated parasites. Necrotic remnants of the plerocercus admixed with finely stippled to coarse, strongly eosinophilic to golden-brown pigmented debris and basophilic pigment (mineralization) were lined by a layer of golden-brown cellular debris and further bounded by thick layers of mature connective tissue. Mineralization has often been described with degenerated cestodes [44,49,59]. It has been said to occur in older infections resulting in the death of the parasite [60]. Dystrophic calcification typically occurs in areas of necrosis when dead and dying cells may no longer regulate the influx of calcium into their cytosol, and calcium accumulates in the mitochondria. Calcium deposition is common with dead parasites. Their significance is that they are an indicator of previous injury to a tissue [61]. This, together with the proliferation of pigmented MA surrounding, and most likely within, the degenerated plerocerci were possibly the cause for the darker coloration observed in the dark brown to black nodules. In line with this, Beveridge et al. [3] remarked that brown and black envelopes contained only remnants of plerocerci and that this dark coloration was likely due to melanisation of the cyst wall. In some species of serranids, fibrotic encapsulation has been associated with what appears to be ceroid, lipofuscin, and melanin pigmentation [61], likely from the proliferation of MA. This seems to indicate that dusky groupers have an immune mechanism to contain, mineralize and eliminate these parasites. The high number of degenerated parasites seems to suggest that fish may develop immunity to these parasites, resulting in the death of the larvae, as previously proposed by MacKenzie [62]. In line with this, Rigby and Dufour [63]

proposed that darker nodules were the result of a host-initiated immune response that isolated and killed the parasite [63]. Based on the numerous degenerated plerocerci encapsulated by thick layers of connective tissue, it has been suggested that plerocerci possibly remain in fish tissues for a long period [64]. Pathogenicity of trypanorhynch larvae appears to be highly associated with the intensity of the infection [10]. Here, we have observed that fish with a higher intensity of infection exhibited a more exuberant pathological response, with extensive fibrosis and visceral adhesion. Butterfish, *Peprilus triacanthus*, with heavy infections with trypanorhynch larvae in the skeletal muscle had weight loss when compared with those with lower burdens [10,65]. In lizardfish, *Saurida tumbil*, high levels of parasitism by *C. gracilis* have been associated with a high mortality rate at one phase of the fish life cycle [66].

Two possible sources of transmission are suggested here: ingestion of infected crustaceans with procercoids or ingestion of smaller fish with the immature plerocercus. In a study with adult whiting *Merlangius merlangus*, Özer et al. [43] postulated that infection with *Grillotia erinaceus* was likely to have occurred from the ingestion of smaller whiting carrying the first developmental stage. Likewise, in a study with halibut *Hippoglossus hippoglossus*, it was suggested that larger fish became infected by feeding on smaller fish containing recently acquired parasites not yet developed beyond the procercoid stage [67].

A crucial requirement for efficient fishery management is knowledge of the health and wellbeing of fish populations and their ecosystems [19]. Knowledge leads to better conservation and sustainable fishery management of the endangered species [19]. In this respect, assessment of the health condition of wild populations is vital, not just for wild stocks, but also for cultured fish that are also susceptible to infectious agents transmitted by broodstock that naturally live in or in the surroundings of the net cages [30]. Even though these infections do not appear to represent a threat for human health, recent research has shown that ingestion of fish with Trypanorhyncha can cause allergic disorders, since immunological hypersensitivity has been demonstrated in studies using murine models [68–70]. In addition, heavy cestode infections may reduce the fish market value by making them unappealing to consumers [8,16].

5. Conclusions

Our findings indicate that Trypanorhyncha are highly prevalent in adult dusky groupers from the Canary Islands. Infections by these parasites induce a progressive and chronic response characterized by an extensive and marked fibrotic reaction with encapsulation of the plerocerci. This suggests that the fish immune system attempts to eliminate the parasites through fibrous encapsulation. However, in fish with high-intensity infections, severe fibrosis with visceral adhesions is common. In these cases, compression and atrophy of the adjacent abdominal organs may occur, eventually leading to organ malfunction and death.

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III. An end to the controversy over the microscopic detection and effects of pristine microplastics in fish organs

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An end to the controversy over the microscopic detection and effects of pristine microplastics in fish organs

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The aquatic environment and the associated fish assemblages are being exposed to an increasing amount of microplastics. Despite the high number of publications on the presence of microplastics in fish, little is known about their uptake, translocation and accumulation within fish organs. Experimental studies on the detection and effects of pristine microplastics in fish have shown controversial and ambiguous results, respectively. Here, we conducted two experiments to detect and assess the impacts of dietary exposure of *Danio rerio* to different types of pristine microplastics. Our results show that *D. rerio* recognizes plastic particles as inedible materials but ingests them when mixed with food or fish oil. Accidental ingestion occurs in fish exposed to relatively small (1–5 µm) microplastic particles without associated food or fish oil. Additionally, *D. rerio* effectively eliminated pristine microplastics 24 h after ingestion; however, retention time was associated with increasing particle size and the intake of additional meals. Clinical signs, such as anorexia and lethargy, are present in fish fed relatively large microplastics (120–220 µm). The ingestion of microplastics does not induce any histopathological changes. To the best of our knowledge, we are able, for the first time, to fully demonstrate the uptake and translocation of plastic microbeads using confocal microscopy. Our results question the findings of previous studies on the detection and effects of pristine microplastics in fish and state that inaccurate interpretations of the histological findings regarding microplastics in fish organs is a prevalent flaw in the current scientific literature.

The ever-growing production of plastics[1,2] and their relatively short lifespan[3], combined with indiscriminate waste-disposal practices and accidental releases[1], have led to the accumulation of plastics in aquatic environments worldwide[4–9]. This situation is especially worrisome due to their long degradation time[2] and potential to be ingested by aquatic organisms[3].

In water, plastics undergo weathering[10] through photolytic, mechanical and biological degradation[11,12]. Under these circumstances, larger plastics degrade into smaller fragments[12], i.e., microplastics (MPs) (< 5 mm)[13]. Another source of MPs in aquatic environments is micro-sized particles intentionally manufactured for use in domestic products (e.g., cosmetics and clothing) and industrial products (media blasting and industrial feedstock)[11,12], which are directly introduced into the environment by human activity[10].

MPs can display a variety of shapes, sizes and colours[12]. In aquatic systems, the predominant shapes of MPs are fibres[14–18], fragments[6,7,19,20] and microbeads[21]. The small size and ubiquity of MPs[22] makes them easily available to aquatic fauna, which are prone to ingest them by confusion with food, accidental ingestion or by transfer through the food chain[23,24]. Several studies have documented the ingestion of plastic and MP particles in aquatic species (invertebrates[10,25–28], amphibians[29], reptiles[30], marine mammals[8,31] and seabirds[32,33]).

In fish, the presence of MPs in the gastrointestinal tract (GIT) has been reported in several marine and freshwater species captured all around the world[34–38]. Overall, the average number of particles found in the GITs of

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fish ranged from 0 to 3 items/fish[34,36–44]. The low number of particles suggests that the potential for accumulation of MPs in the GITs of fish is close to zero and that the presence of MPs in the GITs is indicative of a recent ingestion[23]. However, to confirm this theory, further dietary experimental studies are needed. Knowing the retention times of MPs in fish GITs will help to determine the MP load to which fish are exposed in their lifetime[23]. An experimental study, in which *Carassius auratus* were fed a single intake of MPs (100–500 µm), showed that after 24 h, 90% of the plastic particles had been eliminated, with only 0 to 3 particles being excreted after 144 hours[45]. In another experiment with *Seriolella violacea*, MP (length, 1.2 ± 0.2 mm; diameter, 1.0 ± 0.1 mm) elimination took up to 7 weeks[46]. In *Sparus aurata* fed multiple meals with MPs, most particles were eliminated after a 30-day period of depuration[47]. In general, MPs appear to have a small long-term potential for accumulation, being unlikely to accumulate in the digestive tract[45,47,48]. However, larger particles seem to be retained for a longer time in the GIT. It is also important to bear in mind that the structure of the digestive tract varies among fish species[49] and that additional intrinsic (species, age, and physiology) and extrinsic (habitat, food concentration/availability, and type of MPs) factors have to be taken into account when discussing retention rate patterns for MPs. All these variables highlight the necessity to carry out more studies in different species to better understand the potential for accumulation of MPs in fish.

Despite the apparent effective elimination, a study[24] reported the uptake of MP particles (1–20 µm) by enterocytes in 3 out of 39 fish.

The translocation of MP particles to other tissues, such as the liver[47,50,51] and muscle[37,47], has been described. Avio et al. reported the translocation of plastic particles (200–600 µm) from the digestive tract to the liver in *Mugil cephalus*[50]. Likewise, Collard et al. documented the presence of two particles (39–90 µm) in the livers of *Engraulis encrasicolus*[51], and Abbasi et al. detailed the presence of variably sized MP particles (up to 250 µm) in the livers of four fish species captured in the Persian Gulf[37]. Similarly, Jovanovic et al. observed the presence of < 1 particle (214–288 µm) in the liver of *S. aurata*[47], carefully pointing to the possibility of cross-contamination. The alleged translocation of such large particles is difficult to explain with the current knowledge on translocation pathways for MPs in fish. The plausibility of these reports should be questioned, and cross-contamination should be considered.

Other studies working with relatively small particles have also reported the translocation of MPs to the liver. In a study using 0.5 µm MPs in *Oreochromis niloticus*[52], the photomicrographs available to support the observations merely displayed fluorescence in the liver, while fluorescence was observed diffusely in the remaining organs, which is likely to be attributed to leaching of the fluorescent dye and not necessarily the presence of MP particles[53]. In another study with *D. rerio* using 5 µm MPs[48], the general quality of the photomicrographs did not support the stated results, as previously pointed out by other authors[54].

Even though the direct consequences of MP ingestion have been detailed (i.e., damage and physical blockage of the digestive system and limitation of food intake)[23], our understanding of the impacts of MPs is still limited and often ambiguous. Tissue alterations have been described as a consequence of MP exposure. Pedà et al. reported that pristine MPs induced moderate to pronounced alterations of the distal intestine after 30 days of exposure in *Dicentrarchus labrax*[55]. Thinning of the bowel wall and epithelial damage were described by Qiao et al. in the intestine of *D. rerio* exposed for 21 days[56]. Similarly, Lei et al. showed that MP particles caused cracking of villi and splitting of enterocytes in *D. rerio*[57]. In the same species, Lu et al.[48] reported signs of inflammation and lipid accumulation in the liver after exposure to MPs. In contrast, Asmonaitė et al. found limited impacts on gut integrity in *Oncorhynchus mykiss* exposed to MP particles for 4 weeks[58]. Jovanovic et al. concluded that dietary exposure of *S. aurata* for 45 days to different types of MPs did not cause any damage in the studied tissues[47].

The ambiguity of the results obtained regarding the effects of pristine MPs in fish is largely due to a recurring problem of inaccuracy in the interpretation of the histopathological findings. Similar worries have been expressed by several veterinary pathologists. For instance, multiple errors have been detected in the aforementioned article by Lu et al.[48] (see Comment on "Uptake and Accumulation of Polystyrene MPs in *D. rerio* (*Danio rerio*) and toxic Effects in Liver" by Baumann et al.[54]) as well as in an article by Deng et al.[59] (see Uptake of MPs and related health effects: a critical discussion by Braeuning[50]). The problem with inaccurate data is that it will persist in the literature and will be unequivocally considered reliable. As a consequence, erroneous information will be unintentionally replicated and perpetuated by several authors.

In the present study, two separate experiments were performed using *Danio rerio*, a vertebrate model used for toxicological studies. The acute experiment was conducted to assess ingestion, intestinal retention time, uptake and elimination of MPs in *D. rerio* fed a single intake of pristine MPs. The sub-chronic experiment was designed to evaluate the potential for accumulation and translocation of different types of pristine MPs after prolonged dietary exposure and to determine the microscopic effects of such exposure.

Methods

MP characterization. Green fluorescent spherical microbeads (proprietary polymers of an undisclosed composition) with diameters of 1–5 µm were purchased from Cospheric LLC, USA (Supplementary Fig. S1). The microbeads were dissolved in ultrapure water (density: 1.3/cm^3) to prepare stock solutions and were fluorescently labelled green with excitation and emission wavelengths of 515 and 414 nm, respectively. According to the manufacturer, the fluorescent particles were hydrophilic, and the addition of surfactant was not necessary. Fluorescent particles were used to enable the identification of small MPs (1–5 µm) in fish tissues.

Microfragments of plastic, labelled as polyethylene (PE), were obtained from a cosmetic body cleanser. The content of the cleanser was washed with distilled water and sieved to obtain particles ranging in size from 120–220 µm (mean: 175 ± 42 µm) (Supplementary Fig. S1). White nylon microfibres, obtained from a synthetic textile, were cut under a stereomicroscope to obtain fibres with an average width and length of 13.67 µm and 1.5 mm, respectively (Supplementary Fig. S1).

To determine the composition of the plastic polymers used, Fourier transform infrared (FTIR) spectroscopy was performed. A Bruker IFS 66/S spectrometer (Bruker, Spain) equipped with a deuterated triglycine sulphate (DTGS) detector and a diamond crystal attenuated total reflection (ATR) module was used. FTIR spectra were acquired from an average of 64 scans obtained with an 8 cm^{-1} resolution. The reflectance ratio (R/R_0) was calculated, where R and R_0 are the reflectances measured at the sample and the clean crystal, respectively. Positive bands represent the loss, while negative bands represent the gain of species at sampling. The cosmetic microfragments were confirmed to be PE (Supplementary Data S1). The spectrum obtained by the analysis of the fluorescent microbeads showed a slight similarity to that of polyethylene glycol (< 60%) but was insufficient to determine the polymer composition (Supplementary Data S2).

Ethics approval. All the experimental protocols used in this study were revised and approved by the Animal Ethics Committee of the University of Las Palmas de Gran Canaria and authorised by the competent authority of the Canary Islands Government (Reference number: OEBA-ULPGC 23/2018). GPower3.1 software was used to determine the number of necessary animals per experimental group, with a statistical power of 0.95 and an alpha error of 0.05. All the protocols were designed and performed to result in the death of as few animals as possible and to reduce the duration and intensity of suffering, in accordance with the relevant guidelines and regulations (Directive 2010/63/EU).

Experimental design

During the experiments, three petri dishes with ultrapure water were placed next to each work area and analysed as procedural blanks. The procedural blanks were present at every step of the MP evaluation process to assess sample contamination.

Acute experiment: feeding behaviour, intestinal retention time, uptake and elimination. Seventy *D. rerio* adults of similar weight were purchased from Tropical Centre (ICA Canarias) and kept in acclimation tanks for 4 weeks to adapt to the laboratory conditions. The fish were placed in a semi-static system with tap water conditioned with JBL Biotopol and JBL Denitrol, according to the manufacturer's instructions, at a stocking density of ~ 0.8 fish/dm^3 (0.4 g fish/dm^3) in the animal experimental facility (EGC00616436) at the University of Las Palmas de Gran Canaria. The fish were kept under a natural photoperiod of 12:12 h light:dark cycle. Water chemistry parameters were assessed every two days using a colorimetric test kit (nitrate, 10 mg/dm^3; nitrite, 0 mg/dm^3; pH, 6.8; total hardness, 80–300 mg/dm^3; chlorine, 0 mg/dm^3). Dissolved oxygen (> 6.0 mg/dm^3) and temperature (23–25 °C) were also measured. The water was partly (60%) manually dumped, and debris (uneaten food and faeces) was siphoned from the bottom of the tanks.

During the acclimation period, fish were fed a control diet three times a day (20% of their body weight/day). The control diet was prepared using flake-shaped commercial food for aquarium fish (Basic, DAJANA PET, s.r.o.; 47% protein, 8% water, 7% ash, 5% fat and 2% fibres), which was ground into a fine powder. A gelatine leaf was melted in 27 °C water and mixed with the ground flakes. The final mix was refrigerated at 4 °C until it was solid.

A preliminary study was performed to assess the ability of *D. rerio* to recognize plastic particles as inedible material. Free fluorescent microbeads (0.328 g) were added to an aquarium with five *D. rerio*. Likewise, cosmetic microfragments (0.031 g) were added to another aquarium with the same number of fish. The fish from both groups were sampled 2 h post-feeding (hpf). Euthanasia and necropsies were performed as detailed below.

For the acute experiment, two sets of diets were used. Diet F_A was obtained by adding fluorescent microbeads (18.6×10^{-2} g/cm^3, 16% of the total food delivered) to the control diet. Diet C_A was obtained by mixing cosmetic PE microfragments and textile microfibres (2.1×10^{-2} g/cm^3, 2% of the total food delivered) with fish oil-aromatized gelatine. The gelatine was used as a medium to retain the plastic particles in the food and avoid their separation when incorporated into the water. Both diets were refrigerated at 4 °C until solid.

Following acclimation, the fish were starved for 24 h, randomly collected and then separated into two groups (80 dm^3 per aquaria, n = 30). The fish in group 1 (0.333 ± 0.071 g) were fed a single intake of diet F_A. The sum of the fluorescent MPs provided accounted for 3% of the fish body weight. The fish from group 2 (0.360–0.063 g) were fed a single intake of diet C_A, which accounted for 0.3% of the fish body weight. In both cases, feeding took approximately 30 min. Following this period, to prevent food contamination, the fish from both groups were separated into five aquaria in groups of six. Each aquarium corresponded to a different sampling time (2, 6, 10, 12 and 24 hpf). The fish were closely monitored during these procedures. At every sampling point, the water from each aquarium was filtered, and faeces were recollected and mounted on a slide.

The fish from group 1 and group 2 were sampled 2, 6, 10, 12 and 24 hpf. To avoid contamination, each group had its own handling equipment. Euthanasia was achieved by anaesthetic overdose (0.5–0.6 cm^3/dm^3) through immersion in 2-phenoxyethanol (Sigma-Aldrich). Necropsies were performed under a stereomicroscope (Motic SMZ-161 TL, China). The whole intestine was extracted and fixed in 10% neutral buffered formalin for histology.

Sub-chronic experiment: translocation and toxicity. Seventy-two *D. rerio* adults of similar weight and length were purchased from the same supplier and kept in acclimation tanks under the same conditions as those described for the acute experiment.

Two sets of experimental diets were used. Both were prepared the same way as the control diet to ensure homogeneity throughout the test food. Each test diet was spiked with different types of MPs. Diet F_{SC} was obtained by adding fluorescent microbeads (9.9×10^{-4} g/cm^3, 0.1% of the total food delivered) to the control diet. Likewise, diet C_{SC} was obtained by mixing cosmetic PE microfragments and textile microfibres (3.3×10^{-2} g/cm^3, 3% of total food delivered) with the control diet. A control group, held under identical conditions, was fed a control diet.

After the acclimation period, the *D. rerio* individuals were weighed, and their general body shape and urogenital papilla were inspected to determine their sex. The fish were distributed into two test groups. Fish from group 1 (0.427 ± 0.04 g) were fed the F_{SC} diet, and fish from group 2 (0.568 ± 0.112 g) were fed the C_{SC} diet. All the treatments were carried out in triplicate, and each aquarium comprised an equal number of males (n = 4) and females (n = 4). Each batch (n = 8) was placed in a 20 dm^3 aquarium. A control group (n=24) was added.

The fish from all the groups were fed a control diet three times a day (20% of their body weight) on a fixed schedule. Every two days, the first intake of the control diet was replaced with the F_{SC} diet (0.01% MPs/fish/day) and C_{SC} diet (0.2% MPs/fish/day) in the test groups.

After the second week, the fish from the cosmetic PE group started to manifest anorexia and impaired reactivity to stimuli. The control diet was then reduced to two intakes a day for a week to evaluate the fish response. After a week, the initial feeding routine was resumed.

During feeding time, the fish were monitored for behavioural changes. The feeding experiment lasted 45 days. Mortalities and observable abnormalities regarding both appearance and behaviour were recorded. All the aquaria contained tap water under constant aeration. The filters were regularly washed, and clean water was added.

After 30 and 45 days of feeding, two fish from each replicate aquarium were euthanized. Euthanasia was performed as described for the acute experiment. The weight of each fish was recorded. To prevent contamination with particles, a cut was made in the ventral line. The whole fish were fixed in 10% neutral buffered formalin for 24 h. Whole intestine, liver and muscle sections were then extracted under a stereo microscope (Motic SMZ-161 TL, China). The fish from the control group were similarly dissected. Contamination was prevented and confirmed by the absence of MPs in the control group.

The depuration period was designed to determine the potential for the bioaccumulation of these plastic particles. At the end of the feeding period, the remaining fish were transferred to new aquaria to avoid contamination with the test substance. For 15 days, all the fish were fed only the control diet. At the end of the depuration period, two fish per triplicate were euthanized, weighed and similarly dissected.

Histology. The formalin-fixed tissues were dehydrated, cleared and embedded in paraffin and sectioned at 4 µm. The obtained samples were stained with haematoxylin and eosin (H&E)[61]. Five sections were made from each sample. The slides were mounted and examined with a light microscope (Olympus BX51TF, Japan).

For the assessment of the histological findings in the intestine and liver, the methodology proposed by Saraiva et al.[62] and Bernet et al.[63], respectively, was followed. For each functional unit (i.e., the liver tissue, interstitial tissue and bile duct for the liver and the epithelium and lamina propria for the intestine), pathological changes were classified into five reaction patterns: circulatory disturbances, regressive changes, progressive changes, inflammation and tumour development. The alterations (w) were classified into three important factors: minimal pathological importance (1) if easily reversible, moderate pathological importance (2) and marked pathological importance (3) if generally irreversible. Additionally, a score value (a) ranging from 0 to 6 was used for every alteration, depending on the degree and extent of the alteration: unchanged (0), minimal (1), mild (2), mild to moderate (3), moderate (4), marked (5) and severe (6). Mathematical calculation of lesion indices was performed to assess the degree of damage for each organ separately. An individual description, termed *degree of vacuolation*, was used in addition to the alteration classifications of the reaction patterns. The degree of vacuolation was scored for all the fish using a semiquantitative scale: minimal (1), mild (2), mild to moderate (3), moderate (4), marked (5) and severe (6). For the index calculations, these were not considered, since the changes were already covered by the standardized expressions (plasma alterations; decreased hepatocellular vacuolation) within the respective reaction pattern (Supplementary Tables S1 and S2).

Confocal microscopy. Confocal microscopy was used to assess the presence and uptake of fluorescent MPs by the different tissues. Fluorescence images were acquired with a confocal microscope (Zeiss Confocal LSM800, Germany) at an excitation wavelength of 519 nm and emission wavelength of 543 nm for green and an excitation wavelength of 543 nm and an emitting wavelength of 567 nm for orange. Panoramic images of the whole *D. rerio* intestine were created. To confirm the internalization of the MP particles in tissues, a series of two-dimensional images over the depth ranges of interest (Z-stacks) were performed to obtain a three-dimensional image. The diameter of the microparticles was measured using Zen Blue v2.3 software.

Statistical analysis. For comparisons between groups, Wilcoxon's tests or Student's t-tests were used, and the results are presented as the mean (standard deviation) or median [range]. Differences were considered significant when the two-tailed P value was below 0.05. The statistical analyses were performed by a commercial statistical software package (IBM SPSS Statistics Version 18, SPSS Inc., Chicago, IL).

Results

Acute experiment. *Feeding behaviour.* When exposed to free cosmetic PE MPs, *D. rerio* displayed avoidance behaviour towards food. Two distinct manifestations of avoidance were recorded. In the first case, some fish immediately recognized the MP particles as inedible materials, turning or passing the particles and avoiding contact. In the second case, fish initially moved towards the particles, capturing and nibbling them before spitting them out. In both cases, the fish recognized MP particles as inedible elements.

Occasional fluorescent MPs were observed in the intestinal lumen of the fish when the histological sections were assessed. These observations hint at the possibility of accidental ingestion enabled by the small size of the particles (1–5 µm).

When the MPs were blended with either commercial food (diet F_A) or fish oil (diet C_A), *D. rerio* actively displayed a prey capture behaviour, identifying the food, either visually or by chemosensation, tracking it, with a series of routine bends, capturing it and finally ingesting it.

Intestinal retention time, uptake and elimination. Two hours after the ingestion of diet F_A, 67% of all the fish presented a high number of fluorescent MPs in the lumen of the mid-intestine (Supplementary Fig. S2). Under confocal microscopy, the fluorescent MPs were observed in the villi and in the apical surface of the enterocytes (Fig. 1A). An average of two to three particles per fish was present in both the apical and basal aspects of the enterocyte cytoplasm (Fig. 1B, Supplementary Fig. S2). At 6 hpf, 67% of the fish had fluorescent MPs in the mid- and posterior intestine (Supplementary Figs. S2 and S3). Most fluorescent particles were admixed with the digestive content. Fluorescent MPs were observed inside the goblet cells in 100% of the fish (Fig. 1C, Supplementary Fig. S2). At 10 hpf, fluorescent MPs were present in the lumen of the posterior intestine, admixed with the digestive content (Supplementary Fig. S2). MPs were detected in the enterocyte cytoplasm (Fig. 1D) and inside the goblet cells in 100% of the fish, similar to our observation at 6 hpf. Particles were found in the lamina propria (Fig. 1E) in 67% of the fish (Supplementary Fig. S2). At 12 hpf, most of the fluorescent MPs were in the final portion of the posterior intestine (Supplementary Figs. S2 and S3). In 33% of the fish, particles were visualized within the lamina propria (Fig. 1F, Supplementary Fig. S2). Twenty-four hours after intake, fluorescent MPs were not detected in either the digestive content or the intestinal mucosa (Supplementary Figs. S2 and S3). Overall, the particles that crossed the epithelial barrier had a size ranging from 1.083 to 2.041 µm. The Z-stack photos confirmed the internalization of the MP particles in the cytoplasm of the enterocytes and in the lamina propria (Supplementary Figs. S4–S6). Fluorescent MPs were found in the faeces of the fish 6 hpf and were evident between 6 and 12 hpf, decreasing after 24 hpf (Supplementary Fig. S7).

Cosmetic PE microfragments and microfibres were found in the intestinal lumen of the fish fed the C_A diet. At 2 hpf, 67% of the fish had microfragments and microfibres in their lumen of the mid-intestine (Supplementary Fig. S2). From 6 to 12 hpf, microfragments were detected in the lumen of the middle and posterior segments of the intestine (Supplementary Figs. S2 and S3). At 24 hpf, cosmetic microfragments and microfibres were observed in the last segments of the intestine (Fig. 2, Supplementary Figs. S2 and S3). An average of 15 particles were detected in the faeces collected between 10 and 24 hpf (Supplementary Fig. S8). Cosmetic microfragments and microfibres did not cross the intestinal barrier (Supplementary Fig. S2).

Sub-chronic experiment. *Survival rate, weight gain and feeding behaviour.* Mortalities were recorded for all the test and control groups. Survival rates were 100% in all the test groups. For the control group, the survival rate was 95.8%, as a single fish (1/24) died four weeks after the beginning of the experiment.

Regarding weight gain (weight gain (%) = final wet weight-initial wet weight gain), no significant differences were seen among the different groups after 45 days.

For the first two weeks, once visual contact with food was established, in a sign of recognition, fish from all the test groups displayed an aggregating behaviour towards the food, approaching, capturing, nibbling and chewing the food particles. However, after the second week, the fish in all the replicates fed the C_{SC} diet started to exhibit avoidance behaviour towards food. Anxiety-like behaviours, such as anorexia, hypoactivity and reduced exploration, under-reactivity to stimuli, and a tendency to display an abnormal distribution on the bottom of the tank, were recorded. Additionally, a general change in body colour (i.e., a lightened skin pigmentation) was observed in most animals. Food deposition at the bottom of the aquaria was additionally observed.

After reducing the number of intakes of the control diet for a week, the appetite and activity of the fish fed the C_{SC} diet significantly improved. However, despite their improvements, the fish did not fully return to their initial state of responsiveness. Mild hypoactivity and under-reactivity to stimuli were observed over time. No other clinical signs were observed throughout the experiment.

Particle retention and translocation. Fluorescent MPs were observed in 16.7% (2/12) and 33% (4/12) of the livers of the fish fed the F_{SC} diet thirty and forty-five days after the beginning of the experiment, respectively. MPs particles found in the cytoplasm of the hepatocytes ranged between 1.416 and 1.634 µm (Fig. 3A). A single particle (0.692 µm) was also observed surrounding a blood vessel (Fig. 3B). Serial Z-sections of the liver confirmed the internalization of the MP particles (Supplementary Fig. S9). Fluorescent MPs were not detected in the muscle.

MP particles were not found in any organ apart from the intestinal lumen in the fish fed the C_{SC} diet.

The presence of MPs after 15 days of depuration was also assessed. Fluorescent particles were not observed in the intestinal tract after the depuration period, whereas a small number of cosmetic particles (average 3.3 particles) were detected in the intestinal tract in 50% of the fish (Supplementary Fig. S10). Apart from the intestine, MP particles were not observed in any other tissue.

MPs were not found in the procedural blanks set up to assess contamination.

Histopathological changes. Intestinal samples were assessed following the guidelines proposed by Saraiva et al.[62] as follows (Supplementary Table S1, Fig. 4).

Regarding regressive changes, among the epithelial cells, occasional scattered individual apoptotic cells were observed in fish from all the groups. However, such observations were regarded as McKnight cells, which are often seen in healthy fish in low numbers[64].

Inflammatory changes were not observed, and the number and size of goblet cells did not show any significant variation. For the remaining reaction pattern groups, changes were not observed. Overall, compared with the control group, pathological changes were not observed in any of the test groups during the experimental period.

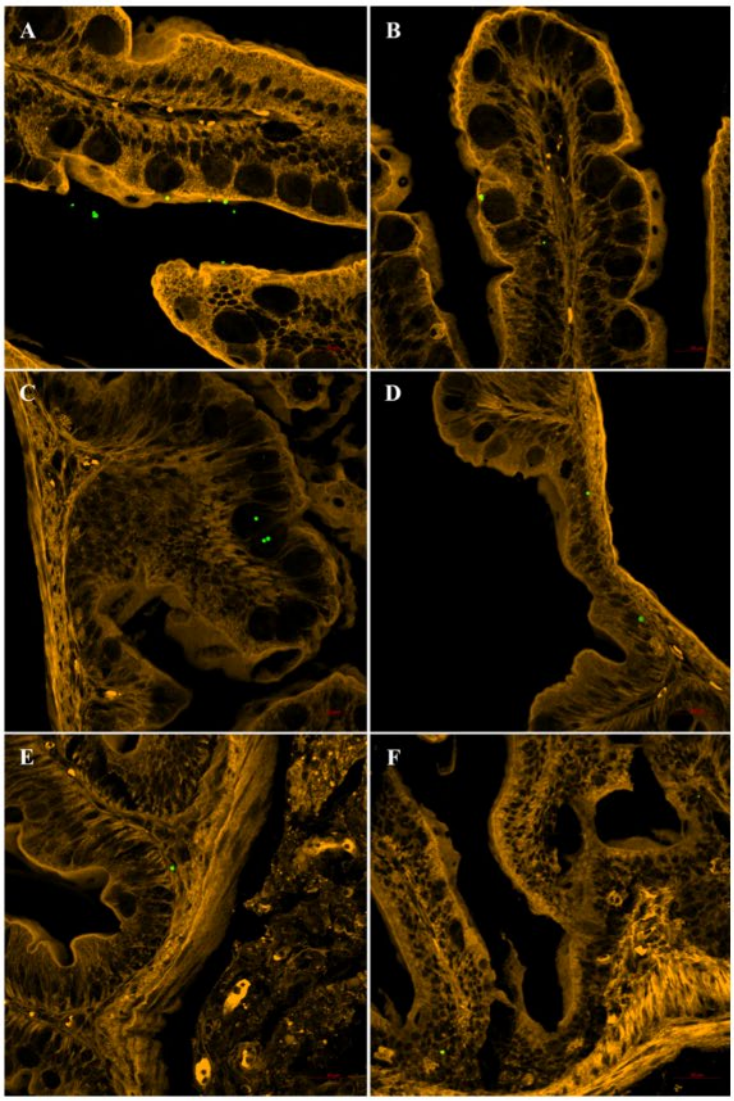

Figure 1. Intestinal segments under confocal microscopy. (**A**) Two hours post-feeding: fluorescent particles in contact with the microvilli on the apical surface of the enterocytes and (**B**) on the apical and basal aspects of the enterocyte cytoplasm. (**C**) Six hours post-feeding: particles inside the goblet cells. (**D**) Ten hours post-feeding: particles in the cytoplasm of the enterocyte and (**E**) in the lamina propria. (**F**) Twelve hours post-feeding: particles within the lamina propria (scale bar = 20 µm).

The liver samples were evaluated according to the guidelines by Bernet et al.[63] as follows (Supplementary Table S2, Figs. 5 and 6).

Regressive changes involving plasma alterations in the liver were noted, particularly vacuolar degeneration. However, decreases in vacuolation were also observed. For that reason, an additional description pattern, termed *degree of vacuolation*, was added. The scoring of this pattern was obtained by comparing both test groups with the corresponding control groups. In the control group, livers from females presented hepatocytes with mottled

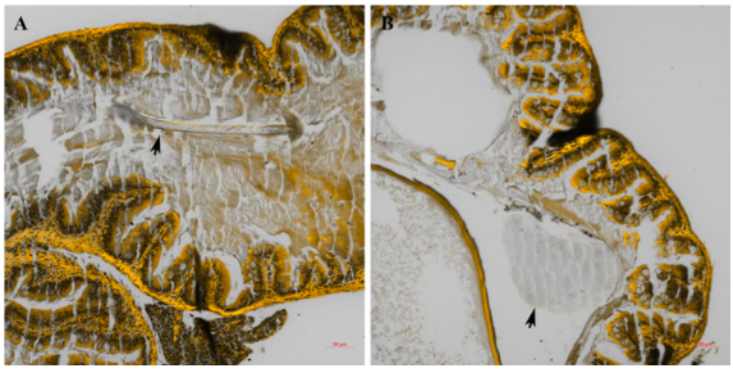

Figure 2. Intestinal segments under confocal microscopy. Twenty-four hours post-feeding: (**A**) synthetic microfibres (arrow) and (**B**) cosmetic PE microplastics in the last segments of the intestine (arrow) (scale bar = 50 µm).

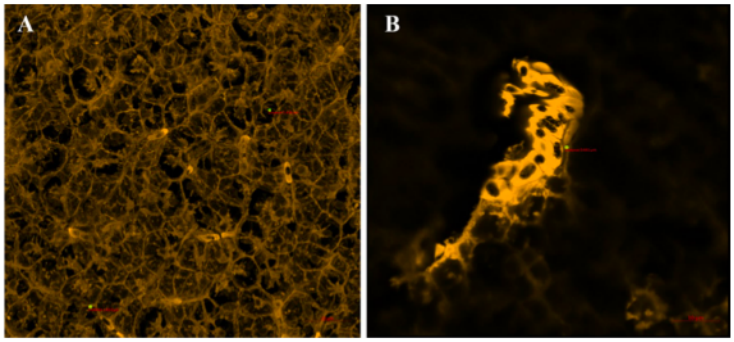

Figure 3. Liver under confocal microscope. (**A**) Fluorescent microspheres within the cytoplasm of the hepatocytes (scale bar = 20 µm) and (**B**) surrounding a blood vessel (scale bar = 10 µm).

cytoplasm, a deeply basophilic appearance in the peri-sinusoidal regions and mild to moderate enlargement due to lipid-like vacuolation (score: 3) (Figs. 5A and 6A). On the other hand, males from the control group showed primarily round to polygonal hepatocytes with clear cytoplasmic vacuoles containing slightly flocculent material with soft margins and centric to paracentric nuclei (score: 3) (Figs. 5B and 6B).

For the test groups, overall, 22.2% (4/18) of the females had minimal to mild vacuolation (score: 1–2) (Fig. 5C, E), while 27.8% (5/18) had moderate vacuolation (score: 4) (Fig. 6C, E). Additionally, 27.8% (5/18) of the males displayed both minimal and mild vacuolation (score: 1–2) (Figs. 5H and 6H), with the remaining males presenting a similar degree of vacuolation as that in the control group (score: 3) (Figs. 5D, F and 6D, F).

When comparing the test groups, in the group fed fluorescent MPs (Figs. 5), minimal to mild vacuolation (score: 1–2) was observed in both females (33.3%) and males (22.2%) (3/9 and 2/9, respectively). A total of 22.2% (2/9) of the females showed moderate vacuolation (score: 4) and no observable changes were recorded in the males. In the cosmetic test group (Fig. 6), 11.1% (1/9) and 33.3% (3/9) of the females and males, respectively, showed minimal to mild vacuolation (score: 1–2). Moderate vacuolation (score: 4) was observed in 44.4% (4/9) of the females, and no observable differences were noted in the males. Over the duration (forty-five days) of the experiment, 33.3% (4/12) of the females presented moderate vacuolation (score: 4), while 25% (3/12) presented minimal to mild vacuolation (score: 1–2). The males did not show differences compared to the control group. However, after the period of depuration, 33.3% (2/6) and 83.3% (5/6) of the females (Figs. 5G and 6G) and males (Figs. 5H and 6H), respectively, presented minimal to mild vacuolation (score: 1–2).

Histologically, moderate vacuolation in both sexes translated into an enlargement of the hepatocyte cytoplasm due to the presence of one or several vacuoles that displaced the nuclei to the periphery. In contrast, when minimal to mild vacuolation was observed, the hepatocytes appeared smaller, and the amount of basophilic and

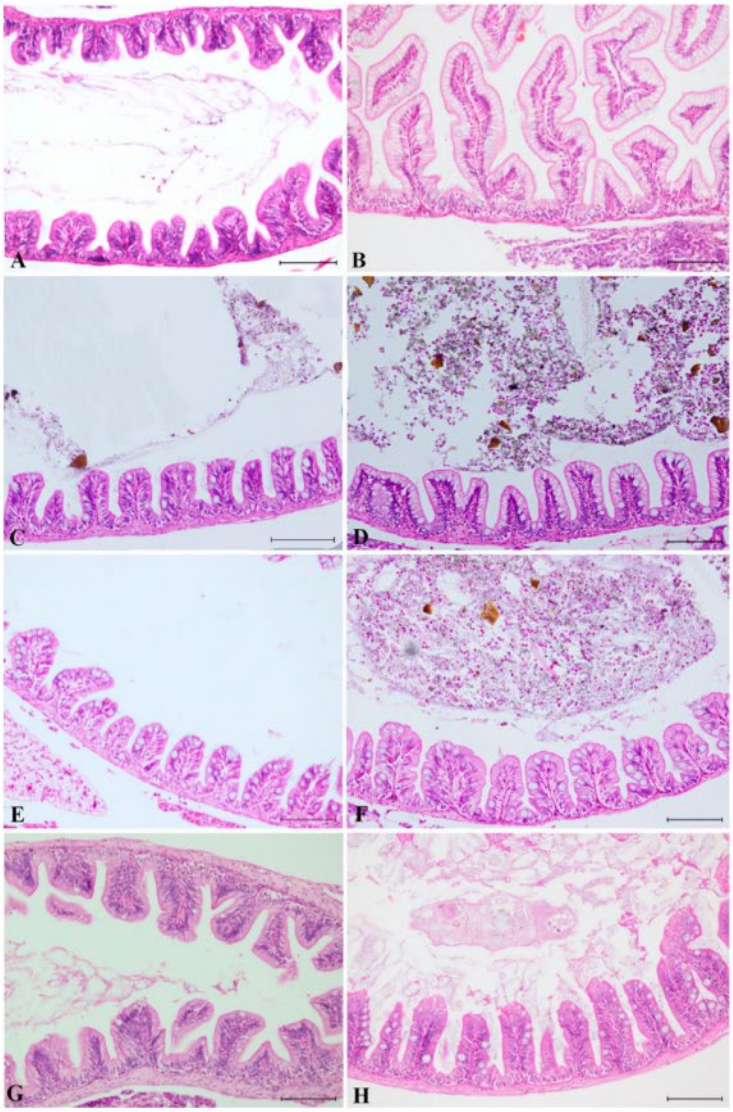

Figure 4. Intestine: (**A, B**) control, (**C, E, G**) cosmetic and (**D, F, H**) fluorescent groups. (**A**) Normal histology of the mid-intestine. (**B**) Specialized enterocytes with supranuclear vacuoles in the posterior region of the mid-intestine. Thirty days after the start of the experiment: intestinal sections from fish fed (**C**) cosmetic MPs and (**D**) fluorescent MPs. Forty-five days after the start of the experiment: intestinal sections from fish fed (**E**) cosmetic MPs and (**F**) fluorescent MPs. Post-depuration: intestinal sections from fish fed (**G**) cosmetic MPs and (**H**) fluorescent MPs (scale bar = 100 µm, **H–E**).

eosinophilic material in the hepatocyte cytoplasm was predominant when compared to the smaller space occupied by the optically empty and irregular vacuoles. Additionally, the nuclei were rounded and centrally located.

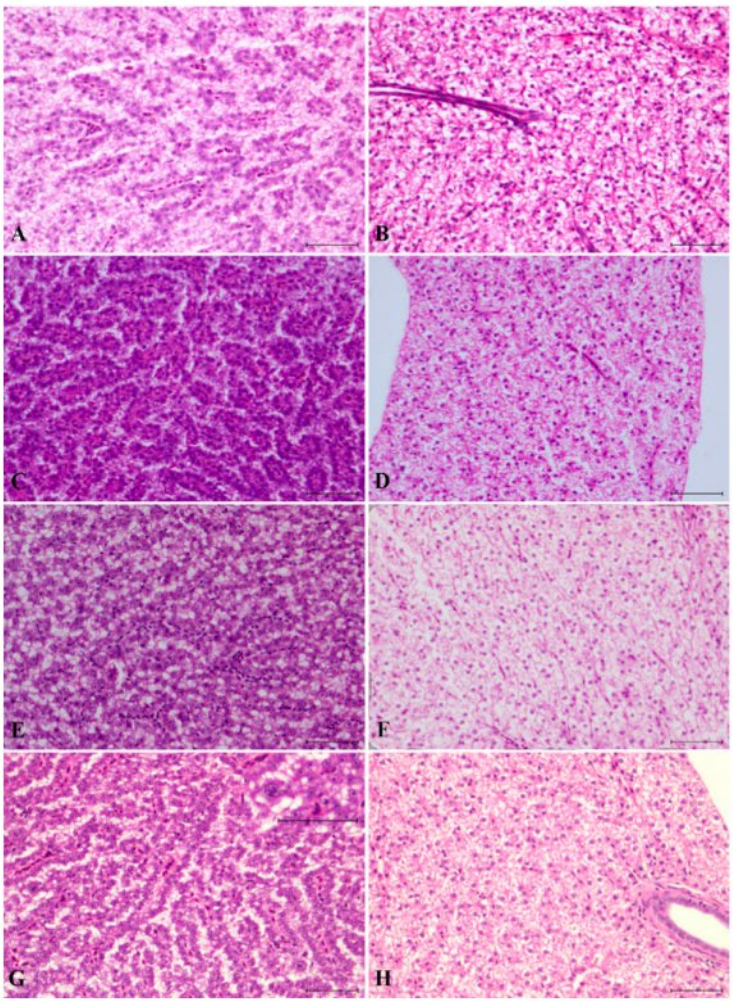

Figure 5. Liver sections from fish fed fluorescent MPs. Control livers were sampled thirty days after the beginning of the experiment from (**A**) a female showing mild to moderate vacuolation and (**B**) a male with mild to moderate vacuolation. Thirty days after the start of the experiment: (**C**) female with minimal vacuolation and (**D**) male with mild to moderate vacuolation. Forty-five days after the start of the experiment: (**E**) females with mild vacuolation and (**F**) females with mild to moderate vacuolation. Post-depuration: (**G**) female with mild vacuolation, showing occasional nuclear enlargement (karyomegaly) (see inset, scale bar = 50 µm), and (**H**) male with mild vacuolation (scale bar = 100 µm, **H**–**E**).

Occasional pyknotic-like hepatocyte nuclei were observed in 100% of the fish in all the groups, including the control group. These findings were attributed to suboptimal fixation of the livers. Pyknosis seldom occurs as an isolated finding and is usually accompanied by other evidence of hepatocyte degeneration or necrosis, such as nuclear karyorrhexis or cytoplasmic hypereosinophilia[65]. Randomly distributed hepatocytes with nuclear enlargement (karyomegaly) (Fig. 5G) were observed in females in both treatment groups as well as in the control group. For that reason, these observations, previously observed in the livers of untreated *D. rerio*, were regarded as an idiosyncratic finding[65].

No further changes were observed.

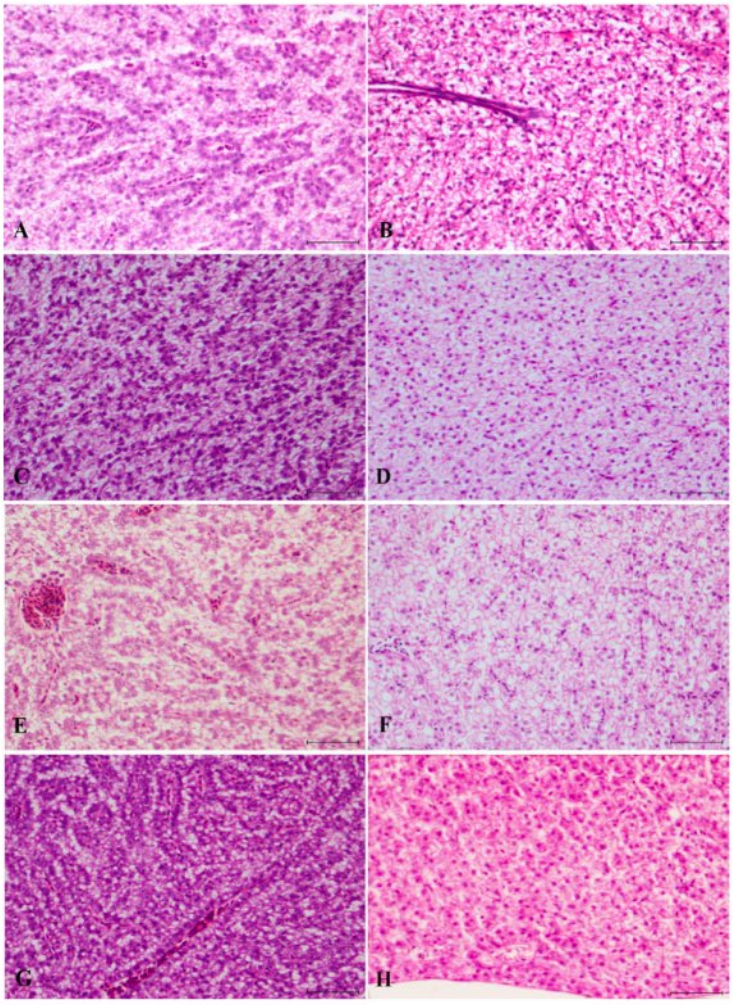

Figure 6. Liver sections from fish fed cosmetic MPs. Control livers were sampled thirty days after the beginning of the experiment from (**A**) a female showing mild to moderate vacuolation and (**B**) a male with mild to moderate vacuolation. Thirty days after the start of the experiment: (**C**) females showing moderate vacuolation and (**D**) males with mild to moderate vacuolation. Forty-five days after the start of the experiment: (**E**) female presenting mild to moderate vacuolation and (**F**) male showing mild vacuolation. Post-depuration: (**G**) female liver with mild vacuolation and (**H**) male with minimal vacuolation (scale bar = 100 µm, **H**–**E**).

Discussion

Propelled by wind, heavy rainfall and tidal currents, MP contamination[12,66,67] has spread to remote lakes[19,68], rivers[14,21,69–71], estuarine regions[72–74], seas[41,75], oceans[76] and even sea ice[77]. As a result, a great number of species are currently at risk of exposure and susceptible to ingestion of MPs[8,10,25–28], either through dietary exposure or by transfer along the food chain.

The predominant types of MPs found in aquatic systems are fibres[14–16,44], microfragments[6,7,19,20] and microbeads[21]. In previous studies, the type of MPs[78,79] as well as shape[80] and size were suggested to influence the level of toxicity inflicted on fish tissues. To replicate those observations, different types (fibres, fragments and beads), shapes (irregular and regular) and sizes of MPs (1–255 µm) were used in the present study.

Acute experiment. *Feeding behaviour.* *Danio rerio* were offered free MPs to determine their ability to recognize plastics as inedible particles, as previously suggested by Kim et al.[81] Following exposure, all the fish displayed a clear refusal behaviour, suggesting that *D. rerio* knowingly recognized plastic as inedible particles. Kim et al.[81] detailed that capture events in *D. rerio* had the lowest rates when fish were exposed only to MPs. Most plastic particles were also quickly rejected in a study by Colton et al.[82] However, despite recognizing plastic as an inedible element, several authors have documented the presence of MPs in the gastrointestinal tract (GIT) of fishes[83–87].

Accidental consumption when foraging on aggregated prey[46] has been observed. Additionally, visual cues that resemble prey, such as colour or shape, may enable the ingestion of smaller particles, hindering the distinction between prey and plastic particles[22,46,88]. Likewise, it has been suggested that odours associated with biofouled plastic debris stimulate foraging behaviour[81,89].

In our study, we observed that free smaller fluorescent MPs were occasionally present in the intestinal lumen. It seems that due to their small size (1–5 µm), these fluorescent MPs were unintentionally ingested. In contrast, larger particles of cosmetic MPs were not observed inside the intestine of *D. rerio*, and accidental ingestion was excluded. Our observations seem to suggest that smaller MPs are more difficult to differentiate from normal prey as previously reported by Critchell & Hoogenboom[22] and/or that low-density MPs of a smaller size are more likely to be passively ingested while gulping air[46,90].

When MPs were mixed with commercial fish food (diet F_A) and fish oil (diet C_A), ingestion rates greatly increased. These observations support previous reports that chemical cues resembling prey[89] and that higher food concentrations are likely to increase MP ingestion[46].

Intestinal retention time, uptake and elimination. The uptake of MP particles was observed in *D. rerio* fed a single meal of the F_A diet. Fluorescent microbeads were detected in the apical and basal surfaces of the enterocytes, inside the goblet cells and in the lamina propria. The internalization of these particles was confirmed by the Z-stack sections. However, despite the uptake of several particles, after 24 h, the MPs had completely cleared from the GITs of *D. rerio* without translocation to other organs. Penetration of individual particles (up to ~70 µm) into the goblet cells has been reported in other species[91]. Endocytosis of luminal material by goblet cells has been described[92], and it has been suggested that some sub-populations of goblet cells may have relatively loose junctions[93]. In a study by Batel et al., the uptake of a few particles was observed in 7.7% of *D. rerio*[24]. However, no further tests were performed to confirm the internalization of the particles. Additionally, the reported incidence was very low.

Conversely, *D. rerio* fed a single intake of the C_A diet presented plastic particles in only the intestinal lumen, and uptake was not observed. In this case, the uptake of microfragments and microfibres seemed unlikely due to their large size. After 24 h, only a microfibre and microfragment remained in the posterior intestine of a single *D. rerio*, indicating a short retention time for these MPs. Similar observations were described in *C. auratus*[45] and *Cyprinodon variegatus*[80] exposed to 5–200 µm and 6–350 µm particles, respectively.

In our study, the retention rates for MPs and food were similar. However, the retention rate for the fluorescent MPs (1–5 µm) was shorter than that for the cosmetic MPs, as after 24 h, all the fluorescent particles had been completely excreted. Despite their larger size and irregular shape, the cosmetic PE microfragments and microfibres were successfully eliminated in most fish after 24 h. The retention rates for the MPs in our study were similar to those observed by other authors. *D. rerio* was shown to rapidly excrete MP particles (70 nm–20 µm), reaching a steady state 48 h after exposure[48], and *C. auratus* eliminated 90% of the particles (50–500 µm) to which it was exposed after 33.4 hours[45]. Similarly, *S. aurata* showed a retention rate close to zero, as 90% of the fish had cleared the MPs (~75 µm) after 24 hours[47]. Despite the similarities, larger particles are likely to take more time to be eliminated. *Seriolella violacea* fed MPs (length, 1.2 ± 0.2 mm; diameter, 1.0 ± 0.1 mm) took an average of 10.6 ± 2.5 days to egest the last MPs[46]. Furthermore, a study by Santos & Jobling performed on *Gadus morhua* fed a single meal with plastic beads (5,000 µm) and MPs (2000 µm) showed a delay in the evacuation of the 5,000 µm beads when compared to the time it took to egest the 2000 µm MPs[94].

Sub-chronic experiment. *Survival rate, weight gain, and feeding behaviour.* After sub-chronic dietary exposure to MPs, the survival rates were 100% in all the test groups. For the control group, the survival rate was 95.8%, as a single fish died at the beginning of the sub-chronic experiment. Our results are in line with previous observations in *D. rerio* exposed to MPs over two[95] and 3 weeks[48]. Conversely, a significant reduction in survival rates was observed in *D. rerio* fed 10 mg/L PP MPs; the fish in this experiment additionally presented swollen abdomens[57].

No significant weight differences were observed between the test groups and the control group. Similar results were reported in *Symphysodon aequifasciatus*[96] and *S. aurata* exposed to MPs for 30 days[97] and 45 days[47]. Likewise, *Acanthochromis polyaccantus* exposed to MPs (average 2 mm diameter) for 42 days did not show significant changes in body condition[22].

After the second week, the fish fed the C_{SC} diet displayed anxiety-like behaviours, such as anorexia and lethargy, and lightened skin pigmentation. Decreases in feeding and swimming activity were reported in *Sebastes schlegelii* after exposure to PS MPs[98] and *Cyprinodon variegatus* after exposure to irregular PE MPs[80]. Lethargy and paling can originate from several factors, such as infections, toxicity, environmental stress, oxygen depletion and starvation[99]. It was also hypothesized that this decreased response occurred upon repeated exposure to MPs and would be the result of habituation to such particles[100]. As in another study with *D. rerio* fed twice a day with nauplii loaded with high concentrations of MPs, there were no observable signs of stress or disease. However, in this case[24], the ingested particles were relatively small (1–20 µm), which could explain the absence of signs of distress after the ingestion of multiple meals with MPs.

Particle retention and translocation. For the F_{SC} diet, fluorescent MPs accounted for 0.1% of the total ingested feed, similar to estimations by Jovanović et al.[47] For the C_{SC} diet, the average density of MPs used was 2,137 items/m^3 (2.49 particles/L). Despite the apparent high number of MP items used, similar or even higher values have been reported in aquatic environments[17,69,101].

In addition, it has been reported that most approaches to estimate the numbers of MPs in aquatic ecosystems lead to underestimations[23]. A common problem with most field studies is that each study uses different mesh sizes, thus having a different cut-off size for the MPs analysed[23,102].

After successive meals of the F_{SC} diet, followed by a period of depuration, *D. rerio* eliminated the particles ingested, as no trace of particles was found in the intestinal tract after the depuration period. In contrast, 50% of the fish fed successive meals of the C_{SC} diet retained an average of 3.3 particles/fish after the end of the experiment. The number of particles found in the intestine after multiple meals is consistent with that found in field studies, in which an average of 0 to 3 particles[34,36–44] was documented. The low number of particles found in the digestive tract may be indicative of the short residence time of MPs within the GITs of fish. These results also appear to indicate that MPs are unlikely to accumulate within the intestine of fish over successive meals. However, particle size is clearly a determining factor when considering clearance rates for plastic particles. *G. morhua* fed multiple meals showed a longer retention rate, as the gastric half-life of the beads was substantially increased with particle size[94]. Our results support the observations by Santos and Jobling[94] that MP retention rate seems to increase with increasing particle size and the intake of additional meals.

Nevertheless, it is important to bear in mind that the structure of the digestive tract varies among fish species[49]. Additionally, other intrinsic (e.g., genetic background, species, age, and physiology) and extrinsic (e.g., habitat, food, type of MPs, and methodology) factors have to be taken into account when discussing clearance rate patterns.

In our study, an average of 1.6 particles/fish was observed in the liver after successive meals of the F_{SC} diet. To validate the process of translocation, the internalization of these particles (up to 1.634 µm) was confirmed by multiple Z-stack sections. The translocation of MP particles to other tissues, such as the liver[47,50,51] and muscle[37,47], has been previously described. Avio et al. reported the translocation of plastic particles (200–600 µm) from the digestive tract to the liver in *M. cephalus*[50]. Likewise, Collard et al. documented the presence of two particles (39–90 µm) in the livers of *E. encrasicolus*[51], and Abbasi et al. detailed the presence of variably sized MP particles (up to 250 µm) in the livers of four fish species captured in the Persian Gulf[37]. Similarly, Jovanovic et al. observed the presence of < 1 particle (214 ± 288 µm) in the liver of *S. aurata*[47], carefully pointing to the possibility of cross-contamination. The alleged translocation of such large particles is difficult to explain with the current knowledge on translocation pathways for MPs in fish; thus, the plausibility of these reports should be questioned.

Other studies working with smaller particles have also reported the translocation of MPs to the liver[48,52]. A study exposing *O. niloticus* to 0.5 µm MPs allegedly observed the translocation of MPs to the liver[52]; however, the photomicrographs used to support those observations barely show fluorescence in the liver, and the fluorescence in the remaining organs is diffusely spread in the tissues. This is likely to be attributed to leaching of the fluorescent dye and not necessarily the presence of MP particles[53]. In another study with *D. rerio* using 5 µm MPs[48], the photomicrographic evidence was inaccurate or poorly presented, as previously highlighted in another publication[54].

As previously declared by Jovanović et al.[47], reports of the translocation of MPs across the fish intestine must be viewed with caution, since the mechanisms of the passage of the plastic material outside of the fish GIT are not yet determined. Two main routes of translocation have been suggested: transcellular and paracellular[18,103]. The transcellular route involves absorption through the microvillous border to the blood[93,103], while the paracellular route occurs through the tight junctions between the cells into the blood[104]. In mammals, transcellular uptake occurs mostly via M cells in Peyer's patches and gut-associated lymphoid tissue (GALT)[105,106]. Fish do not have an organized GALT but instead have lymphoid cells scattered throughout the epithelium and lamina propria and occasional macrophages. Until recently, it was believed that fish lacked M cells[107]. However, recent studies in salmonids[108] and *D. rerio*[109] identified specialized enterocytes with M cell-like activity in the posterior part of the mid-intestine. In fish, these parts of the mid-intestine are the major sites for the uptake of macromolecules and transfer to closely associated intra-epithelial macrophages[109]. In *D. rerio,* these cells are identified by the presence of large, supra-nuclear vacuoles[109], and it has been suggested that these vacuolated cells deliver luminal contents to scattered immune cells present underneath the epithelial layer[110]. However, phagocytic activity is not limited to these cells and was also found in regular enterocytes[109]. The paracellular passage of solid particles through gaps between the enterocytes into the circulatory system has been suggested as the most likely route for MPs, owing to the size range they cover[18]. In cases of severe inflammation and erosion, the passage of particles through the damaged tissue appears to be facilitated[104,111].

MPs could enter the circulatory system by either of these routes and reach the sinusoids through the endothelial fenestrae and the space of Disse. From here, uptake could take place across the basal membrane of the hepatocytes[112]. The fenestrae vary in size, depending on physiological and pathological conditions, controlling what goes in to or out of the space of Disse and what the hepatocytes are exposed to[113]. Latex beads of 1 µm and 100 nm were observed within the hepatic sinusoids in both juvenile and adult *D. rerio*[113].

To the best of our knowledge, our study is the first to confirm the internalization of MP particles in the liver, thus validating the translocation of MP particles. In the present study, the translocation of particles was limited to the liver, as no other organs or tissues showed the presence of MPs, despite other authors having described translocation to the muscle[37,47].

Histopathological changes. The digestive tract of fishes is an extension of the external environment, acting as a critical interface between the internal and external environments[49] and hence being considered a major route of

exposure to MPs. For that reason, intestinal samples were assessed following the guidelines described by Saraiva et al.[62].

Our observations revealed the absence of significant lesions in *D. rerio* from both treatment groups after subchronic dietary exposure. Similar results were reported in *S. aurata*[47] and *Oncorhynchus mykiss*[58] fed different types of pristine MPs (0.1 g/kg bodyweight/day) for 45 days and exposed to both pristine and environmentally deployed PS MPs (10 mg/fish/day) for 4 weeks. Similarly, *Barbodes gonionotus* exposed to PVC fragments (0.2, 0.5 and 1.0 mg/L) for 96 h did not present evident tissue damage[114].

Nonetheless, after an exhaustive review of the scientific literature published on the effects of pristine MPs on fish intestines, we realized that the majority of publications present distinct results. Previous intestinal histological changes reported in fish exposed to pristine MPs included cilia defects in adult *D. rerio*[79]. As ciliated cells are found in only the intestinal epithelia of lampreys, chondrosteans and dipnoids as well as in early life stages in some teleosts[115], they are not present in the intestinal mucosa of most fish species (e.g., *D. rerio*). In most cases, fish have brush border microvilli instead[115], lined by layers of water and mucus[49].

Regressive changes, such as erosion and/or ulceration[78] of the mucosa, have been reported under the following synonyms: epithelial and villi damage[56], cracking of villi, splitting of the enterocytes[57] or breakage of the epithelium[78]. However, such observations must be taken with caution, as erosion and, especially, ulceration are often accompanied by necrosis or haemorrhaging of the mucosa[116]; these changes are not evident in the photomicrographs offered by any of the aforementioned authors. The detachment of the epithelium from the lamina propria has allegedly been observed[55,78,117,118]. However, the photomicrographs given to support these observations show a separation between the mucosal epithelium and the lamina propria, which is a common preparation artefact. Other findings, described as a shortening and fusion of the mucosal folds, have also been documented[55]. Even though persistent toxic damage to the intestinal mucosa and chronic inflammation can produce morphological changes in intestinal folds, such as atrophy and the fusion of adjacent folds, the illusory appearance of these lesions in transverse sections can also be caused by plane-of-section artefacts[64]. This appears to be the case in the aforementioned study[55]. Beheading of villi[55] has also been reported. Is important to bear in mind that examination of the intestine may be problematic since artefacts due to autolysis occur quickly[119]. The autolysis of the tips of mucosal folds is a common artefactual finding and often occurs when whole fish are fixed[72]. Thus, care should be taken when ascribing pathological significance to autolytic changes[72].

Vacuolation of enterocytes has been described[55,79,117]. However, when assessing the vacuolation of the enterocytes in some fish species (i.e., *D. rerio*), the presence of specialized enterocytes in the posterior segment of the mid-intestine with prominent supranuclear vacuoles has to be taken into account[120]. A previous study[79] has characterized the vacuolation of what looks like a normal enterocyte in the posterior segment of the mid-intestine as a pathological change.

Regarding progressive changes, in our study, the number and size of goblet cells did not show significant differences among the test groups. Likewise, a study conducted on *Onchorhyncus mykiss* fed PS MPs showed no significant changes in the numbers of goblet cells observed[58]. Although prior studies[95,118] have mentioned mucous hypersecretion, none of the photomicrographs provided showed a significant presence of mucous secretion. In both cases, the authors pointed out to the goblet cells, but mucous hypersecretion was not evident. Thickness of the mucous layer is also known to change by region, being generally greater in the distal sections of the intestine[121]. Hyperplasia[55,117] and hypertrophy of the goblet cells[78] have also been reported. Mucous cell hyperplasia is generally associated with sources of persistent irritation, such as parasitism[122] and diet[119]. On the other hand, a decrease in the mucus volume[79] and a reduction in the number of goblet cells[118] have been reported. Goblet cells are known to vary along the intestinal length[121], and for many species of teleosts, the posterior intestine contains the highest concentration of goblet cells[123]. Fasting has also been shown to reduce the mucosal mass of the intestine[124]. Another reported finding was hyperplasia of the rodlet cells[55]. However, no evidence of hyperplasia was given by the photomicrographs available, as rodlet cells can be found in low numbers in healthy tissues. Additionally, care should be taken when ascribing pathological significance to the presence or abundance of these cells because no consistent relation has been established between the numbers of rodlet cells and disease[64].

In the present study, inflammation was not observed. Scattered lymphocytes were present in the lamina propria; however, these were considered part of the normal lymphoid tissue. Inflammatory changes[56,78] as well as the presence of neutrophils in the intestinal mucosa[118] and mast cells at the base of the epithelium[79] were observed by other authors. However, these observations are not discernible in the photomicrographs available. The presence of a few leukocytes per se does not necessarily translate to a pathological finding and/or inflammation. In healthy fish, there is a resident population of leukocytes, such as mast cells/eosinophilic granule cells (EGCs), and lymphocytes scattered in the lamina propria[62].

Owing to its large blood supply and marked metabolic capacity, the liver is a target organ for toxicants[112], while also providing pertinent information about general health and revealing the existence of subclinical background diseases[65]. In the present study, liver samples were evaluated separately, according to fish gender, following the protocol proposed by Bernet et al.[63] Specific sex-related differences are characteristic in adult *D. rerio*. In reproductively active, adult oviparous females, an upregulated synthesis of the egg yolk protein vitellogenin often causes the hepatocyte cytoplasm to have a mottled, basophilic appearance, with collapsed sinusoids, owing to the hepatocyte enlargement[64,124]. In contrast, livers from reproductively active males have round eosinophilic hepatocytes with clear vacuoles containing slightly flocculent material and minimal displacement of the nucleus, consistent with glycogen[124].

Overall, no circulatory, proliferative, inflammatory or neoplastic changes were noted in our study. After a thorough review of the scientific literature on the effects of pristine MPs on fish livers, we observed that our findings were contrary to those of previous studies, suggesting the occurrence of several changes as a consequence of MP exposure.

Circulatory changes, namely congestion and hyperemia[78,117] and haemorrhaging[125], have been reported. In a particular case[78], the finding described as congestion is likely intravascular eosinophilic proteinaceous fluid. Similar findings were documented by van der Ven et al.[126], who identified these changes as an accumulation of vitellogenin in vessels. However, the gender of the animals used by Jabeen et al.[78] was not disclosed, and further conclusions cannot be drawn. Overall, when assessing liver congestion and dilated sinusoids, it is important to bear in mind the degree to which the fish was exsanguinated at sacrifice and the amount of care taken to not manually squeeze the liver sample at necropsy[64]. Liver haemorrhaging[125] seems to be characterized by a small number of erythrocytes within the sinusoids, which were likely severed during microtomy. Reports of congestion or dilated sinusoids often are artefacts of tissue collection, preservation or processing[65].

Regarding the regressive changes in our study, 22.2% (4/18) of the females presented minimal to mild vacuolation (score: 1–2), while to 27.8% (5/18) presented moderate vacuolation (score: 4). Additionally, 27.8% (5/18) of the males displayed both minimal and mild vacuolation (score: 1–2). A reduction in vacuolation in both sexes appeared to be time-dependent. Glycogen depletion was similarly described in *Oryzias latipes* exposed to pristine MPs[127]. The loss of hepatocellular vacuolation is a common response of fish livers to toxicity[128]. Furthermore, it is a non-specific finding that can occur as a direct effect of intoxication or secondary to decreased body condition caused by inanition, stress or concurrent disease[64,124]. In our case, vacuolation was not significantly correlated with fish weight, and concurrent diseases were not identified. We believed that the loss of hepatocellular vacuolation might have been caused by stress or even by prolonged exposure to MPs. Paradoxically, toxic exposure can also result in the accumulation of lipids or glycogen in the liver[124].

Increased hepatocellular vacuolation[117] and vacuolar swelling[129] in fish exposed to pristine MPs have also been reported by other authors. However, care must be taken before considering increased hepatocellular vacuolation a pathological change, as it can also be the result of overfeeding an excessively energy-rich diet or lipid peroxidation[124]. It has been suggested that captive marine teleosts may be particularly predisposed to hepatic lipidosis, as observed in *D. labrax* used in a study with MPs[117], owing to a reduced capacity for hepatocyte peroxisome proliferation coupled with the feeding of artificial diets with high proportions of mono-unsaturated fatty acids[124]. Lipid peroxidation in fish may also be toxicant-induced[65,124]. In another study[48], lipid droplets in hepatocytes were reported. However, further histochemical techniques were not performed to confirm the lipid origin of the vacuolation. Additionally, due to artefacts in the control photomicrograph, it is difficult to establish a comparison between the control and test groups to identify a possible increase in hepatocellular vacuolation. Apart from lipid and glycogen vacuolation, there are other potential causes of hepatocellular enlargement, such as vacuolar swelling of the endoplasmic reticulum cisternae (hydropic degeneration)[124]. Vacuolar swelling and hydropic degeneration were allegedly observed in another study[78]. When comparing the control and test group photomicrographs, it can be observed that cells meant to illustrate hydropic degeneration are also present in the control group photomicrograph. As the magnification differs between the images, there is an illusion of larger vacuoles in the test group photomicrograph. The same applies for the reported vacuolar swelling.

Necrosis was also reportedly observed in the livers of fish exposed to MPs[48,117,125]. In one study[125], necrosis was likely due to handling trauma. Clear spaces without tissue are more likely to be artefacts resulting from focal trauma during tissue collection. In other experiments[48,125], there was no evidence of necrosis. Findings classified as hepatocellular necrosis or apoptosis should display cytoplasmic hypereosinophilia with or without condensation; irregular or rounded cytoplasmic margins; nuclear changes, such as pyknosis, karyorrhexis or karyolysis; phagocytosis of necrotic cells or apoptotic bodies; and in the case of necrosis, a potential inflammatory response[65]. Damaged and aggregated nuclei were documented[129]. However, these are likely a proliferation of macrophage aggregates. Starvation, ageing, infectious diseases and toxins are all likely to cause proliferation of macrophage aggregates[124].

Among the progressive changes, alterations in the hepatocyte morphology and hypertrophy[117] were reported. Often, when increased vacuolation results in cytoplasmic enlargement, there is confusion with hepatocellular hypertrophy[65]. However, hepatocellular hypertrophy should be reserved for describing non-vacuolated cells that are enlarged as a consequence of metabolic enzyme induction, which results in an upregulation of organelles[65]. In addition, whether due to physiological or toxicological causes, hepatocyte hypertrophy is often accompanied by basophilia[124].

Inflammation was also described[48,78]. In the first study[48], signs of inflammation were not evident in any of the provided photomicrographs. In turn, the photomicrographs provided in the second study[78] appeared to identify a tubular structure, likely part of the biliary system[130] or, less likely, a vascular structure of a normal hepatic stroma. Even though there might be a few leukocytes in the figure provided[78], rare leukocytes do not necessarily mean inflammation. When assessing inflammation in the liver, one has to take into account that haemopoietic tissue may be present in the periportal areas of the liver in some fish species[131] and that an integral component of inflammation is the infiltration of non-resident leukocytes into the affected site[65].

The specific architectural design of fish livers, having only the basal and basolateral aspects of hepatocytes directly exposed to the sinusoidal perfusion, hinders the uptake of chemicals by fish hepatocytes[124]. This and the lower perfusion rate might help to explain the relative tolerance of fishes to MPs.

Our results show that *D. rerio* individuals recognize plastic particles as inedible materials but ingest them either when they are mixed with food or fish oil or accidentally when exposed to relatively small plastic particles (1–5 µm). Ingested small plastic microbeads (1–5 µm) and medium PE microfragments (120–255 µm) and fibres (average width and length of 13.67 µm and 1.5 mm, respectively) are unlikely to accumulate in the digestive tract of *D. rerio* after one or multiple meals, as MPs were almost completely evacuated after 24 h and only a few particles remained in the digestive tract after sub-chronic ingestion. No mortalities or significant effects on body condition were identified after 45 days of feeding with MPs. However, the fish fed medium-sized, irregular PE MPs showed anorexia and lethargy. The ingestion of particles has been reported to cause physical blockage of

the intestine, causing a false sense of satiety and interfering with feeding[23,34]. In the present case, the relatively large particles are thought to have impaired feeding.

To the best of our knowledge, this is the first study to fully demonstrate the uptake and translocation of plastic microbeads to the liver using confocal microscopy. However, the exact route through which MPs reach the liver is still unknown, and future studies are necessary to determine the mechanisms that allow the uptake and translocation of MPs.

Following sub-chronic dietary exposure to pristine MPs, *D. rerio* did not show any histological lesions in the observed organs. Our results are in contrast to the majority of the scientific literature on the effects of MPs in fish. The differences may be influenced by several elements, such as the species, age, sex, reproductive status of the fish, environment, tested concentrations, size, type, surface chemistry and hydrophobicity of MPs, feeding routine, exposure route, exposure time, number of animals and replicates per treatment group, specimen collection and preparation methods[65]. However, inaccuracy in the interpretation of the histopathological findings may be the main cause for the disparity observed in the results regarding the effects of pristine MPs on fish. A letter[54] written by several veterinary pathologists has highlighted concerns about the recurring problem of inaccurate histopathological data, which is increasingly observed in scientific publications. This situation is especially alarming in cases in which the study conclusions depend heavily on the histopathological results. In addition, such observations will persist in the literature and spawn further misguided research, which is particularly problematic for students and researchers working in fish pathology, expecting to find reliable sources of information in these same publications.

Although pristine MPs per se do not appear to produce imminent damage, most plastics produced are not made entirely of plastic polymers. During the manufacture of plastics, endogenous chemical additives are incorporated into them[18]. MPs are also very efficient in adsorbing persistent organic pollutants already present in water[23]. Therefore, further research is needed to properly identify the effects MPs and their associated contaminants may have on animal health and, consequently, public health.

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Author contributions
C.S.R. conducted the dietary exposure, performed the necropsies, analysed and interpreted the histopathological data, wrote the manuscript, and reviewed the manuscript. Y.B.C. provided advice on the experimental design to ensure that the ethical standards were respected, analysed and interpreted the statistical data, and reviewed the manuscript. A.F. provided funding, analysed and interpreted the histopathological data, and reviewed the manuscript. M.J.C. conceptualized and designed the experiments, conducted the dietary exposure, performed the necropsies, analysed and interpreted the histopathological data, and supervised and reviewed the manuscript.

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The authors declare no competing interests.

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5

CONCLUSIONS

5. Conclusions

1) Despite being a saprophytic fungus and a recognised plant pathogen, *Phoma herbarum* is also an opportunistic fish pathogen. In wild greater amberjack, it causes primary severe granulomatous aerocystitis with complete loss of swim bladder morphology, thus suggesting a hematogenous dissemination route for this fungus in physostome fish.

2) Larvae of Trypanorhyncha are highly prevalent (96.4%) in the coelomic cavity of adult dusky groupers weighing more than 17 kg from the Canary Islands.

3) Trypanorhyncha infections in adult dusky groupers cause a progressive and chronic response characterised by extensive and marked fibrotic reaction with encapsulation of the larvae. The severity and extension of the lesions caused by the larvae are directly proportional to the intensity of the infection. Fish with more larvae (n>30) develop extensive fibrosis of the peritoneum, stomach, and intestine, and show compression of the adjacent abdominal organs.

4) Zebrafish recognise microplastic particles as inedible materials, ingesting them when the microplastics are mixed with food and fish oil, or when exposure to smaller plastic particles (1–5 µm) occurs accidentally.

5) Plastic microbeads (1–5 µm), microfragments (120–220 µm), and textile microfibres (mean of 1500 µm) are unlikely to be retained in the digestive tract in zebrafish after acute oral exposure. However, after sub-chronic exposure, larger microfragments and textile microfibres are retained in the intestines of zebrafish for extended periods. Nonetheless, the potential for retention of MPs is low because an average of only 3.3 particles remains in the intestines of 50% of zebrafish after the depuration period.

6) Plastic microbeads (up to 2.041 µm) can cross the intestinal barrier and enter the cytoplasm of enterocytes, goblet cells, and the *lamina propria*. Two hours after ingestion, microbeads reach the enterocytes and the goblet cells, and after 10 hours, they enter the *lamina propria*.

7) Sub-chronic oral exposure to pristine fluorescent polymer microbeads (9.9×10^4 g/cm^3) or PE microfragments with textile microfibers (3.3×10^{-2} g/cm^3) causes no mortality or significant effects on the body condition in zebrafish. However, ingestion of larger PE microfragments and textile microfibres results in behavioural changes such as anorexia and lethargy, and generalised skin pallor.

8) After sub-chronic ingestion of MPs of different sizes (1–1500 µm), only smaller microbeads (< 1.634 µm) translocate to the cytoplasm of the hepatocytes in zebrafish. This finding suggests that previous evidence on the translocation of MPs up to 600 µm should be further assessed.

9) After a detailed histopathological study of the intestine, liver, and skeletal muscle of zebrafish sub-chronically exposed to different types and sizes of MPs, no significant histopathological findings were observed. Therefore, experimental ingestion of pristine MPs at high concentrations does not cause histopathological changes.

5. Conclusions

6

RESUMEN EXTENDIDO

6.1. Antecedentes y objetivos

El ecosistema marino canario disfruta de una situación privilegiada que genera las condiciones necesarias para la presencia de una gran diversidad de especies endémicas y migratorias, constituyendo así un ecosistema único y de importancia mundial (Gobierno de Canarias - Consejería de Agricultura, 2021; Popescu & Ortega Gras, 2013). Actualmente, se encuentran registradas cerca de 700 especies de peces en la región Atlántico centro-oriental del entorno canario (Espino et al., 2018).

La importancia de las distintas especies marinas para el equilibrio de los ecosistemas es indiscutible, aparte de ser fuente de proteínas y por eso una parte significativa de la dieta del ser humano (FAO, 2020).

En el archipiélago canario, las actividades pesqueras son parte fundamental de su identidad y numerosos municipios dependen en gran medida de este sector (Popescu & Ortega Gras, 2013). En los últimos años se han tomado diversas medidas con el objetivo de asegurar el crecimiento inteligente y sostenible de los recursos marinos. Por otro lado, el desarrollo de actividades turísticas con vistas a impulsar el crecimiento económico, como la pesca-turismo, el turismo acuícola y el turismo marinero y que tienen como objetivo la valorización y difusión de los productos pesqueros, patrimonio y cultura del medio marino, se ven más valoradas por un ambiente marítimo protegido y cuidado (Ley 15/2019, de 2 de mayo, de modificación de la Ley 17/2003, de 10 de abril, de Pesca de Canarias. BOE, nº 141, de 13 de junio, 2019) (Agencia Estatal Boletin Oficial del Estado, 2019).

Se ha reconocido que el aumento de las temperaturas favorece la propagación de vectores a nuevas localizaciones. Descripciones del virus de la lengua azul (Gloster et al., 2008) y hantavirus (Clement et al., 2009; Whitmee et al., 2015) en el norte de Europa; la expansión del virus del dengue en América Central y Norte, y Europa (Watts et al., 2018), o la creciente desaparición de anfibios causada por hongos son algunos de los ejemplos (Pounds et al., 2006). Además, hay que tener en cuenta que el cambio climático no es un simple proceso de calentamiento constante y regular de la tierra, el mar y la atmósfera. Cambios en las precipitaciones y efectos indirectos en la eutrofización, estratificación, capa de hielo, acidificación, niveles de las aguas, caudales, corrientes oceánicas, penetración de la luz

ultravioleta y condiciones climatéricas extremas están ocurriendo igualmente (Johnson et al., 2009; Lõhmus & Björklund, 2015; Marcogliese, 2008; Prowse et al., 2009). Son los efectos acumulativos de diferentes factores estresantes lo que resulta en severas perturbaciones de las cadenas alimentarias y de la pesca, constituyendo una amenaza a los ecosistemas (Marcogliese, 2008).

Para los peces, centinelas del impacto de los múltiples factores estresantes en la biodiversidad (Sebastian & Hering, 2018), el aumento de las temperaturas puede generar estrés fisiológico y comprometer directamente la resistencia del huésped (Genin et al., 2020; Lamb et al., 2018; Marcos-López et al., 2010). El impacto combinado de patógenos virulentos y huéspedes inmunocomprometidos podría ser la base de muchos brotes de enfermedades, además, indudablemente, de reducir el rendimiento reproductivo y/o productivo (Alborali, 2006; Johnson et al., 2009). En salmónidos, se han observado brotes de enfermedad renal proliferativa, causada por el mixozoo *Tetracapsuloides bryosalmonae*, debidos al aumento de la temperatura, y promovidos por la eutrofización (Okamura et al., 2011; Sterud et al., 2007; Tops et al., 2009).

Otro mecanismo relevante para la aparición o brote de enfermedades infecciosas es el efecto del cambio climático en los movimientos de las poblaciones animales. Los cambios en los patrones de migración podrían provocar que los animales migratorios se encuentren y transfieran patógenos a poblaciones de huéspedes previamente no expuestas, o que se expongan a nuevas enfermedades infecciosas (Prowse et al., 2009). Los patógenos introducidos en poblaciones de huéspedes previamente no expuestos pueden propagarse rápidamente, causar altas tasas de mortalidad y conducir a reducciones alarmantes en la abundancia de huéspedes (Lamb et al., 2018; Marcos-López et al., 2010).

La interferencia antropogénica, a través de la pérdida de hábitats y su fragmentación y polución, contribuyen también al cambio climático. Herrera et al. (2018) han publicado un estudio que describe la presencia de microplásticos en caballas capturadas a lo largo de la costa canaria, apuntando la presencia de microplásticos en el 70% de los peces estudiados.

En una época en la que los efectos del cambio climático y de la polución de los océanos son intensamente debatidos y estudiados, es de suma importancia garantizar una comprensión amplia del ecosistema marino. La identificación exitosa de los riesgos sanitarios emergentes está en la base de la protección de la salud pública y el medio ambiente. Así, es esencial tener

un conocimiento más profundo de las enfermedades, no solamente las que pueden tener un efecto nocivo para la salud humana, si no también las que pueden amenazar a las distintas especies de animales que habitan los ecosistemas marinos que circundan las Islas Canarias. En esa línea, queda todavía mucho por hacer para determinar cuales son los patógenos, con qué efecto y durante qué escala de tiempo, cuales tienen potencial de afectar la salud del ecosistema y, como consecuencia, a la seguridad alimentaria. Esto requiere de una estricta vigilancia de los ecosistemas marinos.

El presente trabajo se ha desarrollado con el objetivo de arrojar nuevas luces a las actuales amenazas a las especies de pez salvajes de las Islas Canarias. Lo objetivos genéricos han sido por lo tanto: a) la identificación de la prevalencia de enfermedades en poblaciones de peces salvajes en las Islas Canarias; b) evaluar los cambios patológicos causados por los distintos agentes infecciosos en peces salvajes; c) determinar los distintos contextos en los cuales los peces pueden estar susceptibles a la ingestión de microplásticos; d) evaluar el potencial para la retención y translocación de microplásticos tras su ingestión; e) identificar las consecuencias clínicas y patológicas de la ingestión de microplasticos.

6.2. Resumen de las publicaciones

6.2.1. **Publicación I:** Primera descripción de aerocistitis granulomatosa en un pez limón *Seriola dumerli* salvaje causada por el hongo *Phoma herbarum*

Phoma herbarum (familia Didymellaceae) es un hongo saprófito y un reconocido patógeno vegetal (Aveskamp et al., 2008; Bennett et al., 2018; Kumla et al., 2016; Neumann & Boland, 2002).

En los peces, *P. herbarum* actúa como un patógeno facultativo, causando una micosis visceral crónica progresiva y letal. Publicaciones anteriores detallaron la infección en salmón plateado *Oncorhynchus kisutch*, salmón real *O. tshawytscha* y trucha arcoíris *O. mykiss* en granjas de acuicultura en los Estados Unidos (Boerema et al., 2004; Burton et al., 2004; Faisal et al., 2007; Ross et al., 1975). Además, se ha detallado una micosis visceral con características

similares causada por una especie no identificada del género *Phoma* en ayu *Plecoglossus altivelis* en Japón (Hatai et al., 1986).

La puerta de entrada de *P. herbarum* en los peces aún no ha sido determinada. Una teoría sugerida a menudo es que los conidios ingeridos, a través del alimento o del agua, podrían pasar desde el tracto digestivo hasta la vejiga natatoria a través del conducto neumático (Burton et al., 2004; Faisal et al., 2007; Ochiai et al., 1977). No obstante, la teoría propuesta se basa en infecciones exclusivamente en especies fisostómicas (Burton et al., 2004; Faisal et al., 2007; Ochiai et al., 1977; Ross et al., 1975).

De una forma general, los peces se clasifican en dos tipos, según el tipo de vejiga natatoria que presenten - fisostómicos y fisoclistos. En especies fisostómicas, como los salmónidos, la vejiga natatoria está conectada al esófago por el conducto neumático. Este favorece la entrada de gas en la vejiga natatoria al tragar aire en la superficie (Bone & Moore, 2008; Bruno et al., 2013). Por el contrario, en los peces fisicoclistos, como es el caso de los medregales, la conexión entre la vejiga natatoria y el tracto digestivo deja de existir durante el desarrollo embrionario y los mecanismos de llenado y vaciado de la vejiga natatoria se realizan por difusión en la corriente sanguínea (Bruno et al., 2013; Genten et al., 2009; Hughes et al., 2016).

Una hembra adulta de medregal *Seriola dumerili* fue capturada viva en la región noroeste de Lanzarote, Islas Canarias, a principios del otoño y enviada al Instituto Universitario de Sanidad Animal y Seguridad Alimentaria (IUSA) de la ULPGC en el ámbito del Programa Oficial de Diagnóstico de Ciguatera en Las islas Canarias. Se realizó una necropsia estándar para peces y se tomaron muestras de tejido para su análisis histopatológico.

Los principales objetivos de este estudio han sido 1) identificar las lesiones macroscópicas e histológicas externas e internas y realizar una descripción detallada de las lesiones y los cambios histopatológicos observados; 2) evaluar los cambios patológicos específicos causados por un hongo saprofito oportunista en peces salvajes; 3) determinar la posible puerta de entrada de hongos en peces fisoclistos; e 4) identificar el agente etiológico.

El examen externo no mostró alteraciones significativas. Los hallazgos macroscópicos en los órganos internos se limitaron a la vejiga natatoria que presentaba una pérdida completa de la morfología y aumento difuso de tamaño (36 mm × 16 mm) (Fig. 1). Además, la pared mostraba engrosamiento y opacidad generalizados, con disminución a obliteración del lumen. Numerosas lesiones quísticas multifocales a coalescentes, translúcidas a blanquecinas de tamaño variable (20-80 mm) reemplazaban la estructura normal. Al corte, dichas estructuras contenían un líquido, de transparente a amarillo claro. Alternando entre las lesiones quísticas, de forma multifocal a coalescente, también se observaron nódulos irregulares, naranja oscuro a marrón oscuro, de textura arenosa, de 7 a 20 mm de diámetro. Tanto las lesiones quísticas como los nódulos estaban bien delimitadas y separadas por bandas de tejido conectivo fibroso. También se observaron áreas de hemorragia multifocales, con especial énfasis en el aspecto caudal del órgano.

El examen histológico de la vejiga natatoria reveló múltiples quistes de tamaño variable, distendidos por espacios claros o llenos de líquido proteico homogéneo eosinófilo, y tapizados por hasta doce capas de espesor de células epiteliales cuboidales atenuadas (Fig. 2a). El tejido restante estaba reemplazado por granulomas multifocales a coalescentes, de tamaño variable con un centro de tejido necrótico, a menudo pigmentado y mineralizado, rodeado de macrófagos, células gigantes multinucleadas de tipo cuerpo extraño y linfocitos (Fig. 2b). Los granulomas y las lesiones quísticas estaban separados por prominentes y gruesas bandas de grosor variable de tejido conectivo laxo vascularizado que contenían pequeños, escasos y dispersos agregados de linfocitos y macrófagos. Con frecuencia, dentro de los granulomas, se observó un gran número de hifas fúngicas tabicadas, ramificadas irregulares, filamentosas, de 3-6 µm de ancho, con ocasionales dilataciones bulbosas (Fig. 2c, d).

Las muestras de tejido de otros órganos no revelaron la presencia de infección fúngica y no se observaron otros cambios patológicos, aparte de una leve infección por nematodos (Anisakidae) en la luz del estómago, sin lesiones asociadas.

La detección y secuenciación del ADN fúngico permitió la identificación de la especie como *Phoma herbarum* en las muestras de vejiga natatoria.

Descripciones anteriores de micosis visceral en peces por *P. herbarum* parecen estar restringidas a peces de la familia Salmonidae (Faisal et al., 2007; Ross et al., 1975; Wood et al., 1968). Debido a que la vejiga natatoria era el órgano más afectado, se planteó la hipótesis de

que la infección podría haber resultado del paso del hongo desde el tracto digestivo, después de la ingestión de alimentos o detritus, a la vejiga natatoria, a través del conducto neumático (Burton et al., 2004; Faisal et al., 2007; Hatai et al., 1986).

En especies fisoclistas, esta teoría no se aplicaría debido a la ausencia del conducto neumático (Bruno et al., 2013). En estas especies la transmisión puede ocurrir durante el intercambio de gases por vía hematógena. Cuando el oxígeno disuelto en el agua circundante ingresa al torrente sanguíneo a través de las arteriolas eferentes en las branquias, atraviesa la aorta dorsal y llega a la vejiga natatoria. Ross et al. (1975) y Faisal et al. (2007) observaron vasculitis e invasión fúngica de la aorta dorsal. Igualmente, en un caso de aerocistitis por *Exophiala xenobiotica*, se ha propuesto que la infección pudiera ser el resultado de una invasión vascular en un punto indeterminado, posteriormente diseminada a la rete mirabile de la glándula de gas de la vejiga natatoria (Camus et al., 2014).

A pesar de ser un hongo de distribución ubicua, se han reportado pocas infecciones espontáneas por *P. herbarum* en peces. Esta supuesta baja incidencia apoya la idea de que el efecto patógeno de *P. herbarum* depende en gran medida del estado inmunológico de su huésped (Faisal et al., 2007). Además, en una transmisión experimental *de P. herbarum*, Burton et al. (2004) observaron que las tasas de supervivencia tendían a ser más altas en condiciones ambientales óptimas. En los peces, animales poiquilotermos, su metabolismo es más vulnerable a la temperatura, especialmente en lo que respecta a la inmunidad (Noga, 2010). Los peces de climas templados y fríos son particularmente susceptibles a las enfermedades infecciosas durante la primavera y el otoño, cuando los cambios en la temperatura del agua son más abruptos (Noga, 2010; Plumb & Hanson, 2011). Eventos estresantes, como la temperatura del agua (Ross et al., 1975) y cambios de salinidad (Hatai et al., 1986) o manipulación (Easa et al., 1984; Faisal et al., 2007; Hatai et al., 1986), también han sido descritos antes del desarrollo de las infecciones por *P. herbarum*.

En un ambiente salvaje, la cantidad de factores estresantes que pueden resultar en un mayor riesgo de infecciones es enorme. Varios factores podrían haber provocado la infección. Los patógenos latentes u oportunistas también pueden tener el potencial de causar inmunosupresión, lo que permite el desarrollo de una infección secundaria. En nuestro caso,

la leve infección por nematodos gástricos observada puede haber jugado un papel en la supresión del sistema inmunológico, lo que provocó la infección por *P. herbarum*. Además, un cambio repentino en la temperatura del agua podría afectar negativamente al sistema inmunológico. No obstante, es importante tener en cuenta que *P. herbarum* se caracteriza por causar una enfermedad crónica progresiva y no es fácil determinar el papel definitivo que juegan estos estresores en el momento de inicio de la infección en peces salvajes.

La importancia de este hallazgo radica principalmente en los esfuerzos que se han realizado durante los últimos años para introducir el medregal en la acuicultura. Como *P. herbarum* se ha asociado con grandes pérdidas en los criaderos (Faisal et al., 2007; Ross et al., 1975), es esencial identificar mejor las potenciales enfermedades que pueden representar un cuello de botella para la producción y el rendimiento de los peces, para poder prevenirlas o tratarlas y evitar pérdidas y consecuentes problemas económicos.

6.2.2. **Publicación II:** Estudio patológico sobre los efectos de los cestodos del orden Trypanorhyncha en meros ***Epinephelus marginatus*** **de las Islas Canarias**

La presencia de cestodos del orden Trypanorhyncha en peces marinos ha sido documentada en diversas partes del mundo (Beveridge et al., 2014; Haseli et al., 2011; M. Overstreet, 1978; H. Palm et al., 1994; H. W. Palm, 1997, 2000; Scholz et al., 1993). Los parásitos Trypanorhyncha se caracterizan por un escólex con dos o cuatro botrias (Rhode, 2005) y un aparato tentacular compuesto por cuatro tentáculos retráctiles (Palm et al., 2009). Los tentáculos están armados con numerosos ganchos dispuestos en patrones complejos para adaptarse al sitio de unión en el anfitrión final (Mehlhorn, 2016).

Con un ciclo de vida complejo (Mehlhorn, 2016), la mayoría de las especies de Trypanorhyncha requieren un hospedador definitivo (pez elasmobranquio) que aloja el parásito adulto, un hospedador primario (pequeño crustáceo) donde se desarrolla el procercoide, y un hospedador secundario (pez teleósteo) (Palm et al., 1997; Overstreet, 1978). Cuando el procercoide es ingerido por un hospedador secundario, penetra a través de la pared del estómago y del intestino y se enquista en la cavidad celómica o en los músculos

esqueléticos. Allí, el procercoide se convierte en un plerocerco (plerocercoide o merocercoide, según las especies) (Moser et al., 1984; Palm y Caira, 2008; Roberts, 2012).

A pesar de numerosos estudios que detallan su biología, las descripciones sobre los efectos patológicos causados por estos parásitos en los peces son escasas.

Los objetivos del presente estudio han sido 1) determinar la prevalencia de Trypanorhyncha en meros negros capturados en las Islas Canarias, Océano Atlántico Centro-Oriental; 2) valorar los cambios patológicos producidos por estos parásitos en los peces, especialmente en el mero negro.

Entre 2017 y 2018, 28 ejemplares adultos de mero de las Islas Canarias se enviaron muertos al IUSA, ULPGC en el ámbito del Programa Oficial de Diagnóstico de Ciguatera en Las Islas Canarias. Aunque no se pudo determinar la ubicación exacta de la captura de los peces, se obtuvo la fecha y la ubicación aproximada de la misma. Los meros fueron capturados en las cercanías de Tenerife (n = 5), Gran Canaria (n = 1), Fuerteventura (n = 3) y Lanzarote (n = 19).

Se realizó la necropsia de los peces y se tomaron muestras tisulares representativas para su estudio histopatológico. Las larvas de Trypanorhyncha se lavaron con solución salina y se fijaron en formalina tamponada al 10% para histología y en etanol al 70% para observación directa al estereomicroscopio y microscopio óptico.

El examen macroscópico externo mostró distensión de la cavidad celómica (Fig. 2a).

De los 28 animales muestreados, 27 presentaban en la cavidad celómica abundantes bandas de tejido conectivo fibroso con adherencias intraperitoneales que dificultaban la separación y individualización de los órganos (Fig. 2b, c). Además, se ha observado de forma multifocal, numerosos quistes parasitarios, adheridos o profundamente incrustados dentro del peritoneo y adheridos a las superficies serosas de los órganos viscerales, extendiéndose, a menudo, hacia la túnica muscular y submucosa del estómago y el intestino. Estos quistes eran irregulares, de circulares a ovalados con un extremo estrecho, de tamaño variable (2 mm × 1.5 mm – 6 mm × 3.2 mm), de color amarillo a marrón claro, cada uno conteniendo una larva, con pequeñas cantidades de líquido seroso (Fig. 3). Al mismo tiempo, había numerosos nódulos

oscuros, multifocales a coalescentes (Fig. 3), incrustados adheridos a las superficies serosas de los órganos celómicos, extendiéndose ocasionalmente hacia la túnica muscular y submucosa del estómago e intestino y el parénquima hepático. Estos nódulos eran irregulares, de tamaño variable (10 mm × 25 mm-60 mm × 96 mm), de color marrón oscuro a negro, de firmes a duros, con un núcleo de color marrón claro y arenoso.

Los quistes y nódulos con larvas se observaron principalmente en el peritoneo y la grasa mesentérica (Fig. 4a), particularmente alrededor de los ciegos pilóricos, y en la submucosa, muscular y serosa del estómago (Fig. 4b) e intestino (Fig. 4c). En algunos casos, estas estructuras también estaban presentes en el parénquima hepático (Fig. 4d), adyacentes a las gónadas y adheridas a la pared externa del bulbo arterioso del corazón.

Los parásitos individualizados se caracterizaban por un cuerpo redondo a ovalado, amarillo a marrón claro, blando, con un tamaño medio de 2 mm x 1.5 µm.

Histológicamente, en el peritoneo (Fig. 5a), adherido a las superficies serosas (Fig. 5b) y expandiendo y comprimiendo marcadamente la túnica muscular y la submucosa del intestino (Fig. 5c) y estómago (Fig. 5d), había quistes parasitarios multifocales de tamaño variable (hasta 5.7 mm × 3.9 mm de diámetro), que contenían una sección transversal de una larva de cestodo (plerocercus). Estos estaban rodeados por una fina capa interna intensamente basófila; una capa media, celular, delgada de color marrón dorado y una capa externa, delgada eosinófila, de colágeno con escasos fibroblastos (Fig. 6a-b).

De manera multifocal, mezcladas y englobadas por una cápsula fibrosa, había numerosas larvas degeneradas (Fig. 6a, b), caracterizadas por restos de plerocercos, en ocasiones con fragmentos de tentáculos en forma de gancho, y detritus fina- o gruesamente granulares, en ocasiones intensamente eosinófilos, de color marrón dorado y/o basófilos (mineralización). Alrededor de los quistes de las larvas degeneradas había, frecuentemente, agregados multifocales de macrófagos cargados de pigmento de color marrón dorado (Fig. 6c) y, ocasionalmente, linfocitos dispersos. En ocasiones, en el peritoneo, cuando los quistes estaban rotos, se observaban agregados, de tamaño variable, de linfocitos y macrófagos, rodeando e infiltrando los restos del parásito (Fig. 5a).

Alrededor de los quistes, había abundante proliferación de tejido conectivo fibroso maduro (Fig. 7), caracterizado por fibras de colágeno densamente organizadas y mínima inflamación, con ocasionales y pequeños agregados intercalados de linfocitos y macrófagos de

color marrón dorado y edema. De manera difusa, en las áreas de fibrosis de la submucosa del estómago, había proliferación de vasos sanguíneos de pequeño calibre (Fig. 6c). Además, las arterias de pequeño y mediano calibre dentro del tejido conectivo fibroso tenían paredes marcadamente engrosadas, expandidas por densas capas de tejido conectivo con numerosos vasos sanguíneos de pequeño calibre. Ocasionalmente, en la mucosa del estómago había áreas multifocales de leve necrosis y fibrosis. El hígado no mostró cambios patológicos significativos.

Las secciones de los plerocercos individualizados (Fig. 8) mostraron un tegumento eosinófilo y un parénquima eosinófilo fibrilar con numerosos corpúsculos calcáreos incrustados redondos, de basófilos a transparentes, y un escólex con cuatro bulbos musculares y tentáculos armados. En algunas secciones, en el extremo anterior de los plerocercos se observó el escólex, caracterizado por fibras musculares dispuestas concéntricamente (bulbos musculares) y los tentáculos cubiertos por numerosas columnas de ganchos.

La gravedad y extensión de las lesiones fue directamente proporcional a la intensidad de la infección.

En publicaciones anteriores en meros, las larvas de Trypanorhyncha habían infectado la cavidad celómica, los órganos viscerales y el músculo esquelético. En el músculo esquelético, las larvas causaron infiltración de linfocitos en la zona de inserción con edema focal, degeneración, atrofia y necrosis de las miofibras adyacentes (Hassan et al., 2002; Ibrahim, 2000). En la cavidad celómica, las larvas enquistadas estaban rodeadas por capas concéntricas de tejido conectivo denso y linfocitos (Ibrahim, 2000). También se observó disminución del tamaño del hígado (Ibrahim, 2000). Por el contrario, otros estudios no observaron cambios patológicos en meros infectados con Trypanorhyncha (Rizgalla, 2016; Soliman et al., 2011).

En otras especies de peces se han observado cambios similares con encapsulación de los parásitos por tejido conectivo denso y la infiltración con macrófagos y linfocitos (Abdelsalam et al., 2016; Santoro et al., 2018; Sattari et al., 2014).

Se han descrito respuestas tisulares similares en infecciones de peces con cestodos del orden Diphyllobothriidea, como *Diphyllobothrium* spp. y *Ligula* spp. (Arme & Owen, 1968, 1970; Halvorsen, 1970; McAdam et al., 2015; O'Neill et al., 1988; Otto & Heckmann, 1984; Sharp et al., 1989, 1992; Sweeting, 1977; van Kruiningen et al., 1987; Williams et al., 2012).

Cuando un patógeno infecta a un pez, típicamente ocurre una reacción para eliminar este patógeno (Sharp et al., 1992). Sin embargo, los parásitos como los cestodos suelen ser demasiado grandes para ser fagocitados y, además, pueden poseer mecanismos para evadir el sistema inmunológico del huésped. Esta combinación da como resultado la persistencia de los parásitos en los tejidos, que quedan encapsulados por la respuesta inflamatoria ocasionada. Este tipo de respuesta permite la separación del parásito de los tejidos del huésped, evitando daños mayores. Sin embargo, en infecciones graves, la fibrosis puede producir compresión y atrofia de los órganos adyacentes (Lumsden, 2006), lo que eventualmente conduce a la disfunción del órgano y a la muerte (Ackermann, 2012).

Teniendo en cuenta el gran número y la diversidad de especies del orden Trypanorhyncha, su alta prevalencia en una amplia gama de peces marinos, el impacto que pueden tener en la pesca y la amenaza que pueden representar para las especies de maricultura (Rückert et al., 2008), es primordial el conocimiento sobre los efectos patológicos de Trypanorhyncha en el pescado.

6.2.3. **Publicación III:** Fin a la controversia de la detección microscópica y efectos de los microplásticos vírgenes en los tejidos de peces

La producción cada vez mayor de plásticos (Toussaint et al., 2019; Wright et al., 2013) y su vida útil relativamente corta (Thompson et al., 2009), combinada con prácticas indiscriminadas de eliminación de residuos y pérdidas accidentales (Wright et al., 2013), han llevado a la acumulación de plásticos en ambientes acuáticos en todo el mundo (Carpenter & Smith, 1972; Eriksen et al., 2013; Faure et al., 2015; Lusher, Tirelli, et al., 2015; Reisser et al., 2013). Esta situación es especialmente preocupante debido a su largo tiempo de degradación (Toussaint et al., 2019) y al potencial de ser ingeridos por organismos acuáticos (Thompson et al., 2009).

En el agua, los plásticos sufren los efectos de la degradación fotolítica, mecánica y biológica (Browne et al., 2007; Crawford & Quinn, 2017). En estas circunstancias, los plásticos

más grandes se degradan en fragmentos más pequeños (Crawford & Quinn, 2017), es decir, microplásticos (MP) (<5 mm) (Kemikalieinspektionen (KEMI), 2018). Otra fuente de MP en ambientes acuáticos son las partículas de tamaño micro fabricadas intencionalmente para su uso en productos domésticos (por ejemplo, cosméticos y ropa) y productos industriales (granallado y materia prima industrial) (Browne et al., 2007; Crawford & Quinn, 2017) que se introducen directamente en el medio ambiente por la actividad humana (von Moos et al., 2012).

Los MP pueden exhibir una variedad de formas, tamaños y colores (Crawford & Quinn, 2017). El pequeño tamaño y ubicuidad de los MP (Critchell & Hoogenboom, 2018) los hace fácilmente accesibles para la fauna acuática, que es propensa a ingerirlos por confusión con alimentos, ingestión accidental o transferencia a través de la cadena alimentaria (Batel et al., 2016; Jovanović, 2017).

Teniendo en cuenta este panorama de interacción de los microplásticos con el medio acuático y fauna marina, en este estudio se han marcado los objetivos: 1) evaluar el comportamiento de alimentación después de la exposición a diferentes tipos de PM vírgenes; 2) determinar el tiempo de retención intestinal, captación y eliminación de MP; 3) evaluar el potencial de acumulación de diferentes tipos de PM vírgenes después de una exposición prolongada; 4) evaluar la translocación de MP en los órganos de los peces mediante microscopía confocal; y 5) determinar los efectos patológicos de dicha exposición.

Para llevar a cabo estos objetivos, se realizaron dos experimentos separados utilizando peces cebra *Danio rerio*, un modelo de vertebrado comúnmente utilizado para estudios toxicológicos. El primer experimento - experimento agudo - se realizó para evaluar la ingestión, el tiempo de retención intestinal, la absorción y la eliminación de MP en peces cebra alimentados una sola vez con MP vírgenes. El segundo experimento – experimento sub-crónico - se diseñó para evaluar el potencial de acumulación y translocación de diferentes tipos de MP vírgenes después de una exposición dietética prolongada y para determinar los efectos patológicos de dicha exposición.

En estudios anteriores, se sugirió que el tipo de MP (Jabeen et al., 2018; Qiao, Deng, et al., 2019), así como la forma (Choi et al., 2018) y el tamaño influían en el nivel de toxicidad infligida a los tejidos de los peces. Para replicar esas observaciones, en nuestros experimentos se han usado 3 tipos distintos de partículas plásticas. Microesferas fluorescentes verdes, compuestas de distintos polímeros de composición no identificada y de diámetro entre 1 a 5 µm. Microfragmentos de polietileno (PE) de superficie irregular y un diámetro de 120-220 µm. Microfibras de nylon obtenidas a partir de un tejido sintético, midiendo, de media, 1500 µm x 14 µm.

a) Experimento agudo

Se realizó un estudio preliminar para evaluar la capacidad de reconocimiento de las partículas de plástico por los peces cebra como material no comestible. Para ello, los peces cebra (n=5x2) fueron expuestos a microesferas de plástico fluorescentes y microfragmentos de PE. En ambos casos, los peces rechazaron las partículas de plástico, reconociéndolas como elementos no comestibles.

Para el experimento agudo, se utilizaron dos dietas. La dieta F_A, se obtuvo mediante la adición de microesferas fluorescentes a la dieta de control, y la dieta C_A que se obtuvo mezclando microfragmentos de PE cosméticos y microfibras textiles con gelatina aromatizada con aceite de pescado.

Se separaron los peces en dos grupos y a cada uno se le alimentó con una toma única de MPs. El grupo 1 con la dieta F_A y el grupo 2 con la dieta C_A. Tras este periodo, se separaron los peces de ambos grupos en cinco acuarios en grupos de seis. Cada acuario correspondió a un tiempo de muestreo diferente. Los peces del grupo 1 y del grupo 2 se muestrearon 2, 6, 10, 12 y 24 horas después de la ingesta (hdi). Las necropsias se realizaron bajo un estereomicroscopio. Se extrajo todo el intestino y se fijó en formalina tamponada neutra al 10% para su estudio histopatológico.

Tiempo de retención intestinal, absorción y eliminación.

Para los peces del grupo 1, alimentados con la dieta F_A se observó que, tras 24 horas, apenas quedaban micropartículas fluorescentes en el intestino, ya que la mayoría había sido eliminada. En cambio, para los peces del grupo 2, alimentados con la dieta C_A se observaron ocasionales microfibras y microfragmentos en el intestino posterior 24 hdi. Estos resultados indican que las partículas de mayor tamaño tardan más tiempo en ser completamente eliminadas. A pesar de su mayor tamaño y forma irregular, los microfragmentos y microfibras se eliminaron con éxito en la mayoría de los peces después de 24 horas.

Con relación a la absorción de partículas por las células, se observó 10 hdi en el intestino de los peces alimentados con la dieta F_A, la presencia de partículas fluorescentes en el citoplasma de los enterocitos, "goblet cells" y en la lámina propia. Las partículas observadas median hasta 2.041 µm de diámetro y su internalización en los tejidos se confirmó mediante microscopia confocal (Fig. 1). Por el contrario, en el grupo de la dieta C_A, no se observó la absorción de partículas o fibras por los tejidos. Esto se debe al mayor tamaño de estos MPs, lo que imposibilita su pasaje por la barrera intestinal.

En un estudio anterior (Batel et al., 2016), se observó la absorción de algunas partículas en el intestino de peces cebra. Sin embargo, no se realizaron pruebas adicionales para confirmar la internalización de las partículas.

b) Experimento sub-crónico

Para el experimento sub-crónico se utilizaron igualmente dos dietas experimentales. La dieta F_{SC} se obtuvo añadiendo microesferas fluorescentes a la dieta control, mientras la dieta C_{SC} se obtuvo combinando microfragmentos de PE y microfibras con la dieta control. Adicionalmente, un grupo de control fue alimentado con la dieta control.

Los peces se distribuyeron en dos grupos. Los peces del grupo 1 se alimentaron con la dieta F_{SC} y los peces del grupo 2 con la dieta C_{SC}. Todos los tratamientos se realizaron por triplicado, y cada acuario estuvo compuesto por igual número de machos (n = 4) y hembras (n = 4). Los peces de todos los grupos fueron alimentados con la dieta control tres veces al día en

un horario fijo. Cada dos días, la primera ingesta de dieta control se reemplazó con las dietas experimentales. El experimento de alimentación duró 45 días a los cuales siguió un periodo de depuración de 15 días, durante el cual los peces se alimentaron solo de dieta control. El periodo de depuración permitió determinar el potencial de bioacumulación de estas partículas.

Durante la necropsia, se realizó un corte en la línea ventral y posteriormente se extrajo el intestino, hígado y músculo, bajo un estereomicroscopio y se fijaron en formalina tamponada neutra al 10% para su estudio histopatológico. Los cambios histológicos en intestino y hígado se evaluaron siguiendo las pautas propuestas por Saraiva et al., 2015 y Bernet, 1999, respectivamente.

Tasa de supervivencia, peso y comportamiento

En los grupos experimentales no se observó mortalidad ni cambios de peso estadísticamente significativos. Sin embargo, después de la segunda semana, los peces alimentados con la dieta C_{SC} comenzaron a manifestar inapetencia, reducida reactividad a los estímulos y un cambio general en el color del cuerpo, exhibiendo una pérdida general de color de la piel.

Translocación y retención de partículas

Se observaron MP fluorescentes en el 25% de los hígados de los peces alimentados con la dieta F_{SC}. Las partículas encontradas en el citoplasma de los hepatocitos tenían entre 1.416 y 1.634 µm. También se observó una sola partícula de 0,692 µm rodeando un vaso sanguíneo. La microscopia confocal confirmó la internalización de las partículas de MP (Fig. 3).

La translocación de MP al hígado ha sido descrita en diferentes especies de peces. Sin embargo, el tamaño de las partículas observadas se encuentra en un rango de 100 a 600 µm (Abbasi et al., 2018; Avio et al., 2015; Collard et al., 2017, 2018). La supuesta translocación de partículas de tamaños elevados, como estas, es difícil de justificar con el actual conocimiento sobre las vías de translocación de los MP en los peces. La transferencia de partículas exógenas con 100 a 600 µm de la circulación sanguínea a otros tejidos probablemente causaría una

respuesta inflamatoria del área circundante. Sin embargo, esto no ha sido documentado en ninguno de los estudios antes mencionados (Batel et al., 2016). Además, en dichos estudios no se han realizado pruebas complementarias para confirmar la internalización de las partículas.

Hasta donde sabemos, nuestro estudio es el primero en confirmar la internalización de partículas MP en el hígado, validando así la translocación de MP.

Después 15 días de depuración, no se observaron microesferas fluorescentes en el intestino, mientras que se detectó una pequeña cantidad de microfragmentos en el tracto intestinal en el 50% de los peces (Fig. 2). Aparte del intestino, no se observaron partículas de MP en ningún otro tejido.

Cambios histopatológicos

En el intestino (Fig. 4), en comparación con el grupo control, no se observaron cambios patológicos en ninguno de los grupos de prueba durante el período experimental.

Con relación al hígado (Fig. 5, 6), se observaron cambios regresivos, particularmente degeneración vacuolar hepatocelular. Sin embargo, también se observaron disminuciones en la vacuolización. Los cambios más significativos se observaron en el grupo de la dieta F_{SC}, en el que tras el periodo de depuración se apreció una disminución marcada de la vacuolización.

En estudios anteriores que exponían peces directamente (Ašmonaite et al., 2018; Batel et al., 2016; Jovanović et al., 2018; Rainieri et al., 2018; Romano et al., 2018) o a través de la cadena trófica (Batel et al., 2020) a diferentes tipos de MP, no se observaron cambios histopatológicos significativos. Por el contrario, numerosos estudios han reportado cambios histopatológicos en los peces después de la exposición a MP vírgenes o contaminados. En ensayos experimentales, se esperan diferentes resultados que pueden resultar de variaciones con respecto a la especie, edad, sexo, estado reproductivo de los peces, medio ambiente, concentraciones probadas, tamaño, tipo, química de la superficie e hidrofobicidad de los MP, rutina de alimentación, ruta de exposición, tiempo de exposición, número de animales y réplicas por grupo de tratamiento, recolección de muestras y métodos de preparación (Wolf & Wheeler, 2018).

Sin embargo, la mayoría de los cambios histopatológicos que supuestamente se describen en muchas de esas publicaciones (Hamed et al., 2021; Iheanacho & Odo, 2020b; Jabeen et al., 2018; Karami et al., 2016; Lei et al., 2018; Lu et al., 2016; Qiao, Deng, et al., 2019; Qiao, Sheng, et al., 2019; Yang et al., 2020) son el resultado de interpretaciones inexactas de los datos histopatológicos. Dentro de la comunidad de patólogos (de peces), han aumentado las preocupaciones sobre la calidad general de los hallazgos histopatológicos presentados en la literatura revisada por pares (Baumann et al., 2016). Esta situación es especialmente alarmante en los casos en los que las conclusiones del estudio dependen en gran medida de los resultados histopatológicos. Además, tales observaciones persistirán en la literatura y generarán más investigaciones equivocadas. Esto es particularmente problemático para los estudiantes e investigadores que trabajan en patología de peces, que buscan en estas publicaciones fuentes fiables de información (Baumann et al., 2016). Por lo tanto, como destacaron anteriormente (Batel et al., 2020), es de suma importancia que los métodos dudosos y los resultados inciertos en las publicaciones científicas sean cuestionados y corregidos con trabajos adicionales de alta calidad.

Aunque los MP vírgenes por sí mismos no produzcan daño inminente, la mayoría de los plásticos producidos no están hechos solamente de polímeros plásticos. Durante la fabricación de plásticos, se les incorporan aditivos químicos endógenos (Wright & Kelly, 2017). Los MP también son muy eficientes para adsorber contaminantes orgánicos persistentes que ya están presentes en el agua (Jovanović, 2017). Por lo tanto, se necesita más investigación para identificar adecuadamente los efectos que los MPs y sus contaminantes asociados pueden tener sobre la salud animal y, en consecuencia, la salud pública.

6.3. Conclusiones

1) A pesar de ser un hongo saprofito y un patógeno vegetal reconocido, *Phoma herbarum* también es un patógeno oportunista para los peces. En el medregal salvaje, causa una aerocistitis granulomatosa primaria severa con pérdida completa de la morfología de la vejiga natatoria, lo que sugiere una ruta de diseminación hematógena para este hongo en peces fisóstomos.

2) Las larvas de Trypanorhyncha tienen una alta prevalencia (96,4%) en la cavidad celómica de meros adultos de más de 17 kg de las Islas Canarias.

3) Las infecciones por Trypanorhyncha en adultos de mero provocan una respuesta progresiva y crónica caracterizada por una reacción fibrótica extensa y marcada con encapsulación de las larvas. La gravedad y extensión de las lesiones provocadas por las larvas son directamente proporcionales a la intensidad de la infección. Los peces con un mayor número (n>30) desarrollan fibrosis extensa del peritoneo, estómago e intestino, con compresión de los órganos viscerales adyacentes.

4) El pez cebra reconoce las partículas microplásticas como materiales no comestibles, ingiriéndolos sólo cuando se mezclan con alimentos y aceite de pescado, o cuando se exponen accidentalmente a la ingestión de partículas de plástico de pequeño tamaño (1 a 5 µm).

5) Es poco probable que las microesferas (1 a 5 µm), los microfragmentos de PE (120 a 220 µm) y las microfibras textiles (una media de 1500 µm) de plástico queden retenidas en el tracto digestivo del pez cebra después de una exposición oral aguda. Sin embargo, después de una exposición subcrónica, los microfragmentos PE de mayor tamaño y las microfibras textiles permanecen en el intestino del pez cebra más tiempo en comparación con las microesferas pequeñas (1-5 µm). A pesar de esto, el potencial de retención de MP es escaso, ya que solo un promedio de 3.3 partículas permanece en el intestino del 50% del pez cebra después del período de depuración.

6) Pequeñas microesferas de plástico (hasta 2.041 µm) pueden atravesar la barrera intestinal y entrar en el citoplasma de los enterocitos, las "goblet cells" y la lámina propia. Dos horas después de la ingestión, las microesferas alcanzan los enterocitos y las "goblet cells", y después de 10 horas, también pueden penetrar en la lámina propia.

7) La exposición oral subcrónica a microesferas de polímeros fluorescentes prístinos (9.9×10^{-4} g / cm^3) y microfragmentos de PE mezclados con microfibras textiles (3.3×10^{-2} g / cm^3) no causa mortalidad ni efectos significativos en la condición corporal del pez cebra. Sin embargo, la ingestión de microfragmentos de PE de mayor tamaño y microfibras textiles produce inapetencia y letargo y palidez generalizada de la piel.

8) Después de la ingestión subcrónica de MP con tamaños comprendidos entre 1 µm y 1500 µm, sólo las microesferas más pequeñas (<1.634 µm) penetran en el citoplasma de hepatocito del pez cebra. Esto sugiere que estudios anteriores que reportaron la translocación de MP de hasta 600 µm deberían evaluarse más detenidamente.

9) Después de un estudio histopatológico detallado del intestino, hígado y músculo esquelético de peces cebra expuestos de forma subcrónica a diferentes tipos y tamaños de MP, no se observaron hallazgos histopatológicos significativos. Esto indica que la ingestión de MP vírgenes en altas concentraciones no provoca cambios histopatológicos.

7

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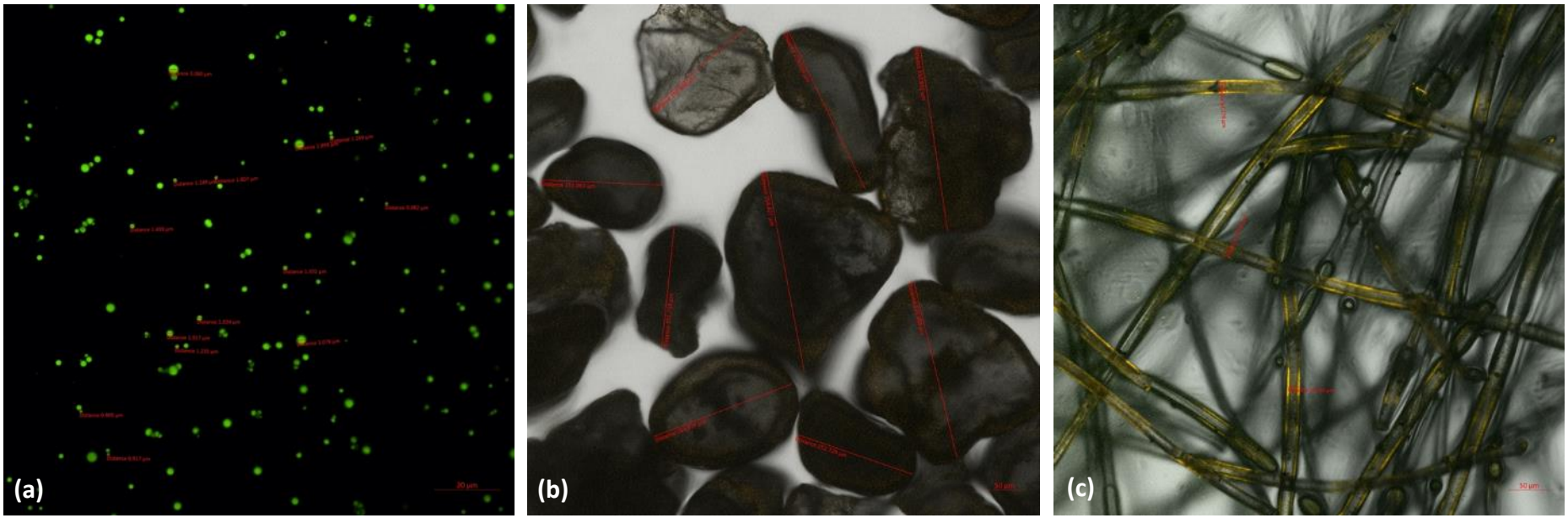
8

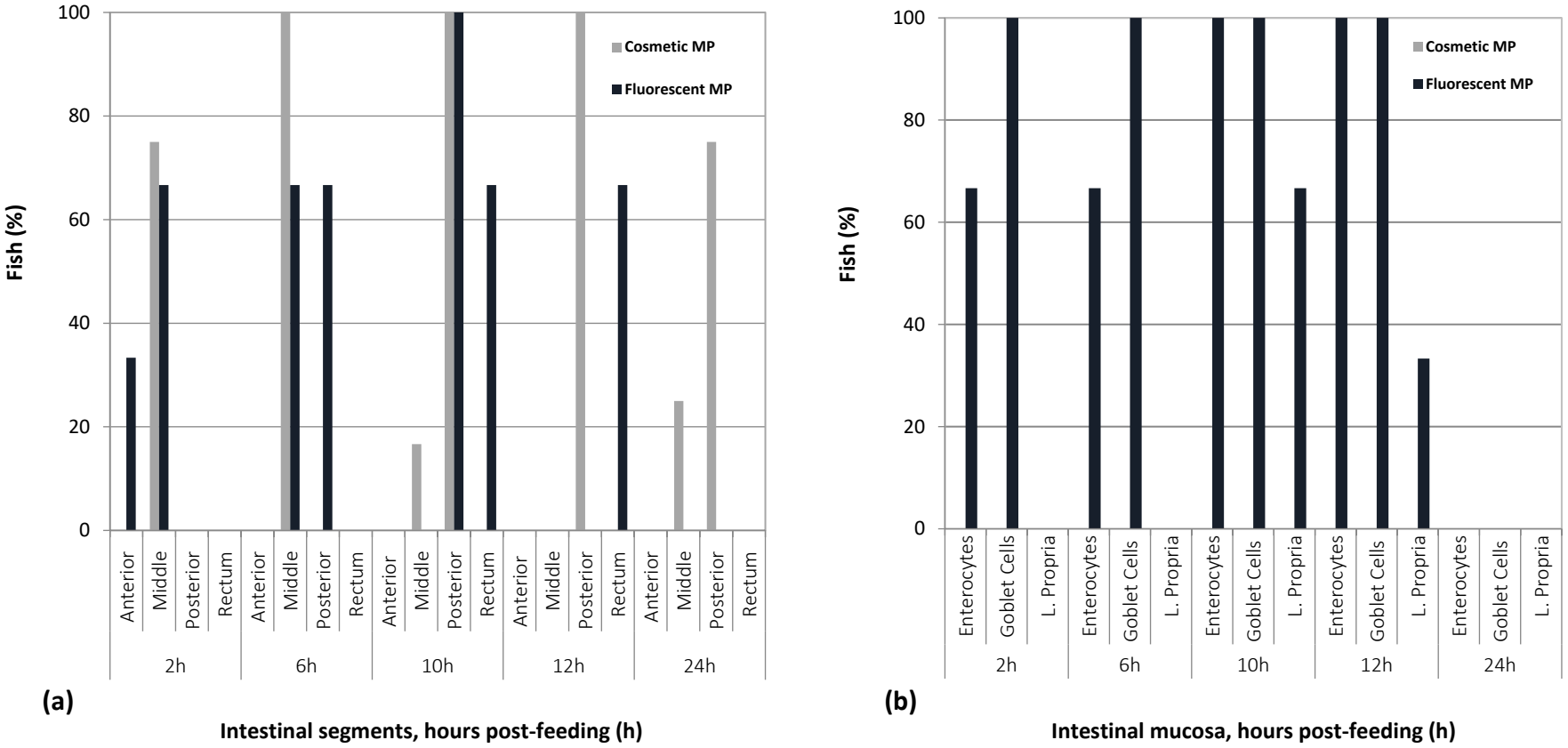
APPENDIX

8.1. Scientific publication III: An end to the controversy over the microscopic detection and effects of pristine microplastics in fish organs.

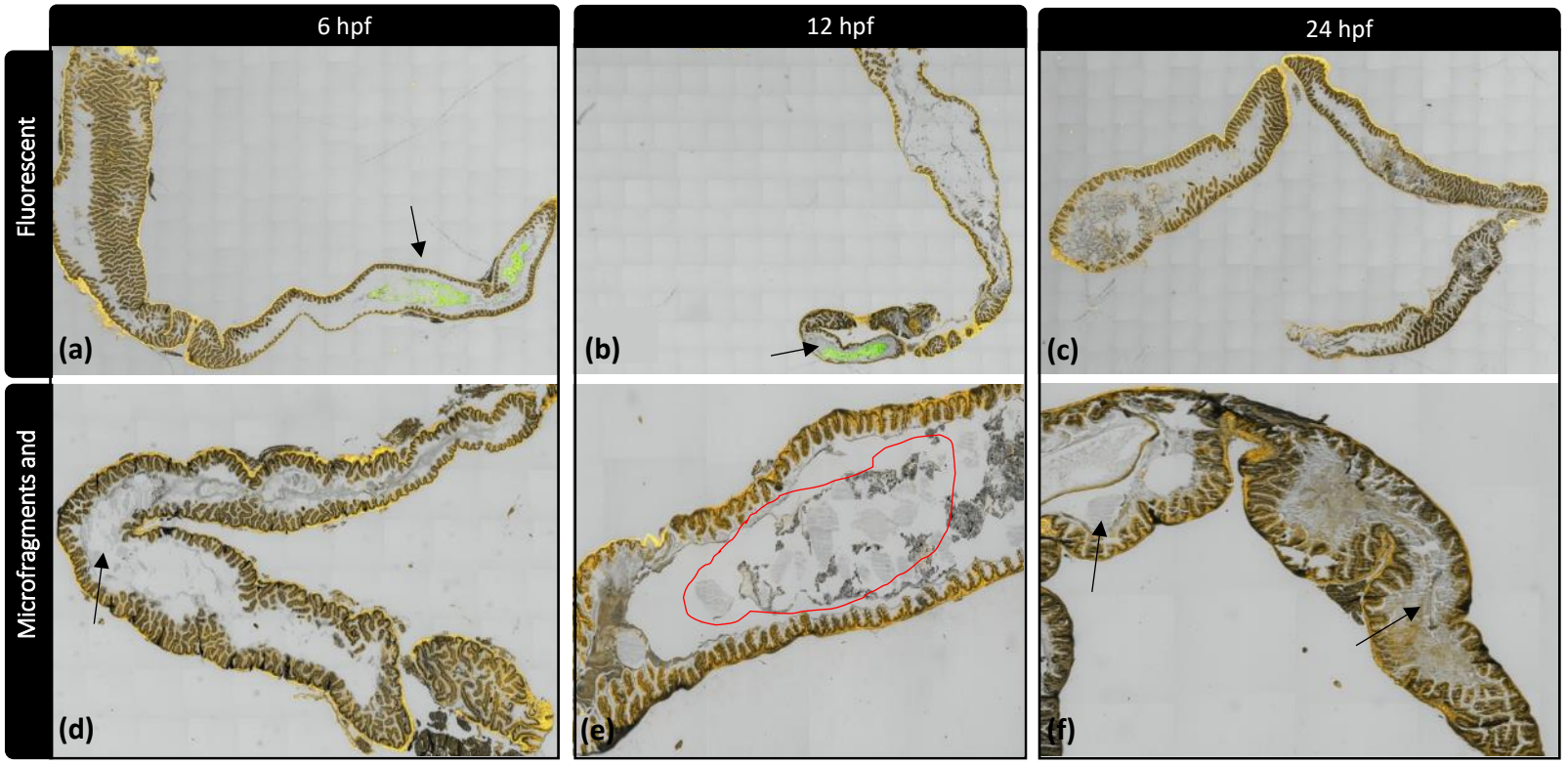
A. Supplementary Figures



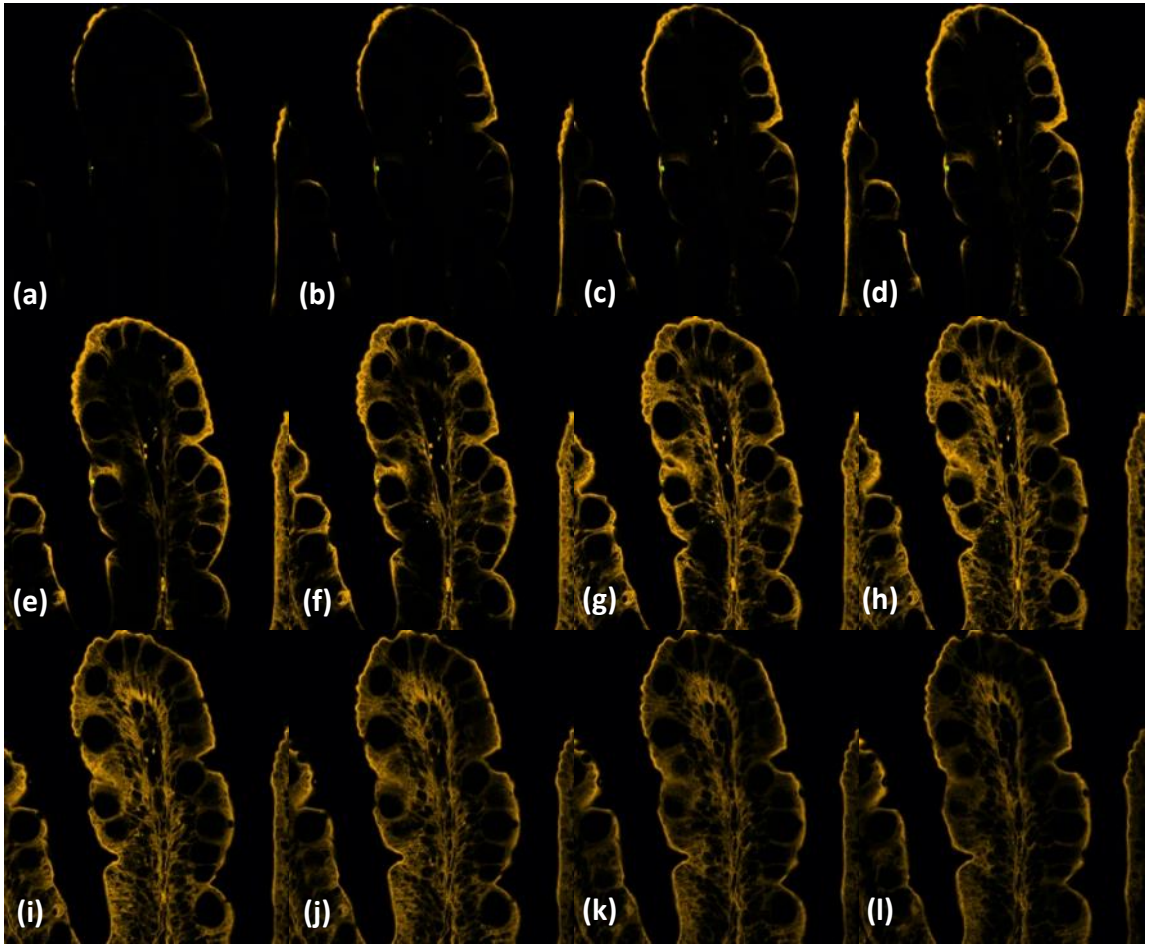
Supplementary Figure S1. Plastic materials by confocal fluorescence microscopy. **(a)** Green fluorescent polymer microbeads, **(b)** PE microfragments from a cosmetic body cleanser and **(c)** synthetic textile microfibers.



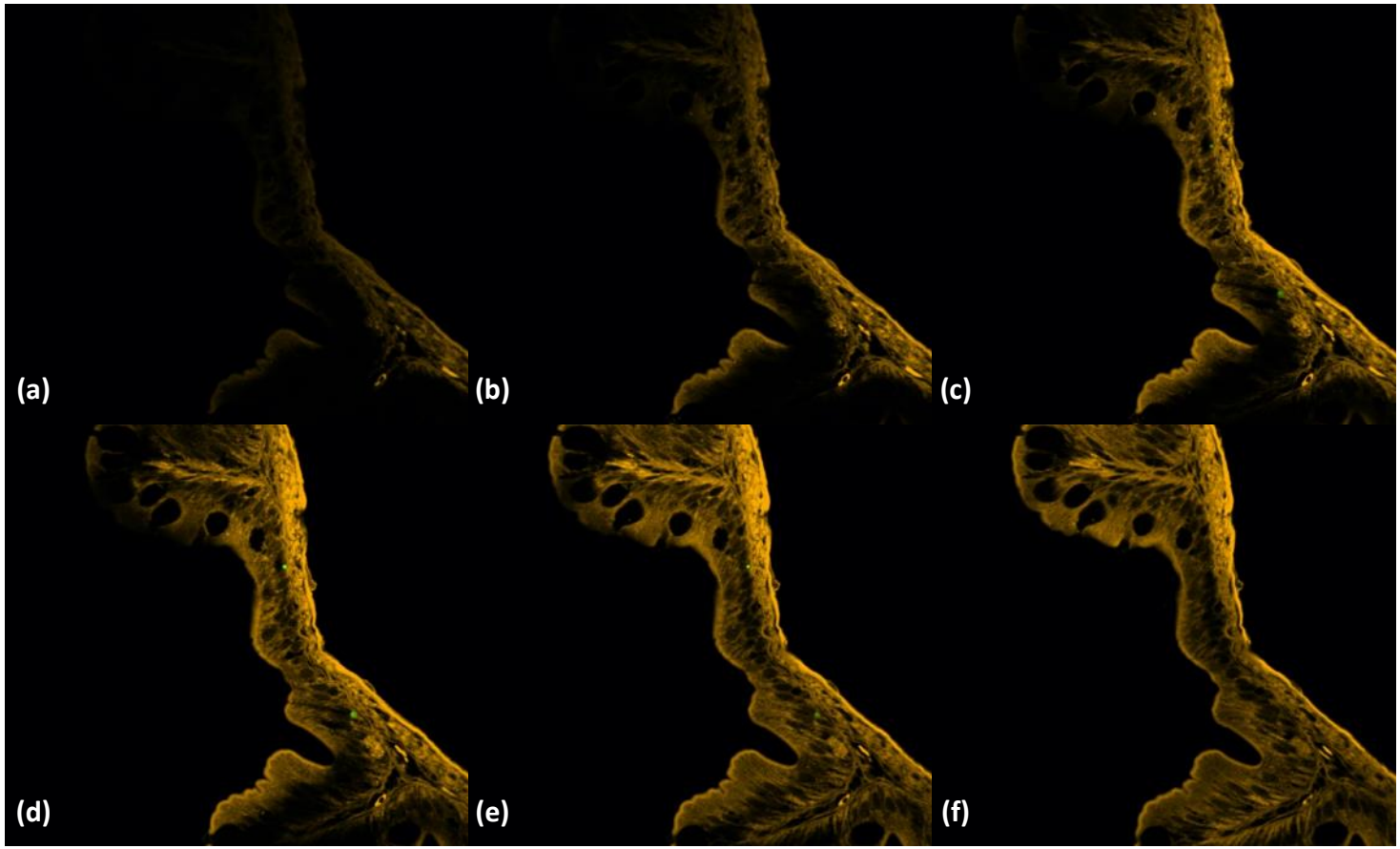
Supplementary Figure S2. Acute Experiment. (a) Cosmetic and fluorescent MPs in the different intestinal segments at different hours post-feeding. (b) Cosmetic and fluorescent MPs in the intestinal mucosa at different hours post-feeding. (*L. Propria: Lamina propria*).



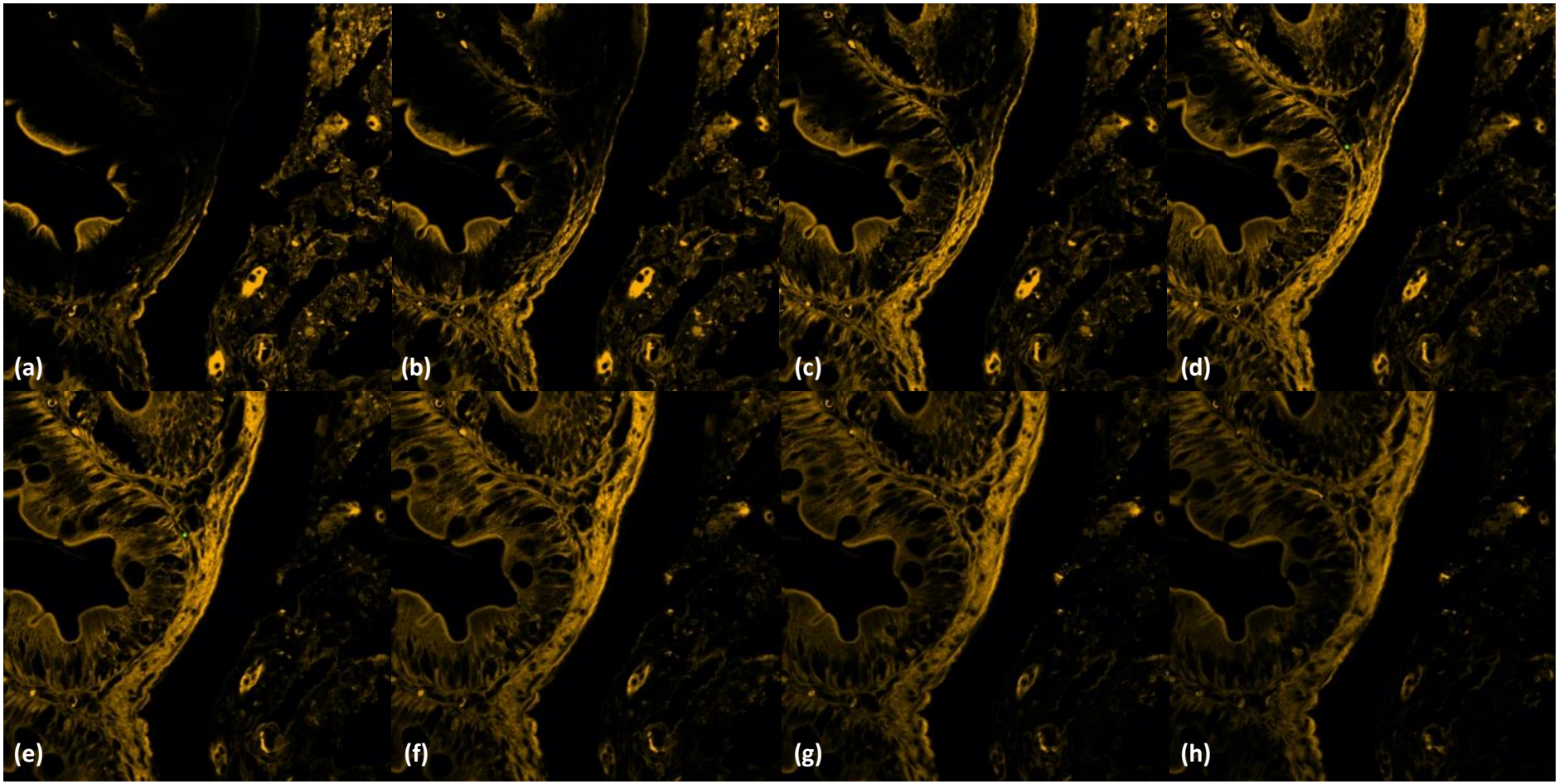
Supplementary Figure S3. Acute Experiment. Panoramic sections of the whole intestine by confocal microscopy. Fish fed **(a-c)** fluorescent MP microbeads, **(d-f)** microfragments and microfibres of MPs. **(a)** Six hours post-feeding (hpf), fluorescent microbeads in the middle and posterior intestine (arrow), **(b)** 12 hpf, microbeads in the final portion of the posterior intestine (arrow), and **(c)** 24 hpf, microbeads absent from the intestine. **(d)** 6 hpf, microfragments in the middle and posterior intestine (arrow), **(e)** 12 hpf, microfragments in the middle and posterior intestine (within the red lines), and **(f)** 24 hpf, rare microfragments and microfibers in the intestinal lumen (arrows).



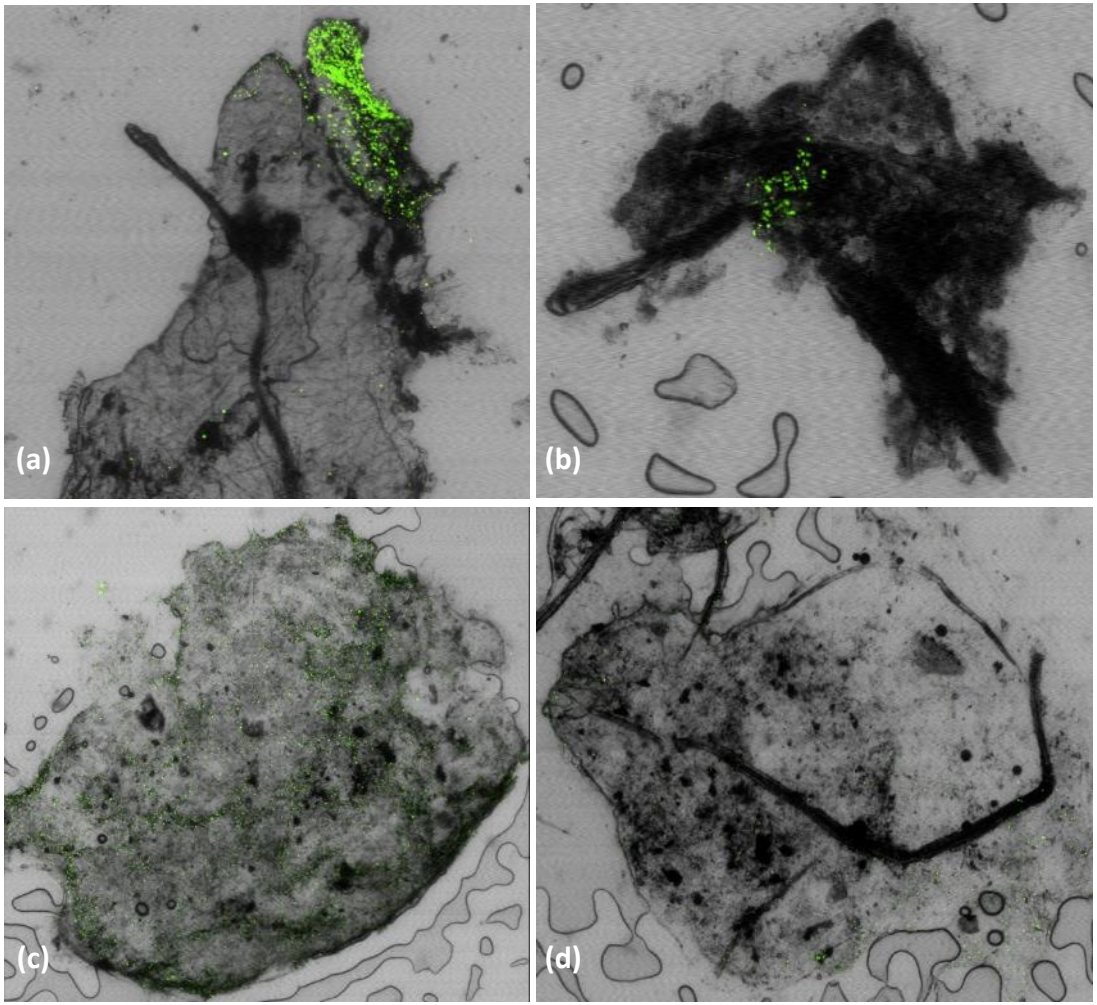
Supplementary Figure S4. Acute Experiment. Confocal microscopy Z-stack images of Figure 1B. A fluorescent particle within the intestinal epithelium (between 2.70 μm and 4.05 μm). **(a)** 0.45 μm, **(b)** 0.90 μm, **(c)** 1.35 μm, **(d)** 1.80 μm, **(e)** 2.25 μm, **(f)** 2.70 μm, **(g)** 3.15 μm, **(h)** 3.60 μm, **(i)** 4.05 μm, **(j)** 4.50 μm, **(k)** 4.95 μm, **(l)** 5.40 μm (Total z-stack = 8.55 μm, scaling=0.45 μm).



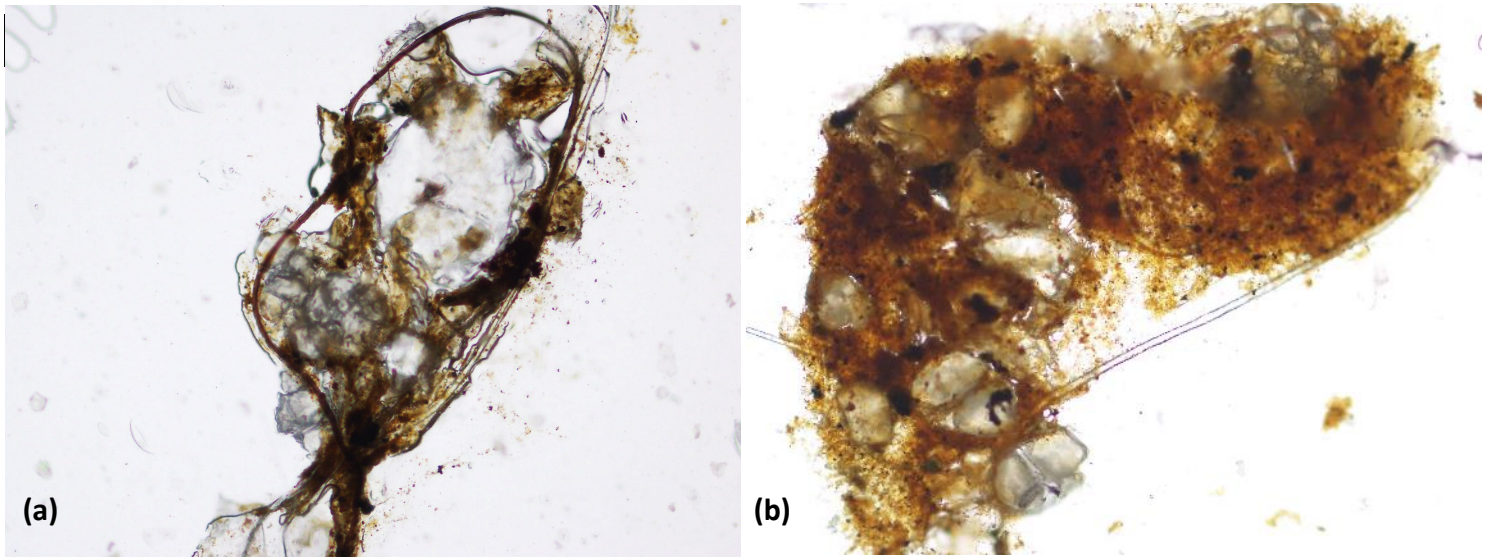
Supplementary Figure S5. Acute Experiment. Confocal microscopy Z-stack images of Figure 1D. Fluorescent particles in the cytoplasm of the enterocytes and lamina propria (between 1.35 μm and 2.25 μm). **(a)** 0.45 μm, **(b)** 0.90 μm, **(c)** 1.35 μm, **(d)** 1.80 μm, **(e)** 2.25 μm, **(f)** 2.70 μm, (Total z-stack = 10.8 μm, scaling=0.45 μm).



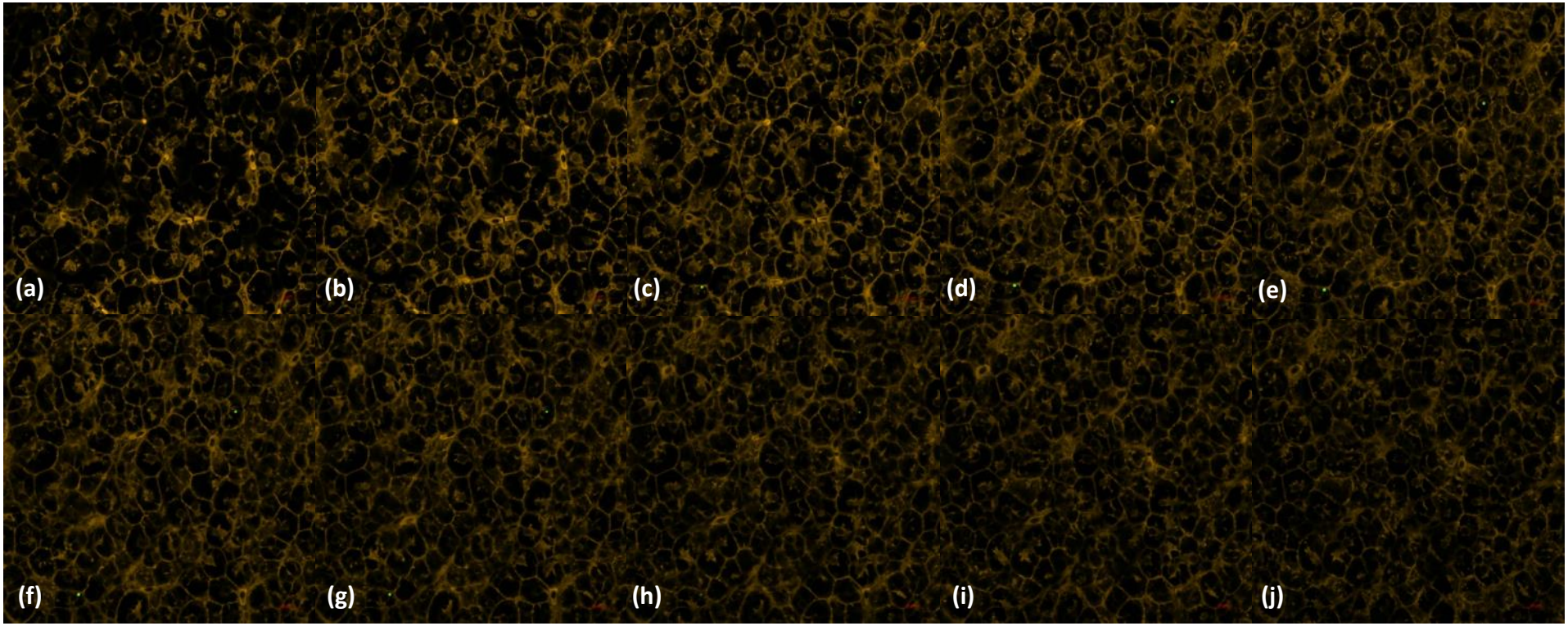
Supplementary Figure S6. Acute Experiment. Confocal microscopy Z-stack images of Figure 1E. Fluorescent particles in the lamina propria (between 1.80 μm and 3.15 μm). **(a)** 0.90 μm, **(b)** 1.35 μm, **(c)** 1.80 μm, **(d)** 2.25 μm, **(e)** 2.70 μm, **(f)** 3.15 μm, **(g)** 3.60 μm, **(h)** 4.05 μm. (Total z-stack=9.9 μm, scaling=0.45 μm).



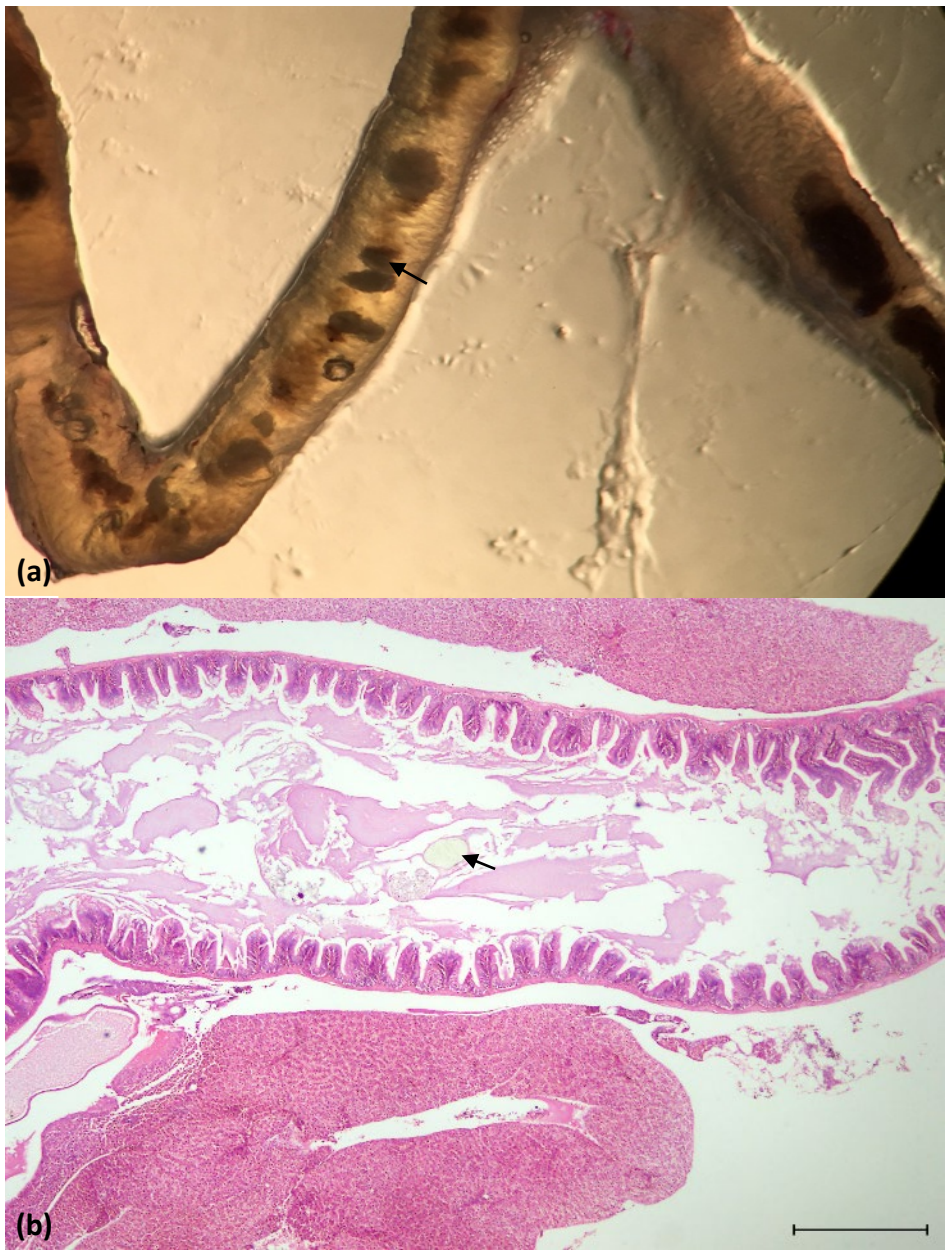
Supplementary Figure S7. Acute experiment. Faces collected from fish fed fluorescent MPs under a confocal microscope. **(a)** 6 hpf, **(b)** 10 hpf, **(c)** 24 hpf, and **(d)** 48 hpf.



Supplementary Figure S8. Acute experiment. Faces collected from fish fed microfragments and fibres under a confocal microscope **(a)** 10 hpf and **(b)** 24 hpf.

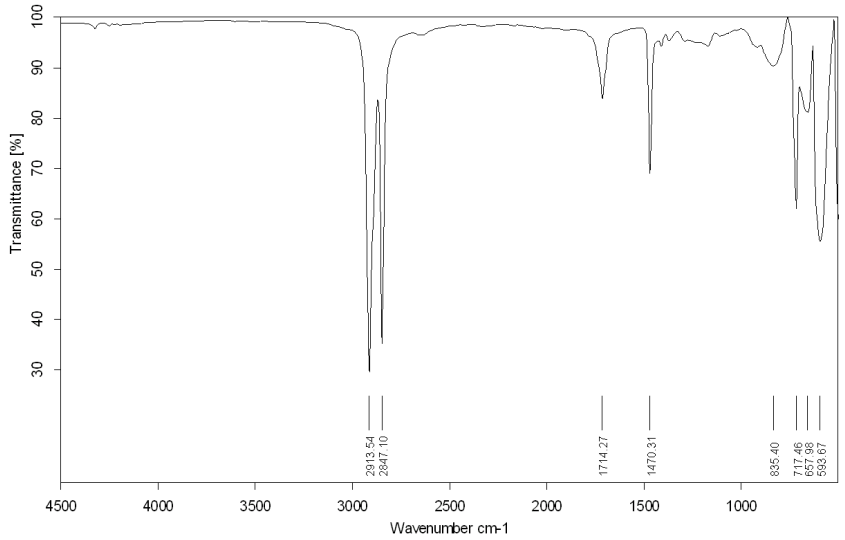


Supplementary Figure S9. Sub-chronic experiment. Confocal microscopy Z-stack images of Figure 3A. Fluorescent particles in the liver (between 4.05 μm and 6.75 μm). **(a)** 3.15 μm, **(b)** 3.60 μm, **(c)** 4.05 μm, **(d)** 4.50 μm, **(e)** 4.95 μm, **(f)** 5.40 μm, **(g)** 5.85 μm, **(h)** 6.30 μm, **(i)** 6.75 μm, **(j)** 7.20 μm (Total z-stack=8.55 μm, scaling=0.45 μm).

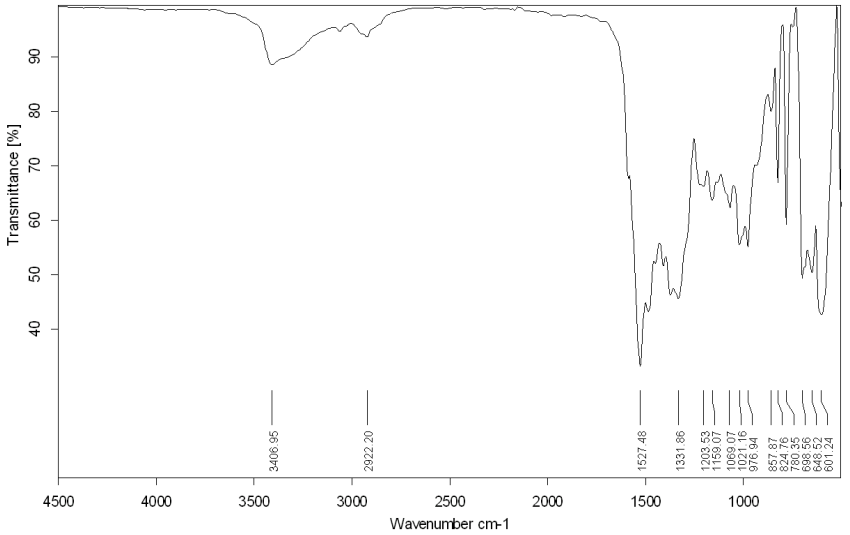


Supplementary Figure S10. Sub-chronic experiment. **(a)** Intestine under a stereomicroscope after 15 days of depuration with microfragments (arrow) in the lumen. **(b)** Histological section of the same intestine with fragments of MPs (arrow) (scale bar = 500 µm, HE).

B. Supplementary Data



Supplementary Data S1. FTIR spectrum of cosmetic plastic samples. Presence of dominant bands in the 3000, 2840, 1714 and 1470 cm^{-1}. These characteristic bands present similarities with PE polymers. https://www.azom.com/article.aspx?ArticleID=12386



Supplementary Data S2. FTIR spectrum of the fluorescent plastic sample. Presence of dominant bands below 1600 cm^{-1} and weak bands in the 2922 and 3406 cm^{-1}. The spectrum shows similarities with PEG polymers. https://www.researchgate.net/publication/274743255_PEGylated_magnetic_nanoparticles_PEGFe3O4_as_cost_effective_alternative_for_oxidative_cyanation_of_tertiary_amines_via_CH_activation. Other common polymers were not detected.

Supplementary Table S1. Histopathological assessment tools for intestine.

				Circulatory Disturbances (rp1)							Regressive changes (rp2)																										
											Epithelium																		*Lamina Propria*								
	D. rerio	Test Group	Sex	Haemorrhage / hyperaemia / aneurysm			Intercelular oedema			I org rp	Architectural and strucutral alterations			Plasma alterations			Deposits			Nuclear alterations			Atrophy			Necrosis			Architectural and strucutral alterations			Plasma alterations			Deposits			Nuclear alterations		
				w	a	a x w	w	a	a x w		w	a	a x w	w	a	a x w	w	a	a x w	w	a	a x w	w	a	a x w	w	a	a x w	w	a	a x w	w	a	a x w	w	a	a x w	w	a	a x w
T0	1	CONTROL	F	1	0	0	1	0	0	0	1	0	0	1	0	0	1	0	0	2	0	0	2	0	0	3	0	0	1	0	0	1	0	0	1	0	0	2	0	0
	2		F	1	0	0	1	0	0	0	1	0	0	1	0	0	1	0	0	2	0	0	2	0	0	3	0	0	1	0	0	1	0	0	1	0	0	2	0	0
	3		M	1	0	0	1	0	0	0	1	0	0	1	0	0	1	0	0	2	0	0	2	0	0	3	0	0	1	0	0	1	0	0	1	0	0	2	0	0
	4		M	1	0	0	1	0	0	0	1	0	0	1	0	0	1	0	0	2	0	0	2	0	0	3	0	0	1	0	0	1	0	0	1	0	0	2	0	0
T30	1	FLUORESCENT	F	1	0	0	1	0	0	0	1	0	0	1	0	0	1	0	0	2	0	0	2	0	0	3	0	0	1	0	0	1	0	0	1	0	0	2	0	0
	2		F	1	0	0	1	0	0	0	1	0	0	1	0	0	1	0	0	2	0	0	2	0	0	3	0	0	1	0	0	1	0	0	1	0	0	2	0	0
	3		F	1	0	0	1	0	0	0	1	0	0	1	0	0	1	0	0	2	0	0	2	0	0	3	0	0	1	0	0	1	0	0	1	0	0	2	0	0
	4		M	1	0	0	1	0	0	0	1	0	0	1	0	0	1	0	0	2	0	0	2	0	0	3	0	0	1	0	0	1	0	0	1	0	0	2	0	0
	5		M	1	0	0	1	0	0	0	1	0	0	1	0	0	1	0	0	2	0	0	2	0	0	3	0	0	1	0	0	1	0	0	1	0	0	2	0	0
	6		M	1	0	0	1	0	0	0	1	0	0	1	0	0	1	0	0	2	0	0	2	0	0	3	0	0	1	0	0	1	0	0	1	0	0	2	0	0
	1	COSMETIC	F	1	0	0	1	0	0	0	1	0	0	1	0	0	1	0	0	2	0	0	2	0	0	3	0	0	1	0	0	1	0	0	1	0	0	2	0	0
	2		F	1	0	0	1	0	0	0	1	0	0	1	0	0	1	0	0	2	0	0	2	0	0	3	0	0	1	0	0	1	0	0	1	0	0	2	0	0
	3		F	1	0	0	1	0	0	0	1	0	0	1	0	0	1	0	0	2	0	0	2	0	0	3	0	0	1	0	0	1	0	0	1	0	0	2	0	0
	4		M	1	0	0	1	0	0	0	1	0	0	1	0	0	1	0	0	2	0	0	2	0	0	3	0	0	1	0	0	1	0	0	1	0	0	2	0	0
	5		M	1	0	0	1	0	0	0	1	0	0	1	0	0	1	0	0	2	0	0	2	0	0	3	0	0	1	0	0	1	0	0	1	0	0	2	0	0
	6		M	1	0	0	1	0	0	0	1	0	0	1	0	0	1	0	0	2	0	0	2	0	0	3	0	0	1	0	0	1	0	0	1	0	0	2	0	0
	1	CONTROL	F	1	0	0	1	0	0	0	1	0	0	1	0	0	1	0	0	2	0	0	2	0	0	3	0	0	1	0	0	1	0	0	1	0	0	2	0	0
	2		F	1	0	0	1	0	0	0	1	0	0	1	0	0	1	0	0	2	0	0	2	0	0	3	0	0	1	0	0	1	0	0	1	0	0	2	0	0
	3		M	1	0	0	1	0	0	0	1	0	0	1	0	0	1	0	0	2	0	0	2	0	0	3	0	0	1	0	0	1	0	0	1	0	0	2	0	0
	4		M	1	0	0	1	0	0	0	1	0	0	1	0	0	1	0	0	2	0	0	2	0	0	3	0	0	1	0	0	1	0	0	1	0	0	2	0	0
T45	1	FLUORESCENT	F	1	0	0	1	0	0	0	1	0	0	1	0	0	1	0	0	2	0	0	2	0	0	3	0	0	1	0	0	1	0	0	1	0	0	2	0	0
	2		F	1	0	0	1	0	0	0	1	0	0	1	0	0	1	0	0	2	0	0	2	0	0	3	0	0	1	0	0	1	0	0	1	0	0	2	0	0
	3		F	1	0	0	1	0	0	0	1	0	0	1	0	0	1	0	0	2	0	0	2	0	0	3	0	0	1	0	0	1	0	0	1	0	0	2	0	0
	4		M	1	0	0	1	0	0	0	1	0	0	1	0	0	1	0	0	2	0	0	2	0	0	3	0	0	1	0	0	1	0	0	1	0	0	2	0	0
	5		M	1	0	0	1	0	0	0	1	0	0	1	0	0	1	0	0	2	0	0	2	0	0	3	0	0	1	0	0	1	0	0	1	0	0	2	0	0
	6		M	1	0	0	1	0	0	0	1	0	0	1	0	0	1	0	0	2	0	0	2	0	0	3	0	0	1	0	0	1	0	0	1	0	0	2	0	0
	1	COSMETIC	F	1	0	0	1	0	0	0	1	0	0	1	0	0	1	0	0	2	0	0	2	0	0	3	0	0	1	0	0	1	0	0	1	0	0	2	0	0
	2		F	1	0	0	1	0	0	0	1	0	0	1	0	0	1	0	0	2	0	0	2	0	0	3	0	0	1	0	0	1	0	0	1	0	0	2	0	0
	3		F	1	0	0	1	0	0	0	1	0	0	1	0	0	1	0	0	2	0	0	2	0	0	3	0	0	1	0	0	1	0	0	1	0	0	2	0	0
	4		M	1	0	0	1	0	0	0	1	0	0	1	0	0	1	0	0	2	0	0	2	0	0	3	0	0	1	0	0	1	0	0	1	0	0	2	0	0
	5		M	1	0	0	1	0	0	0	1	0	0	1	0	0	1	0	0	2	0	0	2	0	0	3	0	0	1	0	0	1	0	0	1	0	0	2	0	0
	6		M	1	0	0	1	0	0	0	1	0	0	1	0	0	1	0	0	2	0	0	2	0	0	3	0	0	1	0	0	1	0	0	1	0	0	2	0	0
DEPURATION	1	CONTROL	F	1	0	0	1	0	0	0	1	0	0	1	0	0	1	0	0	2	0	0	2	0	0	3	0	0	1	0	0	1	0	0	1	0	0	2	0	0
	2		F	1	0	0	1	0	0	0	1	0	0	1	0	0	1	0	0	2	0	0	2	0	0	3	0	0	1	0	0	1	0	0	1	0	0	2	0	0
	3		M	1	0	0	1	0	0	0	1	0	0	1	0	0	1	0	0	2	0	0	2	0	0	3	0	0	1	0	0	1	0	0	1	0	0	2	0	0
	4		M	1	0	0	1	0	0	0	1	0	0	1	0	0	1	0	0	2	0	0	2	0	0	3	0	0	1	0	0	1	0	0	1	0	0	2	0	0
	1	FLUORESCENT	F	1	0	0	1	0	0	0	1	0	0	1	0	0	1	0	0	2	0	0	2	0	0	3	0	0	1	0	0	1	0	0	1	0	0	2	0	0
	2		F	1	0	0	1	0	0	0	1	0	0	1	0	0	1	0	0	2	0	0	2	0	0	3	0	0	1	0	0	1	0	0	1	0	0	2	0	0
	3		F	1	0	0	1	0	0	0	1	0	0	1	0	0	1	0	0	2	0	0	2	0	0	3	0	0	1	0	0	1	0	0	1	0	0	2	0	0
	4		M	1	0	0	1	0	0	0	1	0	0	1	0	0	1	0	0	2	0	0	2	0	0	3	0	0	1	0	0	1	0	0	1	0	0	2	0	0
	5		M	1	0	0	1	0	0	0	1	0	0	1	0	0	1	0	0	2	0	0	2	0	0	3	0	0	1	0	0	1	0	0	1	0	0	2	0	0
	6		M	1	0	0	1	0	0	0	1	0	0	1	0	0	1	0	0	2	0	0	2	0	0	3	0	0	1	0	0	1	0	0	1	0	0	2	0	0
	1	COSMETIC	F	1	0	0	1	0	0	0	1	0	0	1	0	0	1	0	0	2	0	0	2	0	0	3	0	0	1	0	0	1	0	0	1	0	0	2	0	0
	2		F	1	0	0	1	0	0	0	1	0	0	1	0	0	1	0	0	2	0	0	2	0	0	3	0	0	1	0	0	1	0	0	1	0	0	2	0	0
	3		F	1	0	0	1	0	0	0	1	0	0	1	0	0	1	0	0	2	0	0	2	0	0	3	0	0	1	0	0	1	0	0	1	0	0	2	0	0
	4		M	1	0	0	1	0	0	0	1	0	0	1	0	0	1	0	0	2	0	0	2	0	0	3	0	0	1	0	0	1	0	0	1	0	0	2	0	0
	5		M	1	0	0	1	0	0	0	1	0	0	1	0	0	1	0	0	2	0	0	2	0	0	3	0	0	1	0	0	1	0	0	1	0	0	2	0	0
	6		M	1	0	0	1	0	0	0	1	0	0	1	0	0	1	0	0	2	0	0	2	0	0	3	0	0	1	0	0	1	0	0	1	0	0	2	0	0

T0	Beginning of the experiment.
T30	30 days after the beginning of the experiment.
T45	45 days after the beginning of the experiment.
F	Female
M	Male
a	Score value
w	Importance factor
rp	Reaction pattern
I org	Organ index (is the sum of the multiplied importance factors and score values of all changes found within the examined organ).
I org rp	Reaction index of an organ (is the sum of the multiplied importance factors and score values of the alterations of the corresponding reaction patterns).

							Progressive Changes (rp3)																														
							Epithelium						*Lamina Propria*							Inflammation (rp4)										Tumor (rp5)							
Atrophy			Necrosis			I org rp	Hypertrophy			Hyperplasia			Hypertrophy			Hyperplasia			I org rp	Exudate			Activation of RES			Infiltration			I org rp	Benign			Malignant			I org rp	I org
w	a	a x w	w	a	a x w		w	a	a x w	w	a	a x w	w	a	a x w	w	a	a x w		w	a	a x w	w	a	a x w	w	a	a x w		w	a	a x w	w	a	a x w		
2	0	0	3	0	0	0	1	0	0	2	0	0	1	0	0	2	0	0	0	1	0	0	1	0	0	2	0	0	0	2	0	0	3	0	0	0	0
2	0	0	3	0	0	0	1	0	0	2	0	0	1	0	0	2	0	0	0	1	0	0	1	0	0	2	0	0	0	2	0	0	3	0	0	0	0
2	0	0	3	0	0	0	1	0	0	2	0	0	1	0	0	2	0	0	0	1	0	0	1	0	0	2	0	0	0	2	0	0	3	0	0	0	0
2	0	0	3	0	0	0	1	0	0	2	0	0	1	0	0	2	0	0	0	1	0	0	1	0	0	2	0	0	0	2	0	0	3	0	0	0	0
2	0	0	3	0	0	0	1	0	0	2	0	0	1	0	0	2	0	0	0	1	0	0	1	0	0	2	0	0	0	2	0	0	3	0	0	0	0
2	0	0	3	0	0	0	1	0	0	2	0	0	1	0	0	2	0	0	0	1	0	0	1	0	0	2	0	0	0	2	0	0	3	0	0	0	0
2	0	0	3	0	0	0	1	0	0	2	0	0	1	0	0	2	0	0	0	1	0	0	1	0	0	2	0	0	0	2	0	0	3	0	0	0	0
2	0	0	3	0	0	0	1	0	0	2	0	0	1	0	0	2	0	0	0	1	0	0	1	0	0	2	0	0	0	2	0	0	3	0	0	0	0
2	0	0	3	0	0	0	1	0	0	2	0	0	1	0	0	2	0	0	0	1	0	0	1	0	0	2	0	0	0	2	0	0	3	0	0	0	0
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2	0	0	3	0	0	0	1	0	0	2	0	0	1	0	0	2	0	0	0	1	0	0	1	0	0	2	0	0	0	2	0	0	3	0	0	0	0
2	0	0	3	0	0	0	1	0	0	2	0	0	1	0	0	2	0	0	0	1	0	0	1	0	0	2	0	0	0	2	0	0	3	0	0	0	0
2	0	0	3	0	0	0	1	0	0	2	0	0	1	0	0	2	0	0	0	1	0	0	1	0	0	2	0	0	0	2	0	0	3	0	0	0	0
2	0	0	3	0	0	0	1	0	0	2	0	0	1	0	0	2	0	0	0	1	0	0	1	0	0	2	0	0	0	2	0	0	3	0	0	0	0
2	0	0	3	0	0	0	1	0	0	2	0	0	1	0	0	2	0	0	0	1	0	0	1	0	0	2	0	0	0	2	0	0	3	0	0	0	0
2	0	0	3	0	0	0	1	0	0	2	0	0	1	0	0	2	0	0	0	1	0	0	1	0	0	2	0	0	0	2	0	0	3	0	0	0	0
2	0	0	3	0	0	0	1	0	0	2	0	0	1	0	0	2	0	0	0	1	0	0	1	0	0	2	0	0	0	2	0	0	3	0	0	0	0
2	0	0	3	0	0	0	1	0	0	2	0	0	1	0	0	2	0	0	0	1	0	0	1	0	0	2	0	0	0	2	0	0	3	0	0	0	0
2	0	0	3	0	0	0	1	0	0	2	0	0	1	0	0	2	0	0	0	1	0	0	1	0	0	2	0	0	0	2	0	0	3	0	0	0	0
2	0	0	3	0	0	0	1	0	0	2	0	0	1	0	0	2	0	0	0	1	0	0	1	0	0	2	0	0	0	2	0	0	3	0	0	0	0
2	0	0	3	0	0	0	1	0	0	2	0	0	1	0	0	2	0	0	0	1	0	0	1	0	0	2	0	0	0	2	0	0	3	0	0	0	0
2	0	0	3	0	0	0	1	0	0	2	0	0	1	0	0	2	0	0	0	1	0	0	1	0	0	2	0	0	0	2	0	0	3	0	0	0	0
2	0	0	3	0	0	0	1	0	0	2	0	0	1	0	0	2	0	0	0	1	0	0	1	0	0	2	0	0	0	2	0	0	3	0	0	0	0
2	0	0	3	0	0	0	1	0	0	2	0	0	1	0	0	2	0	0	0	1	0	0	1	0	0	2	0	0	0	2	0	0	3	0	0	0	0
2	0	0	3	0	0	0	1	0	0	2	0	0	1	0	0	2	0	0	0	1	0	0	1	0	0	2	0	0	0	2	0	0	3	0	0	0	0
2	0	0	3	0	0	0	1	0	0	2	0	0	1	0	0	2	0	0	0	1	0	0	1	0	0	2	0	0	0	2	0	0	3	0	0	0	0
2	0	0	3	0	0	0	1	0	0	2	0	0	1	0	0	2	0	0	0	1	0	0	1	0	0	2	0	0	0	2	0	0	3	0	0	0	0
2	0	0	3	0	0	0	1	0	0	2	0	0	1	0	0	2	0	0	0	1	0	0	1	0	0	2	0	0	0	2	0	0	3	0	0	0	0
2	0	0	3	0	0	0	1	0	0	2	0	0	1	0	0	2	0	0	0	1	0	0	1	0	0	2	0	0	0	2	0	0	3	0	0	0	0
2	0	0	3	0	0	0	1	0	0	2	0	0	1	0	0	2	0	0	0	1	0	0	1	0	0	2	0	0	0	2	0	0	3	0	0	0	0
2	0	0	3	0	0	0	1	0	0	2	0	0	1	0	0	2	0	0	0	1	0	0	1	0	0	2	0	0	0	2	0	0	3	0	0	0	0
2	0	0	3	0	0	0	1	0	0	2	0	0	1	0	0	2	0	0	0	1	0	0	1	0	0	2	0	0	0	2	0	0	3	0	0	0	0
2	0	0	3	0	0	0	1	0	0	2	0	0	1	0	0	2	0	0	0	1	0	0	1	0	0	2	0	0	0	2	0	0	3	0	0	0	0
2	0	0	3	0	0	0	1	0	0	2	0	0	1	0	0	2	0	0	0	1	0	0	1	0	0	2	0	0	0	2	0	0	3	0	0	0	0
2	0	0	3	0	0	0	1	0	0	2	0	0	1	0	0	2	0	0	0	1	0	0	1	0	0	2	0	0	0	2	0	0	3	0	0	0	0
2	0	0	3	0	0	0	1	0	0	2	0	0	1	0	0	2	0	0	0	1	0	0	1	0	0	2	0	0	0	2	0	0	3	0	0	0	0
2	0	0	3	0	0	0	1	0	0	2	0	0	1	0	0	2	0	0	0	1	0	0	1	0	0	2	0	0	0	2	0	0	3	0	0	0	0
2	0	0	3	0	0	0	1	0	0	2	0	0	1	0	0	2	0	0	0	1	0	0	1	0	0	2	0	0	0	2	0	0	3	0	0	0	0
2	0	0	3	0	0	0	1	0	0	2	0	0	1	0	0	2	0	0	0	1	0	0	1	0	0	2	0	0	0	2	0	0	3	0	0	0	0
2	0	0	3	0	0	0	1	0	0	2	0	0	1	0	0	2	0	0	0	1	0	0	1	0	0	2	0	0	0	2	0	0	3	0	0	0	0
2	0	0	3	0	0	0	1	0	0	2	0	0	1	0	0	2	0	0	0	1	0	0	1	0	0	2	0	0	0	2	0	0	3	0	0	0	0
2	0	0	3	0	0	0	1	0	0	2	0	0	1	0	0	2	0	0	0	1	0	0	1	0	0	2	0	0	0	2	0	0	3	0	0	0	0
2	0	0	3	0	0	0	1	0	0	2	0	0	1	0	0	2	0	0	0	1	0	0	1	0	0	2	0	0	0	2	0	0	3	0	0	0	0
2	0	0	3	0	0	0	1	0	0	2	0	0	1	0	0	2	0	0	0	1	0	0	1	0	0	2	0	0	0	2	0	0	3	0	0	0	0
2	0	0	3	0	0	0	1	0	0	2	0	0	1	0	0	2	0	0	0	1	0	0	1	0	0	2	0	0	0	2	0	0	3	0	0	0	0
2	0	0	3	0	0	0	1	0	0	2	0	0	1	0	0	2	0	0	0	1	0	0	1	0	0	2	0	0	0	2	0	0	3	0	0	0	0

Supplementary Table S2. Histopathological assessment tools for liver

	D. rerio	Test Group	Sex	Circulatory Disturbances (rp1): Haemorrhage / hyperaemia / aneurysm w	a	a x w	Intercelular oedema w	a	a x w	I org rp	Liver tissue: Architectural and strucutral alterations w	a	a x w	Plasma alterations w	a	a x w	Deposits w	a	a x w	Decreased hepatocellular vacuolation w	a	a x w	Nuclear alterations w	a	a x w	Atrophy w	a	a x w	Necrosis w	a	a x w	Degree of vacuolation	Regressive changes (rp2) — Interstitial tissue: Architectural and strucutral alterations w	a	a x w	Plasma alterations w	a	a x w	Deposits w	a	a x w	Nuclear alterations w	a	a x w	Atrophy w	a	a x w	Necrosis w	a	a x w
T0	1	CONTROL	F	1	0	0	1	0	0	0	1	0	0	1	0	0	1	0	0	1	0	0	2	0	0	2	0	0	3	0	0	3	1	0	0	1	0	0	1	0	0	2	0	0	2	0	0	3	0	0
	2		F	1	0	0	1	0	0	0	1	0	0	1	0	0	1	0	0	1	0	0	2	0	0	2	0	0	3	0	0	3	1	0	0	1	0	0	1	0	0	2	0	0	2	0	0	3	0	0
	3		M	1	0	0	1	0	0	0	1	0	0	1	0	0	1	0	0	1	0	0	2	0	0	2	0	0	3	0	0	3	1	0	0	1	0	0	1	0	0	2	0	0	2	0	0	3	0	0
	4		M	1	0	0	1	0	0	0	1	0	0	1	0	0	1	0	0	1	0	0	2	0	0	2	0	0	3	0	0	3	1	0	0	1	0	0	1	0	0	2	0	0	2	0	0	3	0	0
T30	1	FLUORESCENT	F	1	0	0	1	0	0	0	1	0	0	1	0	0	1	2	2	2	0	0	2	0	0	2	0	0	3	0	0	1	1	0	0	1	0	0	1	0	0	2	0	0	2	0	0	3	0	0
	2		F	1	0	0	1	0	0	0	1	0	0	1	2	2	1	0	0	1	0	0	2	0	0	2	0	0	3	0	0	4	1	0	0	1	0	0	1	0	0	2	0	0	2	0	0	3	0	0
	3		F	1	0	0	1	0	0	0	1	0	0	1	0	0	1	0	0	1	0	0	2	0	0	2	0	0	3	0	0	3	1	0	0	1	0	0	1	0	0	2	0	0	2	0	0	3	0	0
	4		M	1	0	0	1	0	0	0	1	0	0	1	0	0	1	0	0	1	0	0	2	0	0	2	0	0	3	0	0	3	1	0	0	1	0	0	1	0	0	2	0	0	2	0	0	3	0	0
	5		M	1	0	0	1	0	0	0	1	0	0	1	0	0	1	0	0	1	0	0	2	0	0	2	0	0	3	0	0	3	1	0	0	1	0	0	1	0	0	2	0	0	2	0	0	3	0	0
	6		M	1	0	0	1	0	0	0	1	0	0	1	0	0	1	0	0	1	0	0	2	0	0	2	0	0	3	0	0	3	1	0	0	1	0	0	1	0	0	2	0	0	2	0	0	3	0	0
	1	COSMETIC	F	1	0	0	1	0	0	0	1	0	0	1	2	2	1	0	0	1	0	0	2	0	0	2	0	0	3	0	0	4	1	0	0	1	0	0	1	0	0	2	0	0	2	0	0	3	0	0
	2		F	1	0	0	1	0	0	0	1	0	0	1	2	2	1	0	0	1	0	0	2	0	0	2	0	0	3	0	0	4	1	0	0	1	0	0	1	0	0	2	0	0	2	0	0	3	0	0
	3		F	1	0	0	1	0	0	0	1	0	0	1	2	2	1	0	0	1	0	0	2	0	0	2	0	0	3	0	0	4	1	0	0	1	0	0	1	0	0	2	0	0	2	0	0	3	0	0
	4		M	1	0	0	1	0	0	0	1	0	0	1	0	0	1	0	0	1	0	0	2	0	0	2	0	0	3	0	0	3	1	0	0	1	0	0	1	0	0	2	0	0	2	0	0	3	0	0
	5		M	1	0	0	1	0	0	0	1	0	0	1	0	0	1	0	0	1	0	0	2	0	0	2	0	0	3	0	0	3	1	0	0	1	0	0	1	0	0	2	0	0	2	0	0	3	0	0
	6		M	1	0	0	1	0	0	0	1	0	0	1	0	0	1	0	0	1	0	0	2	0	0	2	0	0	3	0	0	3	1	0	0	1	0	0	1	0	0	2	0	0	2	0	0	3	0	0
	1	CONTROL	F	1	0	0	1	0	0	0	1	0	0	1	0	0	1	0	0	1	0	0	2	0	0	2	0	0	3	0	0	3	1	0	0	1	0	0	1	0	0	2	0	0	2	0	0	3	0	0
	2		F	1	0	0	1	0	0	0	1	0	0	1	0	0	1	0	0	1	0	0	2	0	0	2	0	0	3	0	0	3	1	0	0	1	0	0	1	0	0	2	0	0	2	0	0	3	0	0
	3		M	1	0	0	1	0	0	0	1	0	0	1	0	0	1	0	0	1	0	0	2	0	0	2	0	0	3	0	0	3	1	0	0	1	0	0	1	0	0	2	0	0	2	0	0	3	0	0
	4		M	1	0	0	1	0	0	0	1	0	0	1	0	0	1	0	0	1	0	0	2	0	0	2	0	0	3	0	0	3	1	0	0	1	0	0	1	0	0	2	0	0	2	0	0	3	0	0
T45	1	FLUORESCENT	F	1	0	0	1	0	0	0	1	0	0	1	0	0	1	0	0	1	0	0	2	0	0	2	0	0	3	0	0	3	1	0	0	1	0	0	1	0	0	2	0	0	2	0	0	3	0	0
	2		F	1	0	0	1	0	0	0	1	0	0	1	0	0	1	1	1	2	0	0	2	0	0	3	0	0	2	1	0	0	1	0	0	1	0	0	2	0	0	2	0	0	3	0	0	3	0	0
	3		F	1	0	0	1	0	0	0	1	0	0	1	0	0	1	0	0	1	0	0	2	0	0	2	0	0	3	0	0	3	1	0	0	1	0	0	1	0	0	2	0	0	2	0	0	3	0	0
	4		M	1	0	0	1	0	0	0	1	0	0	1	0	0	1	0	0	1	0	0	2	0	0	2	0	0	3	0	0	3	1	0	0	1	0	0	1	0	0	2	0	0	2	0	0	3	0	0
	5		M	1	0	0	1	0	0	0	1	0	0	1	0	0	1	0	0	1	0	0	2	0	0	2	0	0	3	0	0	3	1	0	0	1	0	0	1	0	0	2	0	0	2	0	0	3	0	0
	6		M	1	0	0	1	0	0	0	1	0	0	1	0	0	1	0	0	1	0	0	2	0	0	2	0	0	3	0	0	3	1	0	0	1	0	0	1	0	0	2	0	0	2	0	0	3	0	0
	1	COSMETIC	F	1	0	0	1	0	0	0	1	0	0	1	0	0	1	0	0	1	0	0	2	0	0	2	0	0	3	0	0	3	1	0	0	1	0	0	1	0	0	2	0	0	2	0	0	3	0	0
	2		F	1	0	0	1	0	0	0	1	0	0	1	0	0	1	0	0	1	0	0	2	0	0	2	0	0	3	0	0	3	1	0	0	1	0	0	1	0	0	2	0	0	2	0	0	3	0	0
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	4		M	1	0	0	1	0	0	0	1	0	0	1	0	0	1	0	0	1	0	0	2	0	0	2	0	0	3	0	0	3	1	0	0	1	0	0	1	0	0	2	0	0	2	0	0	3	0	0
	5		M	1	0	0	1	0	0	0	1	0	0	1	0	0	1	0	0	1	0	0	2	0	0	2	0	0	3	0	0	3	1	0	0	1	0	0	1	0	0	2	0	0	2	0	0	3	0	0
	6		M	1	0	0	1	0	0	0	1	0	0	1	0	0	1	0	0	1	0	0	2	0	0	2	0	0	3	0	0	3	1	0	0	1	0	0	1	0	0	2	0	0	2	0	0	3	0	0
	1	CONTROL	F	1	0	0	1	0	0	0	1	0	0	1	0	0	1	0	0	1	0	0	2	0	0	2	0	0	3	0	0	3	1	0	0	1	0	0	1	0	0	2	0	0	2	0	0	3	0	0
	2		F	1	0	0	1	0	0	0	1	0	0	1	0	0	1	0	0	1	0	0	2	0	0	2	0	0	3	0	0	3	1	0	0	1	0	0	1	0	0	2	0	0	2	0	0	3	0	0
	3		M	1	0	0	1	0	0	0	1	0	0	1	0	0	1	0	0	1	0	0	2	0	0	2	0	0	3	0	0	3	1	0	0	1	0	0	1	0	0	2	0	0	2	0	0	3	0	0
	4		M	1	0	0	1	0	0	0	1	0	0	1	0	0	1	0	0	1	0	0	2	0	0	2	0	0	3	0	0	3	1	0	0	1	0	0	1	0	0	2	0	0	2	0	0	3	0	0
DEPURATION	1	FLUORESCENT	F	1	0	0	1	0	0	0	1	0	0	1	0	0	1	0	0	1	1	1	2	0	0	2	0	0	3	0	0	2	1	0	0	1	0	0	1	0	0	2	0	0	2	0	0	3	0	0
	2		F	1	0	0	1	0	0	0	1	0	0	1	0	0	1	0	0	1	0	0	2	0	0	2	0	0	3	0	0	3	1	0	0	1	0	0	1	0	0	2	0	0	2	0	0	3	0	0
	3		F	1	0	0	1	0	0	0	1	0	0	1	2	2	1	0	0	1	0	0	2	0	0	2	0	0	3	0	0	4	1	0	0	1	0	0	1	0	0	2	0	0	2	0	0	3	0	0
	4		M	1	0	0	1	0	0	0	1	0	0	1	0	0	1	0	0	1	1	1	2	0	0	2	0	0	3	0	0	2	1	0	0	1	0	0	1	0	0	2	0	0	2	0	0	3	0	0
	5		M	1	0	0	1	0	0	0	1	0	0	1	0	0	1	0	0	1	1	1	2	0	0	2	0	0	3	0	0	2	1	0	0	1	0	0	1	0	0	2	0	0	2	0	0	3	0	0
	6		M	1	0	0	1	0	0	0	1	0	0	1	0	0	1	0	0	1	0	0	2	0	0	2	0	0	3	0	0	3	1	0	0	1	0	0	1	0	0	2	0	0	2	0	0	3	0	0
	1	COSMETIC	F	1	0	0	1	0	0	0	1	0	0	1	0	0	1	0	0	1	0	0	2	0	0	2	0	0	3	0	0	3	1	0	0	1	0	0	1	0	0	2	0	0	2	0	0	3	0	0
	2		F	1	0	0	1	0	0	0	1	0	0	1	0	0	1	1	1	2	0	0	2	0	0	3	0	0	2	1	0	0	1	0	0	1	0	0	2	0	0	2	0	0	3	0	0	3	0	0
	3		F	1	0	0	1	0	0	0	1	0	0	1	0	0	1	0	0	1	0	0	2	0	0	2	0	0	3	0	0	3	1	0	0	1	0	0	1	0	0	2	0	0	2	0	0	3	0	0
	4		M	1	0	0	1	0	0	0	1	0	0	1	0	0	1	2	2	2	0	0	2	0	0	3	0	0	1	1	0	0	1	0	0	1	0	0	2	0	0	2	0	0	3	0	0	3	0	0
	5		M	1	0	0	1	0	0	0	1	0	0	1	0	0	1	0	0	1	2	2	2	0	0	2	0	0	3	0	0	1	1	0	0	1	0	0	1	0	0	2	0	0	2	0	0	3	0	0
	6		M	1	0	0	1	0	0	0	1	0	0	1	0	0	1	0	0	1	1	1	2	0	0	2	0	0	3	0	0	2	1	0	0	1	0	0	1	0	0	2	0	0	2	0	0	3	0	0

T0	Beginning of the experiment.
T30	30 days after the beginning of the experiment.
T45	45 days after the beginning of the experiment.
F	Female
M	Male
a	Score value
w	Importance factor
rp	Reaction pattern
I org	Organ index (is the sum of the multiplied importance factors and score values of all changes found within the examined organ).
I org rp	Reaction index of an organ (is the sum of the multiplied importance factors and score values of the alterations of the corresponding reaction patterns).

																			Progressive Changes (rp3)																			Inflammation (rp4)										Tumor (rp5)							I org
Bile Duct																		I org rp	*Liver tissue*						*Interstitial tissue*						*Bile Duct*						I org rp																		
Architectural and strucutral alterations			Plasma alterations			Deposits			Nuclear alterations			Atrophy			Necrosis				Hypertrophy			Hyperplasia			Hypertrophy			Hyperplasia			Hypertrophy			Hyperplasia				Exudate			Activation of RES			Infiltration			I org rp	Benign			Malignant			I org rp	
w	a	a x w	w	a	a x w	w	a	a x w	w	a	a x w	w	a	a x w	w	a	a x w		w	a	a x w	w	a	a x w	w	a	a x w	w	a	a x w	w	a	a x w	w	a	a x w		w	a	a x w	w	a	a x w	w	a	a x w		w	a	a x w	w	a	a x w		
1	0	0	1	0	0	1	0	0	2	0	0	2	0	0	3	0	0	0	1	0	0	2	0	0	1	0	0	2	0	0	1	0	0	2	0	0	0	1	0	0	1	0	0	2	0	0	0	2	0	0	3	0	0	0	0
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1	0	0	1	0	0	1	0	0	2	0	0	2	0	0	3	0	0	0	1	0	0	2	0	0	1	0	0	2	0	0	1	0	0	2	0	0	0	1	0	0	1	0	0	2	0	0	0	2	0	0	3	0	0	0	1
1	0	0	1	0	0	1	0	0	2	0	0	2	0	0	3	0	0	0	1	0	0	2	0	0	1	0	0	2	0	0	1	0	0	2	0	0	0	1	0	0	1	0	0	2	0	0	0	2	0	0	3	0	0	0	0
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M. Carolina de Sales Ribeiro

A Pathological Study of the Potential Threats to Fish Health in the Canary Islands

M. Carolina de Sales Ribeiro

Klee, 1926