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Valutazione dell'impiego di astaxantina nell'alimentazione di zebrafish (*Danio rerio)* per mitigare gli effetti negative delle microplastiche incluse nella dieta

Evaluation of the use of astaxanthin in zebrafish (*Danio rerio*) feeds to mitigate the negative effects of microplastics included in the diet

Tesi di Laurea Magistrale

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RIASSUNTO

L'inquinamento da plastiche oggigiorno rappresenta un grave problema di natura antropica che minaccia la salute dell'ecosistema e degli organismi che ne fanno parte. L'accumulo continuo e senza precedenti di rifiuti della plastica negli ecosistemi acquatici da parte dell'uomo causa il deterioramento dei servizi ecosistemici con conseguente danno agli organismi e all' uomo stesso. Le microplastiche sono molto pericolose per il benessere degli animali e per la salute umana, in quanto possono entrare nella rete trofica per le loro piccole dimensioni e causare effetti negativi come danni fisici, disturbi del metabolismo, inibizione della crescita, stress ossidativo e genotossicità. Questa problematica è anche presente nell''acquacoltura, uno dei settori principali per l'alimentazione umana stimata a diventare una delle principali fonti di proteine per l'uomo entro il 2030. La contaminazione in acquacoltura da parte delle MP sta diventando sempre più comune, si stima che l'uomo ingerisca da 1 a 30 particelle al giorno a seconda del consumo di pesce. Una delle principali fonti di contaminazione di MP in acquacultura proviene direttamente dai mangimi consumati dagli organismi allevati. Inoltre, la farina di pesce, che è uno dei principali componenti dei mangimi per pesci, deriva direttamente da ambienti di pesca sempre più contaminati dalla presenza di queste particelle. Le MP possono influenzare la qualità e la sicurezza del prodotto ittico d'allevamento, mettendo a rischio la salute non solo del pesce ma anche del consumatore. Dato che non è possibile trovare una soluzione a breve termine per eliminare le MP presenti in mare e nei mangimi, l'utilizzo di molecole bioattive e antiossidanti come l'astaxantina, potrebbe mitigare l'effetto negativo causato dalle MP sul benessere dei pesci allevati.

In questo contesto, l'obiettivo del seguente progetto è stato quello di: formulare diete sperimentali caratterizzate dall'inclusione di due diversi polimeri fluorescenti, polimero A di dimensioni 1-5 µm e polimero B di dimensioni 40-47 µm, a due diverse concentrazioni (50 mg/kg e 500 mg/kg, che rappresentano rispettivamente una concentrazione realistica e una 10 volte superiore per intensificare i possibili effetti); esporre l'organismo modello zebrafish (*Danio rerio*) alle diete contaminate da MPs per un periodo di alimentazione di 6 mesi (dallo stadio di larva a quello di adulto); studiare il possibile effetto benefico di un'esposizione di 1 mese alle diete precedenti con l'inclusione di ASX (7 g/kg) per verificare se la sua aggiunta apporta miglioramenti al benessere degli zebrafish esposti alle MPs.

Al termine del periodo sperimentale (7 mesi), un approccio multidisciplinare che comprende analisi biometriche, istologiche, molecolari, confocali e di microscopia a fluorescenza, ha permesso di valutare gli effetti delle MP sulla crescita, benessere e accumulo delle stesse in relazione alla loro dimensione e concentrazione, nonché i possibili effetti migliorativi dovuti all'inclusione di ASX.

I risultati ottenuti hanno mostrato che le diete sperimentali non hanno influenzato la crescita e la sopravvivenza dei pesci in ogni gruppo. Si è visto che il polimero B non veniva assorbito a livello intestinale ma semplicemente transitava lungo il lume,

causando però un effetto abrasivo sulle pliche intestinali determinato da una riduzione nell'altezza di esse e un aumento nel numero di cellule mucipare di goblet. Nei gruppi a cui è stata somministrata l'ASX però, non è stata riscontrata alcuna diminuzione nell' altezza delle pliche, ma solo un leggero aumento nel numero di cellule di goblet; questo risultato va sostenere l'effetto benefico dell'ASX sulla salute intestinale.

Per quanto riguarda il polimero A, si è visto che può essere assorbito nell' intestino, traslocare nel fegato e successivamente nel muscolo. Le analisi quantitative hanno mostrato come il fegato possa accumulare le MP rappresentando una barriera biologica contro la diffusione di queste particelle verso il muscolo, la parte edibile del pesce. Questo accumulo però ha comportato un aumento nell' espressione dei geni coinvolti nello stress ossidativo. I gruppi nutriti con polimero A ad una minore concentrazione e l'aggiunta di astaxantina hanno permesso di notare livelli più bassi nell' espressione dei geni legati allo stress ossidativo e una diminuizione della quantità di MP presente nei vari organi analizzati, rispetto ai gruppi, nutriti senza ASX. Tuttavia, il gruppo a cui era stata somministrata la dieta con il polimero A ad una maggiore concentrazione e ASX, presenta quantità di MP non ancora sufficientemente basse per avere una riduzione nell' espressione dei geni dello stress ossidativo. Questo risultato è però molto applicativo in quanto la concentrazione più grande di MP utilizzata è una irreale, usata per amplificare gli effetti negativi delle microplastiche.

Dal presente studio quindi si può evincere come l'astaxantina possa essere una soluzione da impiegare nel settore dell'aquacultura per mitigare gli effetti negativi delle microplastiche presenti nei mangimi dei pesci sul benessere animale.

SUMMARY

1. INTRODUCTION

1.1 MPs in marine environment

Plastic pollution is a worldwide problem, and it represents a threat to our planet (Andrady, 2017; Bajt, 2021; Bhuyan, 2022). According to IUPAC (International Union of Pure and Applied Chemistry), plastics are defined as "polymeric materials that may contain other substances aimed at improving their properties and/or reducing their cost". Plastics are crafted by humans in laboratories, they are composites of organic long chain polymers produced by combing chemical monomers often derived from fossil fuels or sometimes biodegradable molecules such as cellulose and poly lactic acid (PLA) (Haider et al., 2019; Mulder, 1998). Finished products may be homogeneous in terms of constituent polymer or contain different types to achieve the desired characteristics (Hale et al., 2020). This type of material began to establish itself in the second half of the nineteenth century when Hyatt brothers from U.S.A. replaced the ivory used to make billiard balls with nitrate cellulose (Rasmussen, 2021). Plastic material has the behaviour to be long-lasting, resistant to degradation, inert, and easy to shape, with very low production costs so, due to its versatility, it began to be used in the manufacture of common objects replacing many naturally derived materials like wood, paper, leather, and glass (Lebreton and Andrady, 2019). From the twentieth century, it has revolutionized the world of industry because it can be moulded into any shape, colour, size, thickness and texture to create very competitive objects (Bajt, 2021). Some of its application in modern society could be single-use food and beverage containers, thermal insulation, home and workplace

furnishings, electrical and electronic devices covertures, vehicle interiors, toys, textiles, surface coatings, and even medical devices (e.g., artificial joints, incubators, intravenous fluid bags, and drug delivery devices). For these reasons, plastics materials have become an indispensable element in everyday life and society became increasingly dependent on them (Andrady and Neal, 2009; Hale et al., 2020).

Despite the countless perks and applications from the industrial, health to the food sector, the disadvantage of using this material is its accumulation directly in the environment with various pollution effects observed in all terrestrial, marine and freshwater ecosystems (Wagner, 2017; Zeng, 2018). Indeed, nowadays, plastics are widespread and ubiquitous in the environment to such an extent that they are used as stratigraphic markers for paleontological dating of sediments to highlight the current geological era, known as the Anthropocene (Zalasiewicz et al., 2016). According to Juan and Martínez (2021), polyethylene (PE) polymer is the most prevalent in the global environment, followed by polypropylene (PP), polyvinylchloride (PVC), polyurethane, polyethylene terephthalate (PET), and polystyrene (PS). The aquatic environment is greatly affected by this type of pollution; Jambeck et al. (2015) suggested that at least 8 million tons of plastic debris per year enter the ocean and they prospected that these inputs would increase ten-fold by 2025. Furthermore, an estimated value of five trillion plastic items has been reported to pollute the world's oceans, consequently posing threat to different aquatic ecosystems (Borrelle et al., 2017; Eriksen et al., 2014; Europe, 2020; Villarrubia-Gómez et al., 2018). Land-based plastic waste is the main source of plastics in the aquatic environment (Lebreton et al., 2017; Schmidt et al., 2017). These wastes come from terrestrial human activities, such as solid or liquid waste discharges into surface waters, leakage or improper disposal of plastic products, excess packaging, and industrial waste (Schreder and La

Guardia, 2014). It is estimated that 79% of plastic waste is released into the environment, 12% is incinerated, and only 9% is recycled (Geyer et al., 2017). Plastics can reach the oceans through waterways like rainwater and rivers that can carry plastic waste accumulated in urban and coastal areas to the sea, promoting large-scale dispersal(Liu et al., 2019; Ma et al., 2020). Lebreton et al. (2017) estimated that 1.15 to 2.41 million tons of plastics are released to the oceans through river-fed estuaries each year. Overall, the transport of plastic debris from land to oceans is a complex process and affected by a variety of factors, including human population density, urbanization, per capita income, hydrological conditions waste management infrastructure, and living standards (Hale et al., 2020). Also, maritime activity, such as shipping and fishing, can contribute to the accumulation of plastics in the ocean (Deshpande et al., 2020). The direct release of plastic materials from ships, such as packaging, consumer items and fishing equipment, can increase the plastic load in marine ecosystems (Laist, 1997). Lost fishing gear in the sea leads to a phenomenon called "ghost fishing" that can negatively affect the biota for years and it is estimated that > 6 million tons of them are lost annually (Wilcox et al., 2015). These gears smother habitats, continue to fish and trap animals, entangle them, and potentially kill marine life (Gilman, 2015). According to Maximenko and Niiler (2008), the five ocean gyres (North Pacific, South Pacific, North Atlantic, South Atlantic, and Indian gyres) are hot spots of plastic accumulation representing the major destinations of marine debris. The "Great Pacific Garbage Patch", for example, is an area of floating trash twice the size of Texas in the Pacific Ocean (Lebreton et al., 2018).

When introduced into the marine environment, buoyant plastic (less dense than marine water), which represents around 60% of the plastic produced (Andrady, 2011), can be transported by surface currents and winds, recaptured by coastlines, degraded into smaller

pieces by the action of sun, temperature variations, waves and marine life (Lebreton et al., 2017). These include low-density polymers used in single-use containers such as polyethylene and polypropylene. However, many other polymers like terephthalate, polycarbonate, and polyvinyl chloride are denser than water and thus are expected to sink. But the fate of both types of plastic is to leave the sea surface (Ballent et al., 2013; Van Sebille et al., 2020) through beaching (Turner and Holmes, 2011) or sinking by biofouling (Chubarenko et al., 2016; Erni-Cassola et al., 2019; Kooi et al., 2018; Ye and Andrady, 1991). Infact, only a very small percentage, around 1%, remains on the surface (Choy et al., 2019; Van Sebille et al., 2015) . At the end they reach the benthic compartment where, low or no light penetration and low temperature conditions promote the resistance of plastics to degradation, allowing them to remain in the environment for a long time (Bajt, 2021; Galgani et al., 2015). Environmental half-lives of plastics vary by polymer type and ambient conditions but range from days to centuries (Ward et al., 2019), for example, a plastic bottle can remain in the environment for up to 400 years before it degrades completely (Barnes et al., 2009). Due to chemical, physical and biological processes such as exposure to UV light, hydrolysis, wave action and currents, resuspension phenomena and microbial activity, macroplastics are subject to fragmentation and degradation (Barnes et al., 2009; De La Fuente et al., 2021) leading to the formation of smaller particles termed microplastics (MPs). It is widely accepted that plastic items smaller than 5 mm are termed MPs (Canning-Clode et al., 2020; IMO/FAO/UNESCO-IOC/UNIDO/WMO/IAEA/UN/UNEP/UNDP/ISA, 2019; Thompson et al., 2004), although there is still no globally common definition for the lower limit (Frias and Nash, 2019; Van Cauwenberghe et al., 2015). MPs can be classified into primary and secondary ones (Cole et al., 2016): primary MPs are those manufactured originally in small size,

like microbeads for cosmetics products and industrial abrasives (Fendall and Sewell, 2009; Gregory, 1996); secondary MPs, far more abundant than primary ones, are formed by the breakdown of larger plastic particles through weathering processes. MPs particles are among the most important contributors to marine plastic pollution (Andrady, 2011), they are found in diverse forms, such as spheres, fragments, and fibres. MPs can be fragmented into ever-smaller debris over time, eventually becoming nanoplastics <1 μm (Hartmann et al., 2019; Lambert and Wagner, 2016). Dris et al. (2016) calculated atmospheric deposition of synthetic fibres on the city of Paris, which amount is from 3 to 10 metric tons each year, thereby suggesting potential human exposure. Moreover, their small size and relatively low density contribute to their long-range transport (Barboza et al., 2019; Cózar et al., 2017) and global distribution (Auta et al., 2017; Cózar et al., 2014). Studies suggested that some MPs may be transported thousands of kilometres through the action of sea currents and wind; they have also been discovered in presumably pristine areas, including Arctic Sea ice (Peeken et al., 2018), the Antarctic (Waller et al., 2017), and deep ocean trenches (Jamieson et al., 2019). Moore et al. found that, trawling the water's surface with 333 μm mesh nets, the mass of microplastic in samples from the North Pacific gyre was six times that of coincident plankton.

MPs also represent a great concern for biodiversity and organisms' health: studies show that more than 80% of reported incidents between marine debris and organisms were associated with plastic, while 11% of all investigated encounters are with MPs (Wright et al., 2013). Ingestion of MPs by aquatic biota has been widely surveyed (Cauwenberghe and Janssen, 2014; Lusher et al., 2013; Possatto et al., 2011). Some studies have recovered the high amounts of MPs in zooplankton of the lower trophic levels (Amin et al., 2020; Botterell et al., 2020b). The MPs ingested by zooplankton could be transferred to other

organisms of the trophic chain, including the filter-feeding and carnivorous fish (Auta et al., 2017; Botterell et al., 2020a; Vroom et al., 2017). Furthermore, since MPs are similar in size, shape, and colour to planktonic organisms (Botterell et al., 2020b), they are mistakenly ingested by invertebrates and fish in the marine environment thus entering the food web. Organisms can bioaccumulate MPs (when the uptake is greater than the ability of an organism to egest the contaminant) (Wang et al., 2016) and consequently transfer them to higher trophic levels in a phenomenon called biomagnification (Kelly et al., 2007). Nowadays, MPs have been found in the marine organisms over 233 species worldwide, including many of commercial interest that are consumed by human as food such as fish, bivalves, and crustaceans (Avio et al., 2015; Bellas et al., 2016; Cauwenberghe and Janssen, 2014; Lusher et al., 2013). The occurrence of MPs in some edible fish species (*Mullus barbatus* and *Merluccius merluccius*) has been measured in three different geographical subareas of the Mediterranean Sea (Giani et al., 2019) in which plastic fragments were detected in 23.3% of all fish, with fibres as the main source. A study performed at the Northern and Central Adriatic Sea on the benthic flatfish *Solea solea* recorded the presence of MPs in 95% of the sampled fish, with more than one MPs item found in around 80% of the examined specimens (Grati et al., 2018). MPs in the sea can also act as a vector of other pollutants (such as styrene, toxic metals, phthalates, bisphenol A - BPA, polychlorinated biphenyls - PCB, polycyclic aromatic hydrocarbons - PAHs, pathogens, and alien species) (Barnes et al., 2009; Galloway and Lewis, 2016; Wright and Kelly, 2017). All these evidence indicate that in the environment, especially in areas with high anthropogenic impact, marine organisms populations may be negatively affected by plastic pollution and they could decrease over time with potentially

adverse consequences for environmental health, biodiversity conservation, ecosystem services, human food security, and reduced food availability for the human population.

1.2 MPs in aquaculture

Consumption of aquatic products is an attribute of many socio-cultural group of people because of its excellent nutritional profile and numerous health benefits (Chen et al., 2020). Aquatic products are rich sources of protein, essential fatty acids, and other essential micronutrients (Iheanacho et al., 2023). Fisheries and aquaculture sectors have been increasingly recognized for their essential contribution to global food security and nutrition in the twenty-first century (FAO 2018). According to FAO total fisheries and aquaculture production reached a record of 214 million tonnes in 2020, comprising 178 million tonnes of aquatic animals and 36 million tonnes of algae. Due to the rapid expansion of the global population, aquaculture is becoming an increasingly important approach for supplying market demand. In 2019, aquaculture provided 52 % of fish production for human consumption with a value of 250 billion USD (FAO, 2020). Aquaculture can be used to farm a variety of species, including macroalgae, crustaceans and molluscs, although finfish dominates global production contributing >54.3 million tonnes of food worth 139.7 billion USD (FAO, 2020). Aquaculture is a fast-growing sector, and FAO 2022 data indicate that it will account for 50% of world fish production. In addition, the sudden increase in demand for marine products and the inability of the only fishing industry to meet this demand, are contributing to the expansion of aquaculture sector (Merino et al., 2012). Growth forecasts according to the FAO will lead the aquaculture sector to match the productivity of the fisheries sector as early as 2030.

The steady growth of aquaculture has led the scientific community to become interested in the problems associated with it, like the MPs one (Chen et al., 2018; Mathalon and Hill, 2014; Oliveira et al., 2016). In fact, besides to fished fish species, the presence of this pollutant is documented extensively in farmed species (Vázquez-rowe et al., 2021).

The presence of MPs in mariculture is widely documented (Chen et al., 2018; Rasyid, 2019): 16.4/m^3 of microplastics were found in the mussel-farming region in Jurujuba Cove, while the total average abundance of MPs in Xiangshan Bay was 8.9 ± 4.7 items/ m^3 in seawater (Castro et al., 2016; Chen et al., 2018). Moreover, land-based aquaculture can be also subjected to MPs pollution: one of the main sources of contamination may be the water used for the aquaculture facilities, which depend on groundwater, rivers and lakes, springs, coastal and marine waters, treated rainwater and wastewater from urban, agricultural and industrial sources (Lebel and Chuah, 2019). Other sources are represented by aquaculture facilities and gears like aerators, water pumps, electrical cables, and others (Mnyoro et al., 2021). In addition, also biofilters and some components of recirculating aquaculture systems (RAS) are made of plastic materials that can be degraded and form MPs fragment (Lopardo and Urakawa, 2019). Personal protective equipment (PPEs) such as nose cum face masks, hand gloves, and aprons are made of plastic polymers which can contribute to the volume of MPs detected in aquaculture facilities (Iheanacho et al., 2023). Finally, feed is another important source of MPs in the aquaculture system and MPs can be introduced into it during its production and packaging processes (Wright et al. 2013; Van Cauwenberghe et al. 2015). Just like every other cultured animal, formulated diets are fed to cultured specimens especially under semi-intensive or intensive systems to support growth and survival. These feeds are compounded using fish meal as one of the main components (Boyd et al., 2022). For fish

meal, feedstock derives from targeted capture of small marine fish such as Peruvian anchoveta (*Engraulis ringens),* Pacific sardine (*Sardinops sagax*), and Atlantic herring (*Clupea harengus*), by-catch, and by-products (i.e. offal, trimmings) from the processing of larger commercial fish species (Cashion and Manach, 2017). A global study on MPs investigation in fish meal products showed that MPs were detected in fish meal samples from ten countries, with the highest levels (5.5 ± 1.6 items/g) detected in China, Peru, and Myanmar (Wang et al., 2022). Finally, fish medicines and animal protection products are another important source of MPs in the aquaculture environment (Han et al., 2018). All these evidences demonstrate the presene of MPs in aquaculture systems and how they reach them; the issue is that MPs can stress reared organisms and be the cause of a wide range of negative effects (Fig. 1).

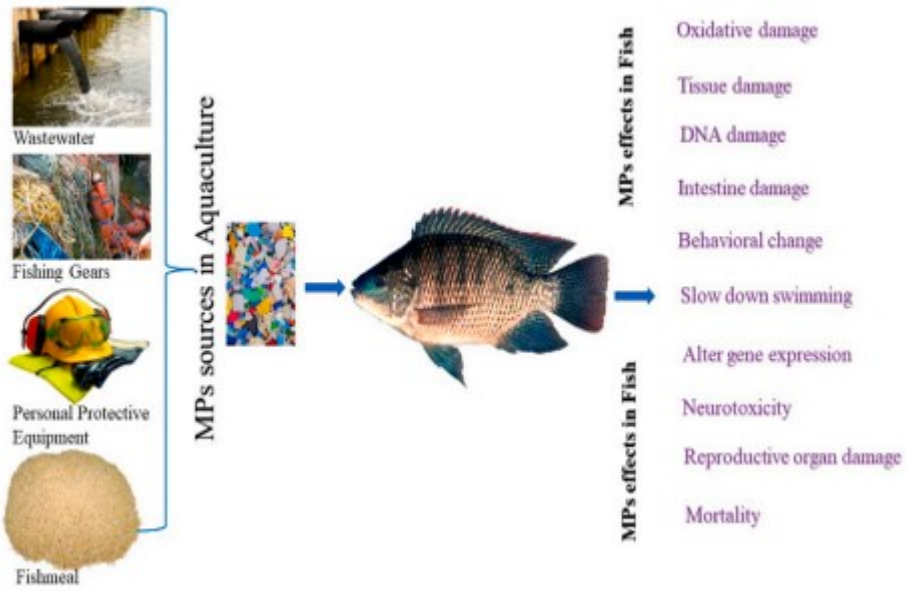

Fig. 1: Sources of MPs in aquacuture and studied effect in raered fish (Iheanacho et al., 2023)

1.3 MPs and reared fish welfare

Due to the rapid expansion of the aquaculture sector in the last decades, welfare of farmed fish species has received greater attention. For animal welfare the ability of an animal to adapt to its environment and remain in good health, live a natural life, and express its natural behaviour is intended (Ashley, 2007). It is important to guarantee this condition because it will affect the quantity and quality of final product, and to ensure an ethic value to the aquaculture practice that unfortunately is not always respected, due to the increase of the global demand. Many studies reveal that MPs can directly harm the health of farmed animals through the ingestion of the particles, or indirectly by feeding on contaminated prey (Gabriel et al., 2020; Jeong and Choi, 2019; Zhou et al., 2022).

The effects of MPs can be classified as physical, chemical, and biological (Iheanacho et al.,2023). Physical effects depend on the size and shape of the particle (GESAMP 2015). Once ingested MPs can cause obstruction of the gastrointestinal tract with consequent inflammatory responses. Such blockages can lead to internal damage, including intestinal perforation and ulcerative lesions with potentially gastric rupture and deformation that can leads to the death of the organism (Colferai et al., 2017). Chemical effects are those caused by the composition of the polymeric chain, the presence of additives, or others persistent organ pollutants (POPs) such as polychlorinated biphenyls (PCBs), organochlorine pesticides, polycyclic aromatic hydrocarbons (PAHs) and heavy metals which can be adsorbed on the surface of MPs when moving in the aquatic environment

(Juan and Martínez, 2021; Koelmans et al., 2016). Some plasticizers such as phthalates, bisphenol A (BPA), and polybrominated compounds (flame retardants) are known as endocrine disruptors with the capacity to influence estrogenic activity (Bajt, 2021). Mercury is of special relevance because it is a global pollutant and a common contaminant in the marine environment, it is highly toxic to animals and its organic forms (particularly methylmercury) biomagnifies in trophic webs (Eagles-smith et al., 2018). POPs even at non-lethal concentrations can alter key aspects of behaviour (Ferreira, et al. 2016) such as reduction in predation and result in adverse health outcomes (Rochman, 2016).

Regarding biological effects, studies on fish like gilthead seabream (*Sparus aurata*), European seabass (*Dicentrachus labrax*), and zebrafish (*Danio rerio*) exposed to MPs have shown reduction in feeding rate (Colferai et al., 2017), body mass, and metabolic rate, as well as a decreased allocation of energy for growth (Farrell and Nelson, 2013), reduced predatory (Luís et al., 2015) and swimming activity (Barboza et al., 2018), changes in behavioural responses (Yin et al., 2018), decreased fertilization and larval abnormalities (Le and Gom, 2017), neurotoxicity due to acetylcholinesterase inhibition and oxidative stress (Avio et al., 2015; Barboza et al., 2018; Oliveira et al., 2013), induction of inflammatory responses, histological changes, DNA damage, cytotoxicity, and mortality events (Chen et al., 2021; Hale et al., 2020). The MPs absorption pathways of fish can occur through oral, gill and skin absorption, and can rapidly accumulate in gills and intestine (Bhagat et al., 2020). However, only MPs with size ≤ 20 μm would be able to penetrate organs while the smallest fraction (< 10 μm) would be able to access all organs, cross cell membranes, the blood-brain barrier and the placenta (Borrelle et al., 2017; Burkhardt-holm, 2012; Galloway and Lewis, 2016). Infact the distribution of MPs in secondary tissues, such as liver, muscle, and brain, is widely documented (Wright and

Kelly, 2017). A previous work by Cattaneo et al. (2023) conducted on zebrafish larvae and juveniles supports how larger particles (40-47 μm) only transit in the intestinal lumen, while smaller particles (1-5 μm) are absorbed by intestinal mucosal folds and can be transferred to other organs like liver. In addition, the liver was recognized by the authors as an organ in which MPs are trapped, preventing the translocation to other tissue like the striated muscle. However, MPs that enter the fish circulatory system can interact with blood proteins, including albumin and globulin, to form protein-plastic compounds that potentially impact blood circulation. In addition, MPs can be internalized in the blood, damaging red blood cells by mechanical, osmotic, and oxidative stress, which can impact haematological properties such as RBCs (red blood cells count) (Qian et al., 2020). Studies conducted on juveniles of zebrafish show that MPs caused behavioural and morphological changes, such as upward or downward curvature of the tail (vascular defect), deformation of the mandible, pericardial edema, string heart, decreased heart rate and swimming competence (Mak et al., 2019; Qiang and Cheng, 2019). MPs that have penetrated cell membranes can cause greater toxicological implications (Triebskorn et al., 2019) than those that are too big to be absorbed that can may be quickly egested; in fact, the smaller particles can alter cellular functioning (Burkhardt-holm, 2012). Studies documented changes in trans-membrane gradients alteration on toxin excretion rate, induction or inhibition of enzymes connected to carbohydrate and lipid metabolism, and changes in steroid hormone levels, resulting in variation of triglyceride and cholesterol levels (Banaee et al., 2019; Bednarz et al., 2020). It is shown that MPs can induce a series of immune responses after entering the tissues and organs of aquatic animals causing a possible oxidative damage (Espinosa et al., 2019). Espinosa et al. (2018) observed an activation of the response mediated by head kidney leucocytes in seabream (*Sparus*

aurata) and sea bass (*Dicentrarchus labrax*) using 40–150 μm polyethylene and polyvinyl chloride particles. Veneman et al. (2017) tested developmental effects of polystyrene (0.7 μm) in early stage of zebrafish, and they noted interactions with phagocytic cells (including neutrophils) and activation of the complement system and neutrophils. Significantly increased mRNA and protein levels of genes linked to the innate immune system (*il1a*, *il1b*, and *ifn*) have been demonstrated (Chen et al., 2018). MPs toxicity in organisms can also occurs through the formation of free radicals and can lead to genomic instability and changes in pathology, physiology, and biochemistry, which is also associated with carcinogenesis (Gideon and Faggio, 2019).

Specifically, MPs toxicity in fish cells mainly results from oxidative stress, including disturbance to redox balance, injury to cellular components, and excessive reactive oxygen species (ROS) production (Cap et al., 2020). An increase in ROS and oxidative stress in cells has been implicated in serious diseases and disorders (Suzuki et al., 2012). Organisms have developed an antioxidant defence system that maintains the level of reactive oxygen species (ROS) and protects vital biomolecules from free radical damage. Superoxide dismutase (SOD), catalase (CAT), glutathione-transferase (GST), glutathione (GSH), glutathione peroxidase (GPX), and glutathione reductase (GR) are important and essential biomarkers for measuring oxidative stress in an organism and they represent the main defence mechanism against ROS (Bhagat et al., 2020). Umamaheswari et al. (2021) reported that ROS and lipid peroxidation (LPO), a chain reaction triggered by free radicals which can compromise cell membrane integrity, significantly increased in *D. rerio* exposed to MPs. MPs induce the ROS production in fish or inhibit the antioxidant ability, leading to a state of susceptibility to oxidative stress. Production of ROS is also associated with DNA damage in zebrafish embryo exposed to polystyrene MPs (Özkaraca

and Bu, 2020). Damage to DNA and alternation in the expression of a gene encoding DNA repair pathways provide a crucial basis to measure the effect of MPs in zebrafish. Cytochrome P450 (CYP) is a phase I enzyme that plays an important role in metabolizing aryl hydrocarbon receptor (AhR) compatible toxicants and is encoded by the *cyp1a* gene. AhR is a nuclear receptor that is activated by environmental toxicants and regulates the expression of xenobiotic metabolism genes. The upregulation of *cyp1a* was reported in the liver tissue of zebrafish exposed to MPs (Mak et al., 2019). Increased ROS levels stimulate lipid peroxidation, producing (4-hydroxynonenal (HNE) and MDA (malondialdehyde), which damage the structure of the cell membrane. Raised LPO in *D. labrax* exposed to MPs indicates that oxidative stress and lipid injury are induced (Barboza et al., 2018). Solomando et al. (2020) reported that MPs exposure of *S. aurata* caused protein damage and increased LPO oxidative stress, even when the antioxidant reaction and detoxification systems were activated, including SOD, CAT, GST, and GSH. Lipid oxidative damage can lead to a wide range of adverse effects. Gill lipid peroxidation damage may compromise respiration and biotransformation of xenobiotics in gills among other crucial processes (Pandey et al., 2008). Lipid peroxidation in muscle may disrupt muscular (e.g. cellular energy production) and neuromuscular functions resulting in deficit of energy, problems of movement coordination, decrease of the swimming performance, and several other adverse effects (Vieira et al., 2009). Lipid peroxidation damage in the brain may cause the disruption of membranes of presynaptic vesicles containing neurotransmitters resulting in increased levels of neurotransmitters into synaptic clefs (Hil et al., 1999), among other types of neurotoxicity (Fitzgerald et al., 2021). In terms of brain function, studies have shown that small MPs particles affect the development and functioning of fish brain (Mattsson et al., 2017), causing a structural

damage (Wan et al., 2019), and a decrease of acetylcholinesterase activity (Barboza et al., 2018). Acetylcholinesterase (AChE) activity helps in regulating the brain function in zebrafish and is regarded as an important biomarker for neurotoxicity. A decrease in AchE activity was found in zebrafish exposed to MPs (Sarasamma et al., 2020) and it leads to severe neurotransmission disorders, motor dysfunction, and behavioral abnormalities (Banaee et al., 2019; Barboza et al., 2018; Iheanacho et al., 2023; Umamaheswari et al., 2021). MPs and their additives also have adverse effects on the reproduction of aquaculture products (Le and Gom, 2017).

Moreover, some studies have investigated the influence of MPs on organismal microbiomes. Microbes and other organisms that have been found on plastic debris, generally described as the "plastisphere" (Zettler et al., 2013), are of particular concern regarding the spread of exotic invasive species and pathogens. Some of these communities have been found to include pathogenic organisms, such as *Vibrio* spp. (Tender et al., 2015), *Escherichia coli, Stenotrophomonas maltophilia, Bacillus cereus* (Barboza et al., 2018), and *Aeromonas salmonicida* (Kova et al., 2017). The importance of microbiomes to nutrition and disease protection is well recognized in fishes (Adamovsky et al., 2018) and humans (Shreiner et al., 2015). In a study conducted on juvenile sea bass, it was shown that MPs provided in the diets, with a size range of 1-5 μm, can translocate into the fillet shifting attention to potential human health risks (Zeytin et al., 2020). MPs are resistant to chemical degradation; if ingested, their biological persistence are important factors leading to their risk (Wright and Kelly, 2017). The Peyer's patches of the human ileum (third portion of the small intestine) are considered the major sites of uptake and translocation of particles (Wu et al., 2023). Studies have confirmed that non degradable particles such as aluminosilicate and TiO2 accumulate in

large quantities in the basal phagocytes of Peyer plaque (Powell et al., 2010). Both are non-degradable particles, MPs will also deposit in this range and hijack the absorption pathway of endogenous particles, thus interfering with immune sensory and monitoring and damaging human immunity (Wright and Kelly, 2017). Studies have shown that MPs can penetrate into mammalian blood vessels and lymph nodes at the villus tips of the desquamation zones (Wright and Kelly, 2017), then induce inflammation and immune response (Powell et al., 2010). MPs also cause hemolysis after entering the blood. Studies have shown that PS MPs with diameter less than 5 μm can cause about 4 % hemolysis in humans after entering the blood compared with relative controls (Wu et al., 2023). MPs such as PP, PE, PS and PU are also detected in human placenta and meconium (Braun et al., 2021), which suggests the presence of MPs poses a potential health threat to developing fetuses. It has been confirmed that some additives in MPs do serious harm to human body including reproductive toxicity (e.g., DEHP and BPA), carcinogenicity (e.g., vinyl chloride and butadiene), and mutagenicity (e.g., benzene and phenol) (Powell et al., 2010). MPs can carry pollutants including HOCs and heavy metals (such as cadmium, zinc, nickel and lead) (Rochman et al., 2014). In *vitro* studies showed that the release of Cr loaded on MPs in human digestive system was higher compared to the one presents in water environments (Wu, 2023). This means that the human inner environment promotes the release of pollutants loaded on MPs and increases the health risks posed by MPs. Knowledge about the effects of MPs on the human health through the consumption of fish is still atthe frist steps and requires further investigation, but some remedies are required to control the MPs pollution problem (Law and Thompson, 2014; Gabriel et al., 2015; Rist et al., 2018).

1.4 Remedies and solutions against MPs effects

As MPs pose a potential treat to animal, environmental and human health, the global pollution by MPs and its effects should be addressed according to the World Health Organization (WHO) 'One Health' approach. Its vision is based on the recognition that human, animal, and ecosystem health are inextricably linked.

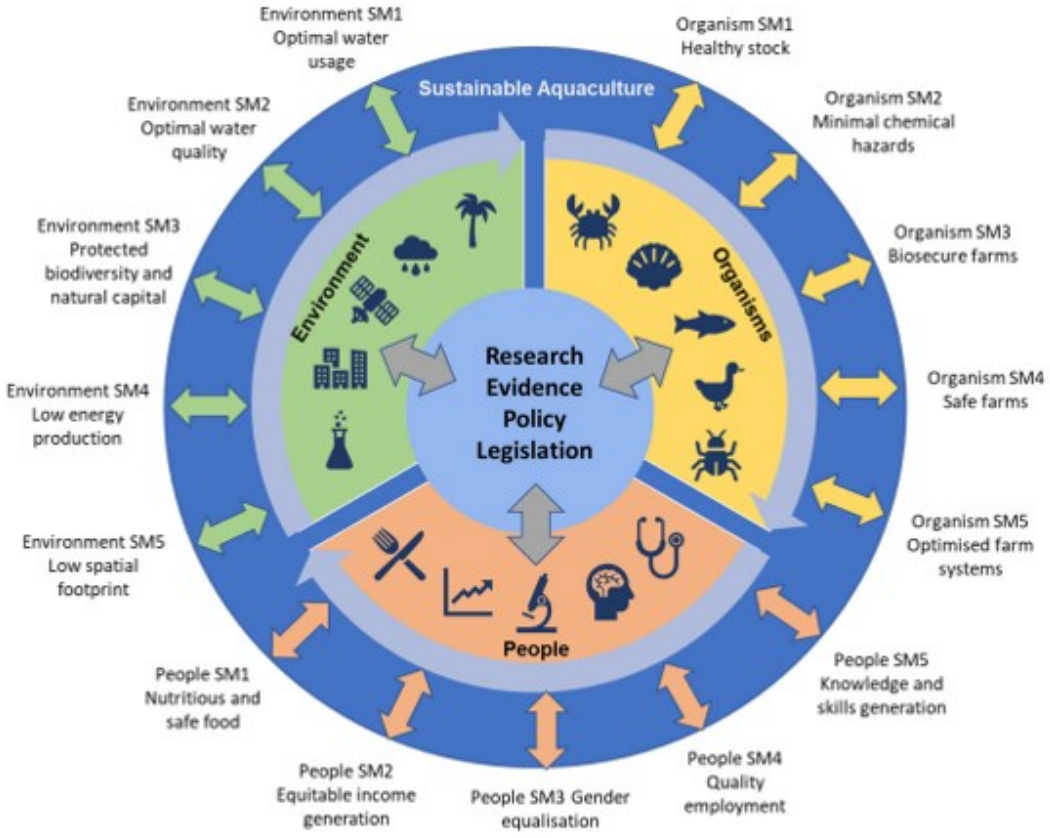

Fig.2 The One Health Aquaculture approach to design of a sustainable aquaculture sector. The approach proposes 15 success metrics spanning environment-, organism- and human-health; fulfilment of which are underpinned by the availability and application of research, evidence, policy, and legislation (Stentiford et al., 2020).

The One Health Aquaculture approach (Fig. 2) is only a starting point for more meaningful societal discourse around aquatic food supply. It is built upon the recognition that food systems, including those in water, are increasingly considered as a conduit around which biodiversity may be lost and gained, climate change mitigation strategies may succeed or fail and, human communities may be well nourished or, impoverished (https://www.iss.it/one-health). Sustainability is a key concept in aquaculture, as the industry must balance growth in marine food production with conservation of marine resources and protection of aquatic ecosystems. From an environmental point of view, sustainability in aquaculture aims to minimize the negative impact on the marine environment, including the prevention of plastic pollution.

Marine pollution by MPs requires a multifactorial approach, involving both actions at the individual level and interventions at the policy and international levels to try to diminuish the quantity of plastic in the environment. The presence of laws that regulate the plastic use, production and elimination are essential: International Convention for the Prevention of Pollution from Ships (MARPOL) Annex V entered into force in 1988 to reduce the discharge of garbage (including plastic) to the sea. The 2005 MARPOL Annex IV instead restricts the discharge of sewage from ships and allow dumping of sewage when the ship is in operation and has an approved sewage treatment plant or when the ship is discharging comminuted and disinfected sewage using an approved system more than three nautical miles from the nearest land. Single-use plastics may include plastic bags, water bottles, straws, and other plastic materials that are used once and then discarded. Most countries have imposed a tax or limits on the use of bags made with plastic (Biswas and Hartley, 2017). It's known that plastic bags have already been banned in many cities, regions, and

countries all over the world (Iheanacho et al., 2023). Furthermore, the exclusion of MPs as microbeads from cosmetic products and other consumables have been advocated for by many countries through legislation (United States Congress, 2015; Canada Gazette, 2016). Attempts have also been made to remove plastic microbeads from personal care products. In the United States of America, the Illinois Environmental Council (IEC) collaborated to draft the legislation to ban plastic microbeads in Illinois (Microbead-Free Waters Act of 2015, 114th United States Congress (2015–2016) becoming effective in 2017 (Wu et al., 2017). Netherland also banned the use of microbeads in rinse-off personal care and cosmetic products (Nikiema and Asiedu, 2022). Other countries that have banned microbeads are Canada, France, Italy, the UK, Thailand, and New Zealand (Guerranti et al., 2019). Treatment of wastewaters from industrial sewages, has been identified as very important for the reduction or elimination of plastic and MPs pollution (Nikiema & Asiedu, 2022). This is because MPs get into the environment especially the aquatic ecosystem from wastewater treatment plants (Hirai et al., 2011). Replacement of plastic polymers with natural materials is a great solution to diminuish the quantity of plastic in the environment; biodegradable (BPs) materials have been suggested to replace plastic-based materials such as polyester and thermo-plastics (Cerbule et al., 2023). Use of microorganism can make easier and more efficient the elimination platics as suggested by different studies that identified several strains of microbes capable of biodegrading petrobased synthetic polymers like polyethylene and PVC (Shah et al., 2008). For example, it has been observed that the gut bacteria of mealworms can slowly breakdown polystyrene (Hale et al., 2020). Replacement of fish meals with alternative protein sources in livestock and fish diets has been suggested as a potential way to mitigate MPs exposure to farmed organisms (Hanachi et al., 2019). A paradigm shift to plant-based protein such

soybean has been suggested to be an effective mitigation strategy to curb MP contamination in fish diet and fish (Zhou et al., 2021). The adoption of single-cell proteins (algae, fungi, yeast, bacteria) may also stand out as an excellent alternative for fish meal in aquafeed, considering their nutritional qualities. All of these are solutions that could decrease the amount of plastics in the environment in a long term period, however at the present day, where the presence of plastics in the environment is still huge, it is necessary to change approach and focus directly on solutions that can directly improve fish welfare and mitigate the negative effects of microplastics on organisms.

On this regard, antioxidants can be defined as molecules that, at low concentrations, delay or prevent oxidation, acting at biological membranes, or at intracellular levels, therefore protecting the cells of different organs and diverse biological systems (Cornelli, 2009). These molecules, like carotenoids, can protect cells from oxidative processes mediated either by light, free radical-mediated peroxidation, or singlet oxygen (Khademalhosseini et al., 2023).

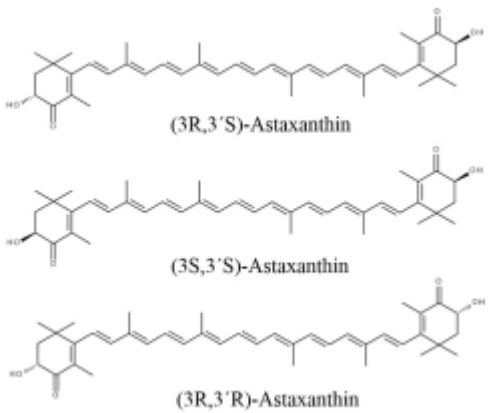

Fig. 3: Stereoisomers of astaxhantin (3,3′-dihydroxy-β,β′-carotene-4,4′-dione)

Among them, astaxanthin (ASX) (fig. 3), 3,3′-dihydroxy-β, β′-carotene-4,4′-dione, is a red C40 xanthophylle and one of the most abundant aquatic carotenoids (Winny Routray Deepika Dave and Pohling, 2019). It is standing out within its chemical family as it has been shown to have the highest oxygen radical absorbance capacity, with a 100–500 times more antioxidant capacity than α-tocopherol (Vitamin E), a well-known and commonly used antioxidant (Nguyen, 2013). Several sources of natural ASX have been reported, including the microalgae *Haematococcus pluvialis*, *Chlorella vulgaris*, *Chlorella zoofingiensis* and *Chlorococcum sp*. It is also naturally synthesized by the red yeast *Phaffia rhodozyma* (Higuera-Ciapara et al., 2006). Among these, *H. pluvialis* is currently the only natural source of ASX approved for human consumption (Aneesh et al., 2022). ASX can also be included indirectly in our diet by consuming crustaceans (e.g., copepods, shrimp, and krill) and salmonid (e.g., salmon, rainbow trout) species, whose diets include natural sources of astaxanthin. The ASX molecule has two asymmetric carbon atoms at positions 3 and 3’ (Fig. 3). Consequently, there are different possible optical isomers or enantiomers: 3S, 3’S; 3R, 3’R; and 3R, 3′ S. Natural ASX, isomers with a chirality 3S, 3’S, or 3R, 3’R, are found to be more biologically active than synthetic one (Deng et al., 2023) Synthetic ASX consists in a combination of 3R,3’R; 3R, 3′ S; and 3R,3’R isomers (1:2:1) (Liu et al., 2014); nowadays, synthetic methods to develop ASX from ketoisophorone (C9H12O2) are used to meet the commmercial requirement of ASX (Aneesh et al.,2022). ASX contains conjugated double bonds, hydroxyl, and keto groups, showing both lipophilic and hydrophilic properties (Higuera-Ciapara and Goycoolea, 2006). The conjugated double bonds at its center are responsible for its red color and, most important, for its high antioxidant capacity, as it donates the electrons that react with free radicals to convert them into more stable products blocking free radical chain

reactions (Ambati et al., 2014). ASX can also trap free radicals in its terminal ring moiety, in which the hydrogen atom at the C3 methine has been suggested to be a radical trapping site (Raza et al., 2021). As ASX shows both lipophilic and hydrophilic properties, this molecule is exposed to both the inside and outside of the cell, where it can scavenge radicals from the surface of the cell and at the interior of the phospholipid membrane. This feature makes ASX unique when compared to other antioxidants, such as β-carotene, zeaxanthin, lutein and vitamin C, which can only reside within or outside the lipid bilayer membrane, respectively (Cao et al., 2021). Given its unique features, ASX has been widely studied in the last years, both in animal and human models, showing neuroprotective, cardioprotective, and antitumoral properties, moreover, it's declared safe for use and hence classified as "pure antioxidants" (Huang et al., 2023). As supplements to food sources, astaxanthin products have already entered the market mainly in the form of soft gel, capsule, cream, energy drink, oil, and extract (Lu et al., 2021) Studies carried out in different species of fishes found that ASX can also prevent tumor, increase the number of B and T cells through lymphocyte proliferation, and improve the immune resistance and growth performance of fish species (Donoso et al., 2021). As reported by Cheng et al. (2018), ASX had promoted the growth and antioxidant defense system of pufferfish (*Takifugu obscurus*) under high temperature stress. Diatoms *Amphora coffeaeformis* as feed additives could compensate for the negative effects of MPs exposure on Nile tilapia (*Oreochromis niloticus*) such asoxidative stress (Ismail et al., 2021). A recent study performed on discus fish *Symphysodon aequifasciatus* higlights that ASX could play an antioxidant role to combat oxidative stress caused by MPs (Huang et al., 2023). Considering the global pollution by MPs, the toxic effects that have been found in animals, and the potential risks to humans, more research on the toxicity of these

particles to humans are urgently needed (Patel et al., 2022)ASX could be a way to ensure greater animal welfare for farmed species and a way to limit the negative effects created by plastic pollution.

2. Aim of the thesis

Plastic pollution is recognized as a severe anthropogenic issue in the coastal and marine ecosystems across the world (Thushari and Senevirathna, 2020). Unprecedented and continuous accumulation of growing plastic contaminants into any respective aquatic ecosystem by the anthropogenic sources causes direct and/or indirect interruption to ecosystem structure and functions, consequently harm to the organisms (Galgani et al., 2015). MPs are very dangerous to animal welfare and human health as they can enter the biotic component because of their small size and cause negative effect such as physical damage, liver metastasis, metabolism disorders, growth inhibition, oxidative stress, and genotoxicity (Bajt, 2021; Bhuyan, 2022; Zhou et al., 2021). Aquaculture is one of the main sectors for human nutrition and is estimated to be able to supply about 62 % of the seafood by 2030 going to be one of the main sources of protein for human (Mimako Kobayashi Siwa Msangi and Anderson, 2015). Contamination of aquaculture by MPs is becoming increasingly common. It is estimated a range from 1 to 30 particles per day (Van Cauwenberghe et al., 2015) are ingested by humans depending on consumption of fish. One of the main sources of this contamination comes directly from the feed consumed by farmed organisms. The feed production process is very complex, and MPs

are easily introduced through the processes of manufacturing, processing, production, transportation, storage, and sale (Wright et al. 2013; Van Cauwenberghe et al. 2015). In addition, fishmeal, that is one of the main component of fish feeds, is derived directly from fish caught environments increasingly contaminated by the presence of these particles. MPs can potentially influence the quality and safety of the farmed fish product, that are closely related to the health not only of the fish but also of the consumer. The use of antioxidant molecules such as astaxanthin (ASX) could mitigate the negative effect of MPs on farmed fish.

In this context, the aim of the following project, is to: (i) produce experimental diets characterized by the inclusion of two different fluorescent MPs polymers (amino formaldehyde polymer of size 1-5 μm and polyethylene of size 40-47 μm) at two different concentrations (50 mg/kg and 500 mg/kg, representing a realistic and a 10x higher concentration to intensify possible effects, respectively); (ii) exposing the model organism zebrafish (*Danio rerio)* to the MPs contaminated diets over a 6 months feeding trail (from larvae to adult stage); (iii) investigating the possible beneficial effect of 1 month exposure to the previous diets with an inclusion of ASX (7 g/kg) to verify if its addition provides welfare improvements in zebrafish exposed to MPs.

At the end of the feeding trial (7 months), the effects of MPs on growth, welfare, and accumulation of MPs in relation to their size and concentration, as well as the possible ameliorative effects due to ASX inclusion, were assessed through a multidisciplinary approach including biometric, histological, molecular, confocal, and fluorescence microscopy analyses.

3. Material and methods

3.1 ethics

All the experimental procedures involving animals conducted in the present study were performed in accordance with the Italian legislation on experimental animals and were approved by the Ethics Committee of the Marche Polytechnic University (Ancona, Italy) and the Italian Ministry of Health (Aut. n. 391/2023-PR). The suffering of the animals was minimized by using an anaesthetic (MS222; Merck KGaA, Darmstadt, Germany).

3.2 experimental model

Fig. 4: *Danio rerio*

Danio rerio, known as zebrafish (Fig.4), is a freshwater teleost (3-4 cm) belonging to the family Cyprinidae and native to Southeast Asia. The body is tapered and laterally

compressed with the mouth pointing upward and the lower jaw protruding forward. The livery has alternating thicker blue and thinner white horizontal lines. The tail and anal fin show the same coloration as the rest of the body, while the other pectoral fins are olive in color. It lives in tropical areas subject to typical monsoon weather fluctuations, inhabiting rivers, small streams, other channels, stagnant or slow-moving pools near streams, and rice fields (Mc Clure and Hall, 2006). It is an omnivorous organism, feeding on zooplankton, insects, algal and plant compounds (Spence et al., 2008). In captivity, this fish is fed rotifers, naupli of *Artemia salina* and dry granular food (Ribas et al., 2014). Zebrafish has been emerging in recent years as a model organism in the field of basic research due to a number of characteristics that favor its wide use: ease of breeding, low maintenance costs, simplicity in induction of reproduction, great eggs and embryos production which are practically transparent, and reaching adulthood in 3-4 months (Spence et al., 2008). Zebrafish reproduce readily in captivity, females continuously lay eggs at frequent but irregular intervals, and a single female can produce, under optimal conditions for reproduction and growth, several hundred eggs in a single spawning event (Spence et al., 2008). The eggs are demersal and nonadhesive, with a diameter of about 0.7 mm, and once released are immediately fertilized by males. Special precautions must be used in captivity to avoid cannibalism towards the eggs (Ribas et al., 2014). Only 18 hours after fertilization, zebrafish embryos show well-developed eyes and muscle and brain segments; after about 24 hours, segmentation is completed, after 48-72 hours hatching occurs and the larva emerges. The newly hatched larva is about 3 mm long, does not have a fully developed gut, and the insufflation of the swim bladder occurs about two days after hatching (Spence et al., 2008). In the first few days of life, the larvae grow rapidly, the yolk sac is consumed, and the intestine, which was previously tubiform, folds

over taking an "S" shape. After the second day after hatching, along with the development of the swim bladder that allows them to swim actively, the larvae begin to breathe from their gills and move their jaws leadingto a change in prey seeking and feeding (Kimmel et al., 1995). In addition to ease in breeding, another reason why zebrafish is an excellent model organism is that its entire genome has been sequenced, reporting a high similarity to that of humans. In fact, it shares 70% of the genomic makeup with humans, and genes associated with disease development show about 84% homology with those of humans. Zebrafish has been found to be useful in understanding the mechanisms of tumor initiation, growth and spread, since the biology of neoplasms in fish and humans is the same. Zebrafish is also widely used in many fields of aquaculture for the study and identification of genes involved in mechanisms that preside over nutrient metabolism, muscle development, and stress (Khan and Alhewairini, 2019).An important feature, useful for the development of biological studies, is the fact that embryos and larvae are completely transparent. Thus, it is possible to directly observe the development of various organs in addition to visualization of vessels and circulatory flow through the use of fluorescent markers (Ribas et al., 2014).

3.3. MPs features

Two different fluorescent microbeads (MPs) were purchased from Cospheric LLC (Goleta, CA, USA): (i) Polymer A: amino formaldehyde polymer (FMV-1.3), with a size range of 1-5 μm and an emission peak of 636 nm when excited at 584 nm; (ii) Polymer B: polyethylene (UVPMS-BR-0.995), with a size range of 40-47 μm and an emission peak of 607 nm when excited at 575 nm. Before being included during the experimental diets preparation at the above-mentioned concentrations, MPs fluorescent microbeads,

hydrophobic in their pristine state, were resuspended, according to the company's technical support suggestion, in a 0.1% tween-80 solution as a surfactant (Merck KGaA), and then rinsed with deionized water for three times; low tween-80 concentrations are non-toxic for zebrafish.

3.4 experimental diets

Five test diets were prepared at the Department of Agriculture, Food, Environmental and Animal science of University of Udine (Italy). A control MPs-free diet (named Control) was formulated to resemble the proximate composition of a commercial standard diet available for zebrafish (Zebrafeed; Sparos LDA, Olhão, Portugal) and that one used in a previous study performed on zebrafish (Zarantoniello et al., 2020). Four experimental diets containing MPs were prepared by adding at two different concentrations (mg/kg of feeds) the fluorescent polymers A or B to the control mixture diet: (i) 50 and 500 mg/kg of polymer A (diet A50 and A500, respectively); (ii) 50 and 500 mg/kg feed of polymer B (diet B50 and B500, respectively.

In particular, all powdered ingredients used for the test diets production, were well mixed (GastroNorm 30C1PN, ItaliaGroup Corporate Srl) for 20 min and then oil and water were added to the mixture to attain appropriate consistency for pelleting. During the Control diet preparation, the added water was MPs-free; while for the test contaminated diets, the water was used to carry into the moist blend the A or B polymers. Pellets were obtained by using a 3-mm-die meat grinder, dried at 37 °C for 48 hours in a ventilated heater and then ground and sieved through a battery of sieves to obtain particles of 400-600 μm and 600-1000 μm diameter. Diets were subsequent stored in vacuum bags and shipped to Department of Life and Environmental Sciences, Marche Polytechnic University (Italy).

Feed samples were analysed for dry matter, crude protein, ether extract, and ash contents according to AOAC (2016). The ingredients, MPs concentrations and proximate composition of experimental diets are reported in Table 1.

Table 1. Ingredients (g/kg), MPs concentrations (mg/kg feed), and proximate composition (% of DM) of experimental diets used in the present study.

	Control	**Diet A50**	**Diet A500**	**Diet B50**	**Diet B500**
Ingredients (g/kg)					
Fish meal [1]	490	490	490	490	490
CPSP 90 [2]	123	123	123	123	123
Wheat gluten meal [3]	120	120	120	120	120
Pea protein concentrate [4]	120	120	120	120	120
Wheat starch [5]	55	55	55	55	55
Fish oil	60	60	60	60	60
Soya lecithin	8	8	8	8	8
Mineral and vitamin supplements [6]	14	14	14	14	14
Binder (sodium alginate) [7]	10	10	10	10	10
MPs concentrations (mg/kg feed)					
Polymer A (size: 1-5µm)	-	50	500	-	-
Polymer B (size: 40-47µm)	-	-	-	50	500
Proximate composition (%)					
Dry matter	94.2				
Crude protein	58.3				
Crude lipid	14.0				
Ash	12.5				

[1] Fish meal (61% CP, 11% CF), kindly provided by Skretting Italia, Mozzecane (VR, Italy). [2] Soluble fish protein concentrate (82% CP) (Sopropêche, France). [3] Wheat gluten meal (CP, 81%), kindly provided by Skretting Italia. [4] Pea protein concentrate (CP 69%) (Lombarda trading srl, Cremona, Italy). [5] Wheat starch: pre-gelatinized wheat starch kindly provided by Skretting Italia. [6] Mineral and Vitamin supplement composition as reported in (Zarantoniello et al., 2021). [7] Sodium alginate (Merck KGaA, Darmstadt, Germany). For proximate composition, values are reported as mean ± standard deviation of triplicate analyses.

3.5 Experimental design

Zebrafish embryos (wild type strain AB) were obtained from the broodstock colony of Università Politecnica delle Marche and maintained in a Tecniplast system (Varese, Italy)

for 48 hours, with a photoperiod of 12 hours light and 12 hours dark, under optimal water conditions: 28 ± 0.5 °C, pH 7 ± 0.1, ammonia and nitrite concentrations < 0.01 mg/L, and nitrate concentration < 10 mg/L. Then, the embryos were collected, selected under a stereomicroscope, and randomly divided into five experimental groups according to the 5 dietary treatments. A total number of 15000 embryos was used.

After hatching, zebrafish larvae were initially reared in thirty 20 L tanks (6 tanks per experimental group; 500 larvae per tank) with the same water conditions of the brood-stock' tanks. Water was gently replaced 10 times a day by a dripping system and the sides of each tank were provided with black panels to reduce light. After 20 days post fertilization (dpf), fish of each tank were transferred in 100 L tanks (6 tanks per experimental group) equipped with mechanical and biological filtration (Panaque, Roma, Italy).

Starting from 5 dpf to the reach of adult phase (6 months), zebrafish were fed the experimental diets as follows: (i) Control group, fed on CTRL diet; (ii) A50 group, fed on diet containing 50 mg/kg of polymer A (range size: 1-5 μm); (iii) A500 group, fed on diet containing 500 mg/kg of polymer A (range size: 1-5 μm); (iv) B50 group, fed diet contain-ing 50 mg/kg of polymer B (range size: 40-47 μm); (v) B500 group, fed on diet containing 500 mg/kg of polymer B (range size: 40-47 μm). The experimental design is shown in figure 5.

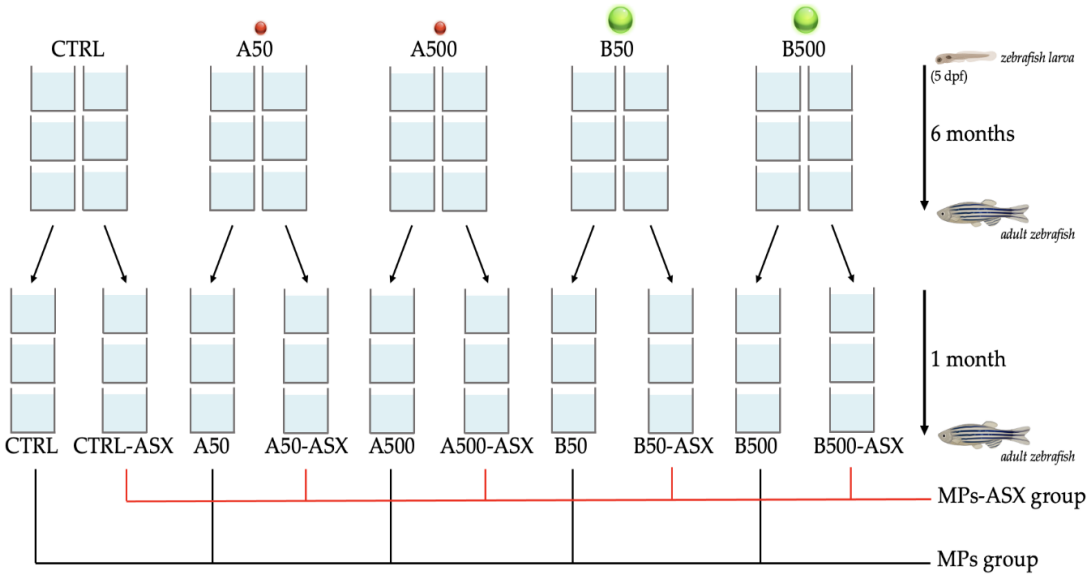

Fig. 5: experimental design

The feed particle size was adapted in relation to fish growth during the feeding trial: < 100 µm from 5 to 15 dpf, 101-200 µm from 16 to 30 dpf, 201-400 µm from 31 dpf to the end of the experiment (60 dpf) following the pro-cedures of (Zarantoniello et al., 2021). Each experimental group was fed at a feeding rate of 3% body weight, di-vided into two equal amounts (one in the morning and one in the afternoon). In addition, from 5 to 10 dpf, zebrafish larvae in all the experimental groups were fed the rotifers *Brachionus plicatilis* (5 individual/mL) according to (Zarantoniello et al., 2021).

After this 6-month period, each experimental group (composed by six tanks) was split in two subgroups for the second part of the trial which lasted 30 days: three tanks continued to be fed the experimental diets, meanwhile the other three tanks were fed the same diets with the addition of ASX (7 g/kg) and groups were named as CTRL-ASX, A50-ASX, A500-ASX, B50-ASX and B500-ASX, respectively.

Uneaten feed and dead specimens, if present, were siphoned 30 min after feeding from all the experimental tanks and recorded.

At the end of the trial (7 months), the required number of fish per tank, of each group, were sampled, after a lethal dose of MS222 (0.3 g/L) (for details, see next sections).

3.6 Biometry

Standard length and fresh weight were measured for each sampled specimen. Standard length was determined with Measy 2000 Typ 5921 caliper (Switzerland), accuracy: 0.1 mm. Weight was evaluated with OHAUS Eplorer analytical balance (Greifensee, Switzerland), accuracy: 0.1 mg.

3.7 Fate and quantification of MPs

3.7.1 Detection of Mps

During sampling, 5 intestine, 5 livers, and 5 muscle samples were taken per each tank (15 samples per experimental group of each tissue), which were fixed in PFA solution for 24 hours. Subsequently, the solution was changed to PBS 1x until analysis. All samples were immersed in a 50% PBS 1x- 50% glycerol solution for microscopic observation. The presence of fluorescent MPs microbeads in the collected samples was assessed with a Nikon A1R confocal microscope (Nikon Corporation, Tokyo, Japan). Samples were exited with 561/647 nm wavelengths simultaneously and emissions were collected at 615 and 670 nm, to visualize respectively the MPs (red) or tissue texture (blue). Images were processed with NIS-Element software (manufacturer Nikon).

3.7.2 Quantification of MPs

Digestion of 5 intestine, 5 liver and 5 muscle samples per each tank (15 samples per experimental group of tissue) was performed at the Reproductive and Developmental Biology Laboratory of the Department of Life and Environmental Sciences (DISVA) of the Università Politecnica delle Marche (Ancona, Italy). Samples were stored at -20°C and weighed before digestion. Digestion was done by adding the samples to a pre-filtered 10% KOH solution (glass fiber filter, 0.7 µm pore size, Whatman 30 GF/A) made with deionized water and KOH tablets (Sigma-Aldrich). The solution was added to each sample (1:10 w/v ratio) and incubated at 40 °C for 48 h by modifying the existing protocol described by Di Renzo et al. (2021). Then, the digestion products were filtered on glass fiber filters (0.7 µm pore size, Whatman GF/A) using a vacuum pump connected to a filter funnel. The filter membranes were dried at room temperature and placed in glass Petri dishes until visual inspection for identification and characterization of MPs particles. To avoid plastic contamination, plastic materials were avoided whenever possible, and work surfaces and laboratory instruments were thoroughly and repeatedly washed with pre-filtered 70% ethanol throughout the digestion and filtration process. Cotton gowns, face masks and disposable latex gloves were worn during all procedures.

The glass fiber filters, once dry, were analyzed individually using a Zeiss Axio Imager.A2 fluorescence microscope (Zeiss, Oberkochen, Germany) so as to quantify fluorescent MPs particles from the experimental diets ingested by the fish.

3.8 Histological analyses

At the end of the experiment, five fish per tank (15 per experimental group) were randomly taken and intestine and liver were collected. The samples were immersed entirely in Bouin's solution for fixation. After 24 hours at 4 °C, the samples were washed three times with 70% ethanol and kept in this solution. After the dehydration process in decreasing dilution solutions of ethanol (80%, 95%, 100%), the samples were washed with xylene (Bio-Optica, Milan, Italy) and included in paraffin (Bio-Optica). The solidified paraffin blocks were cut with a microtome (Leica RM2125 RTS, Nussloch, Germany), and the 5-µm sections were stained with Mayer hematoxylin and eosin Y (Merck KGaA) or Alcian Blue depending on the objective of the analysis. Sections were observed using a Zeiss Axio Imager.A2 microscope (Zeiss, Oberkochen, Germany), and images were acquired using a combined color digital camera (Axiocam 105, Zeiss) and analyzed using ZEN 2.3 software (Zeiss).

In samples stained with Mayer hematoxylin and eosin Y (Merck KGaA), morphometric assessment of intestinal mucosal fold height and submucosal thickness was performed. While in the samples stained with Alcian Blue (Ab), the presence and relative abundance of Ab-positive goblet cells was evaluated.

3.9 Molecular analyses

3.9.1 RNA extraction and cDNA synthesis.

Total RNA was extracted from liver and intestine samples taken from 15 specimens per experimental group using RNAzol TM reagent (Merck KGaA). The extracted total RNA was diluted in 20 µL of RNAse-free water (Qiagen). Final RNA concentration was determined by the use of a NanoPhotometer P-Class spectrophotometer (Implen,

München, Germany), and RNA integrity was verified by GelRedTM by highlighting 28S and 18S ribosomal RNA bands on 1% agarose gels. RNA was stored at -80 °C until use. cDNA synthesis was performed using the High Capacity cDNA Retrotranscription Kit (Bio-Rad, Milan, Italy) following the manufacturer's instructions using 1 µg of total RNA.

3.9.2. Real-Time PCR

PCR analyses were performed with the SYBR green method in an iQ5 iCycler thermal cycler (Bio-Rad). The thermal profile for all reactions was: 3min at 95 °C, followed by 45 cycles of 20s at 95 °C, 20s at different Melting Temperature depending on the gene (see TM in Table 3) and 20s at 72 °C. The fluorescent signal was detected at the end of each cycle, and melting curve analysis was performed to confirm that only one PCR product was present in each reaction. Negative controls revealed no amplification products, and no primer dimer formation was evident in the experimental blanks.

Relative quantification of the expression of genes involved in stress response [glucocorticoid receptor (*nr3c1*), heat shock protein 70 (*hsp70.1*)], fish growth [insulin-like growth factor-1 (*igf1*)] and oxidative stress response [superoxide dismutase 1 (*sod1*), superoxide dismutase 2 (*sod2*), catalase (*cat*)] was performed on liver samples. Relative quantification of the expression of genes involved in the inflammatory response [interleukin-1β (*il1b*), interleukin-10 (*il10*), TNF factor (*litaf*)] and appetite [ghrelin (*ghrl*)] was performed on gut samples. The ribosomal protein L13 (*rpl13*) and actin-related protein 2/3 complex subunit 1A (*arpc1a*) genes were used as housekeeping genes to standardize the results. The sequences of the primers used are shown in Table 2. The data obtained were analyzed using iQ5 version 2.0 software (Bio-Rad), which includes the GeneEx Macro iQ5 Conversion and GeneEx Macro iQ5 files.

Gene	Forward Primer (5′-3′)	Reverse Primer (5′-3′)	AT (°C)	ZFIN ID
il1b	GCTGGGGATGTGGACTTC	GTGGATTGGGGTTTGATGTG	54	040702-2
il10	ATTTGTGGAGGGCTTTCCTT	AGAGCTGTTGGCAGAATGGT	56	051111-1
litaf	TTGTGGTGGGGTTTGATG	TTGGGGCATTTTATTTTGTAAG	53	040704-23
nr3c1	AGACCTTGGTCCCCTTCACT	CGCCTTTAATCATGGGAGAA	58	050522-503
hsp70.1	TGTTCAGTTCTCTGCCGTTG	AAAGCACTGAGGGACGCTAA	58	990415-91
sod1	GTCGTCTGGCTTGTGGAGTG	TGTCAGCGGGCTAGTGCTT	60	990415-258
sod2	CCGGACTATGTTAAGGCCATCT	ACACTCGGTTGCTCTCTTTTCTCT	60	030131-7742
cat	CCAAGGTCTGGTCCCATAA	GCACATGGGTCCATCTCTCT	60	000210-20
rpl13	TCTGGAGGACTGTAAGAGGTATGC	AGACGCACAATCTTGAGAGCAG	59	031007-1
arpc1a	CTGAACATCTCGCCCTTCTC	TAGCCGATCTGCAGACACAC	60	040116-1

Table 2. Sequences, identification numbers (ZFIN ID), and annealing temperature (AT) of primers used in the present study

3.10. Statistical analyses

All data were checked for normality (Shapiro–Wilk test) and homoscedasticity (Levene's test). All the data were then analysed through one-way analysis of variance (ANOVA) followed by Tukey's multiple comparison post hoc test, performed using the software package Prism 8 (GraphPad software version 8.0.2, San Diego, CA, USA). Significance was set at $p < 0.05$.

4. Results

4.1 Growth and survival

Survival rate was about 94 ± 3 % in all the experimental groups. Figure 6 shows the wet weight of the adult zebrafish fed the different experimental diets. No significant differences were evident among the experimental groups fed the A-diets (A50, A50-ASX, A500, A500-ASX) and those fed the Control ones (CTRL and CTRL-ASX) (Fig. 6a) as well as among experimental groups fed B-diets (B50, B50-ASX, B500, B500-ASX) and those fed the Control ones (Fig. 6b).

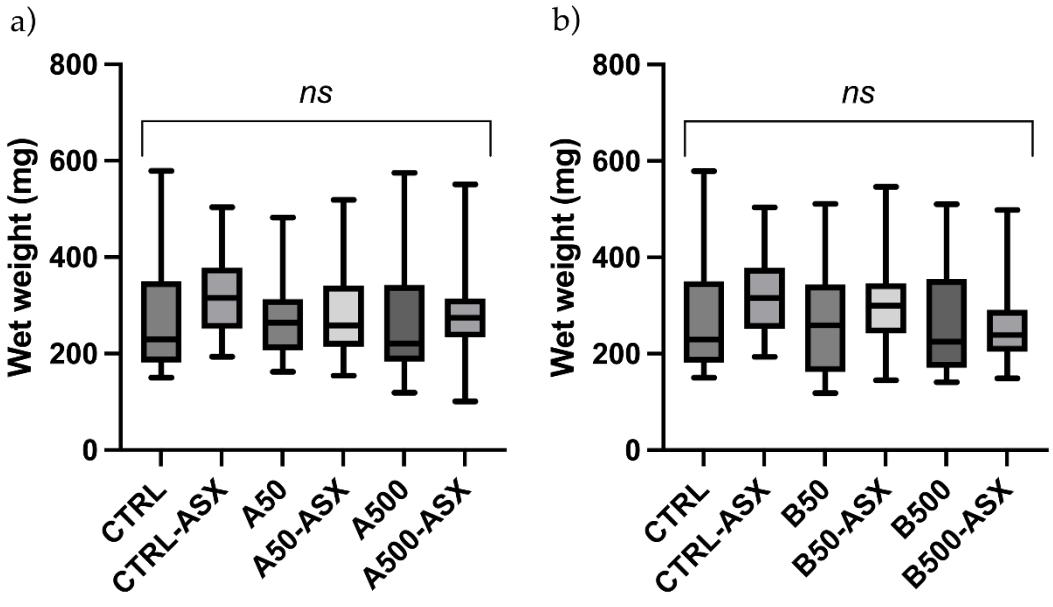

Fig. 6: Wet weight (mg) of adult zebrafish fed the experimental diets. (**a**) groups fed A-diets (A50, A50-ASX, A500, A500-ASX) compared to those fed the Control ones (CTRL and CTRL-ASX); (**b**) groups fed B-diets (B50, B50-ASX, B500, B500-ASX) compared to those fed the Control ones. Boxplots show minimum and maximum (whiskers), first quartile, median and third quartile (box) (n = 60). *ns*, no significant differences among the experimental groups ($p > 0.05$).

4.2 MPs detection

Analysis by confocal microscopy in zebrafish gut samples (Fig.7) revealed the presence of MPs (1-5 µm in size) in the A50 (Fig.7b), A500 (Fig.7c), A50 A (Fig. 7g) and A 500 A (Fig. 7h) groups. While MPs of size 40-47 µm were not present.

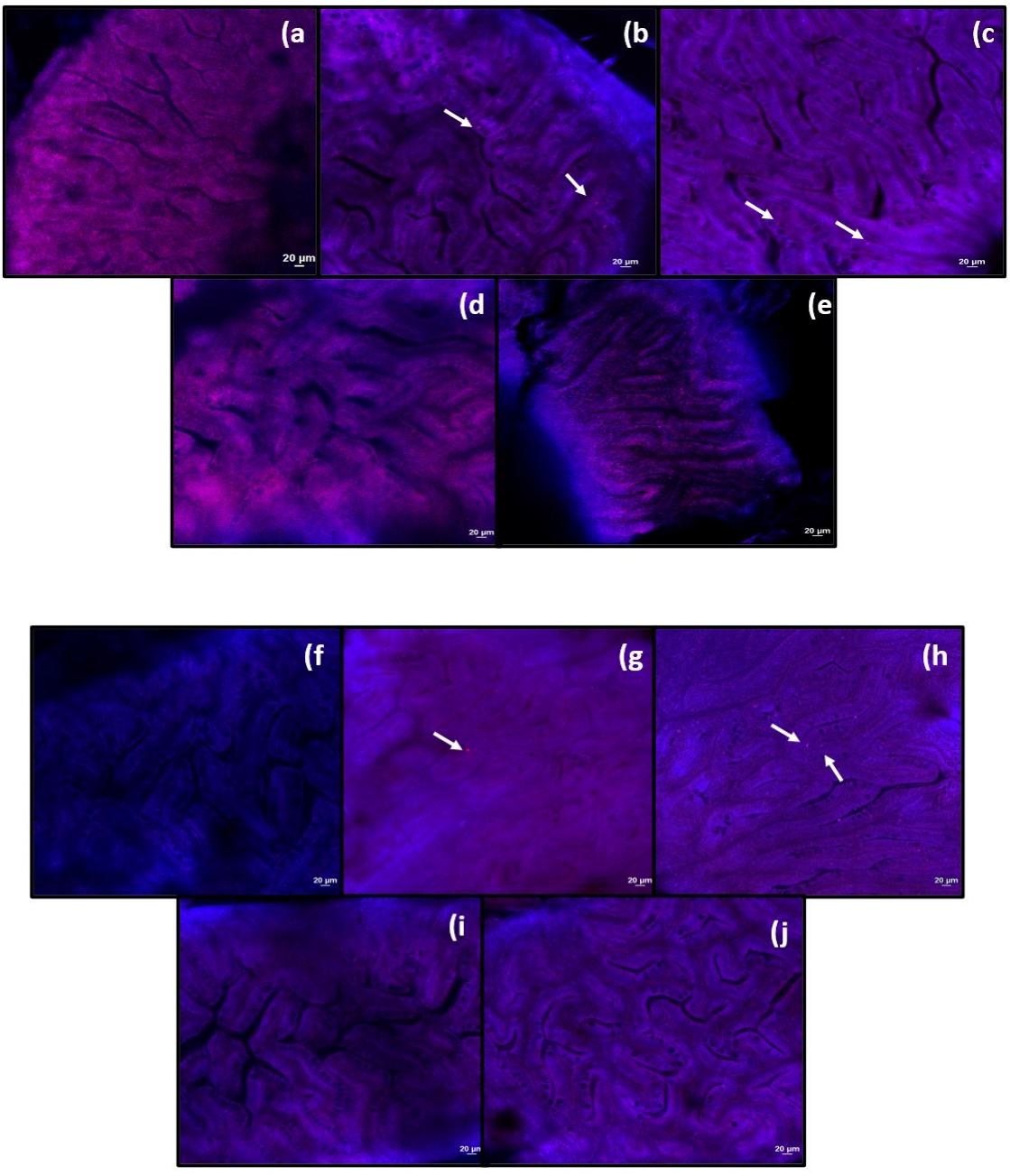

Fig. 7. Representative images of intestine samples of zebrafish fed on experimental diets: (a) CTRL, (b) A50, (c) A500, (d) B50, (e) B500, (f) CTRL-ASX, (g) A50-ASX, (h) A500-ASX, (i) B50-ASX, (j) B500-ASX. Scale bar = 20 µm. MPs are represented by the red dots (indicated with white harrows).

Analyses performed on liver parenchyma (Fig.8) showed similar results evidencing that only polymer A was able to accumulate. Polymer A MPs were detected in A50 (Fig.8b), A500 (Fig.8c), A50-ASX (Fig. 8g) and A500-ASX (Fig.8h) groups. Conversely, polymer B is not found in any groups fed with B-diet.

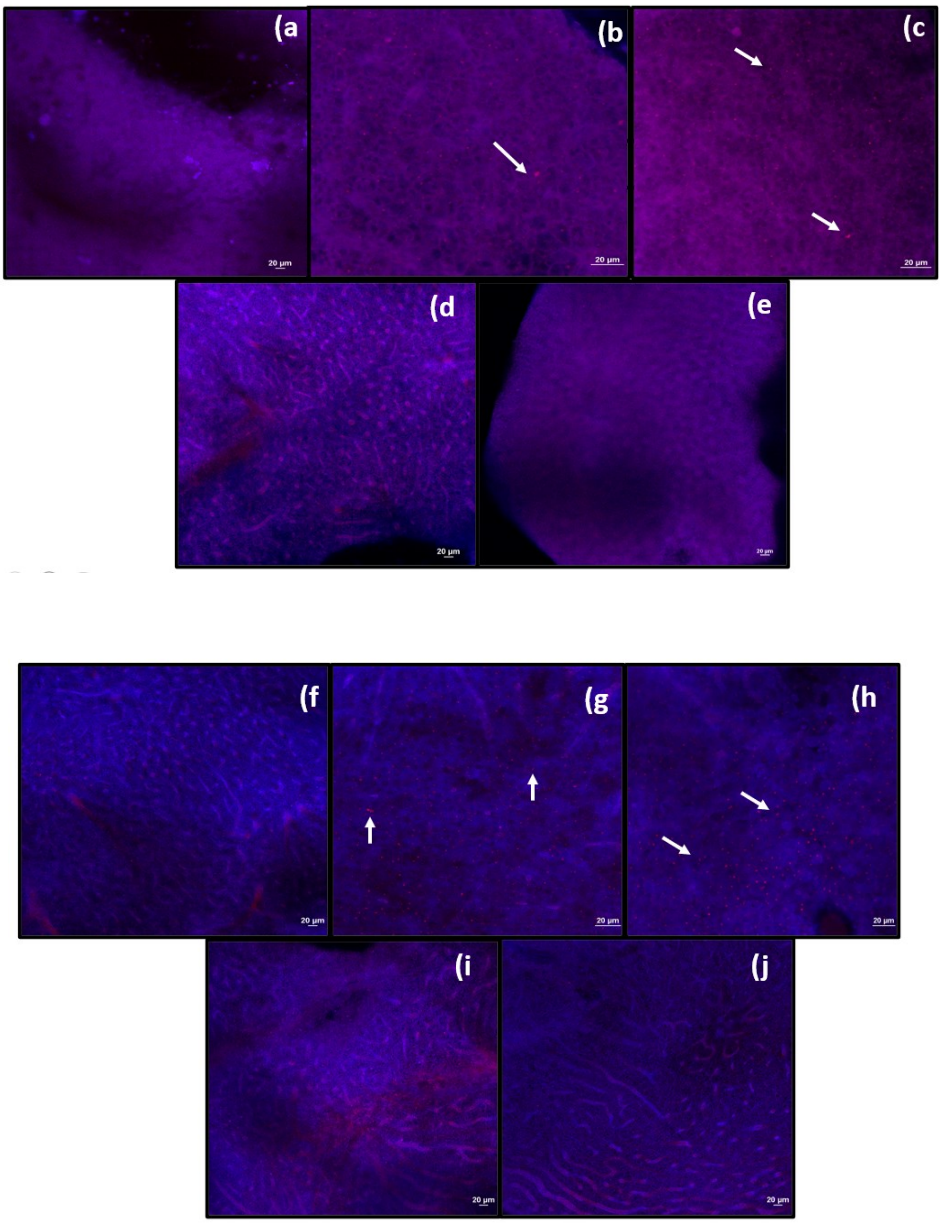

Fig. 8. Representative images of hepatic parenchyma samples of zebrafish fed on experimental diets: (a) CTRL, (b) A50, (c) A500, (d) B50, (e) B500, (f) CTRL-ASX, (g) A50-ASX, (h) A500-ASX, (i) B50-ASX, (j) B500-ASX. Scale bar = 20 µm. MPs are represented by the red dots (indicated withwhite harrows)

A similar scenario was evident also in the muscle samples analysed (Fig.9): MPs of size 1-5 µm were found in the A50 (Fig. 9b), A500 (FIG. 9c), A50-ASX (Fig. 9g), A500-asx (Fig.9h) groups, while MPs of size 40-47 µm were not present

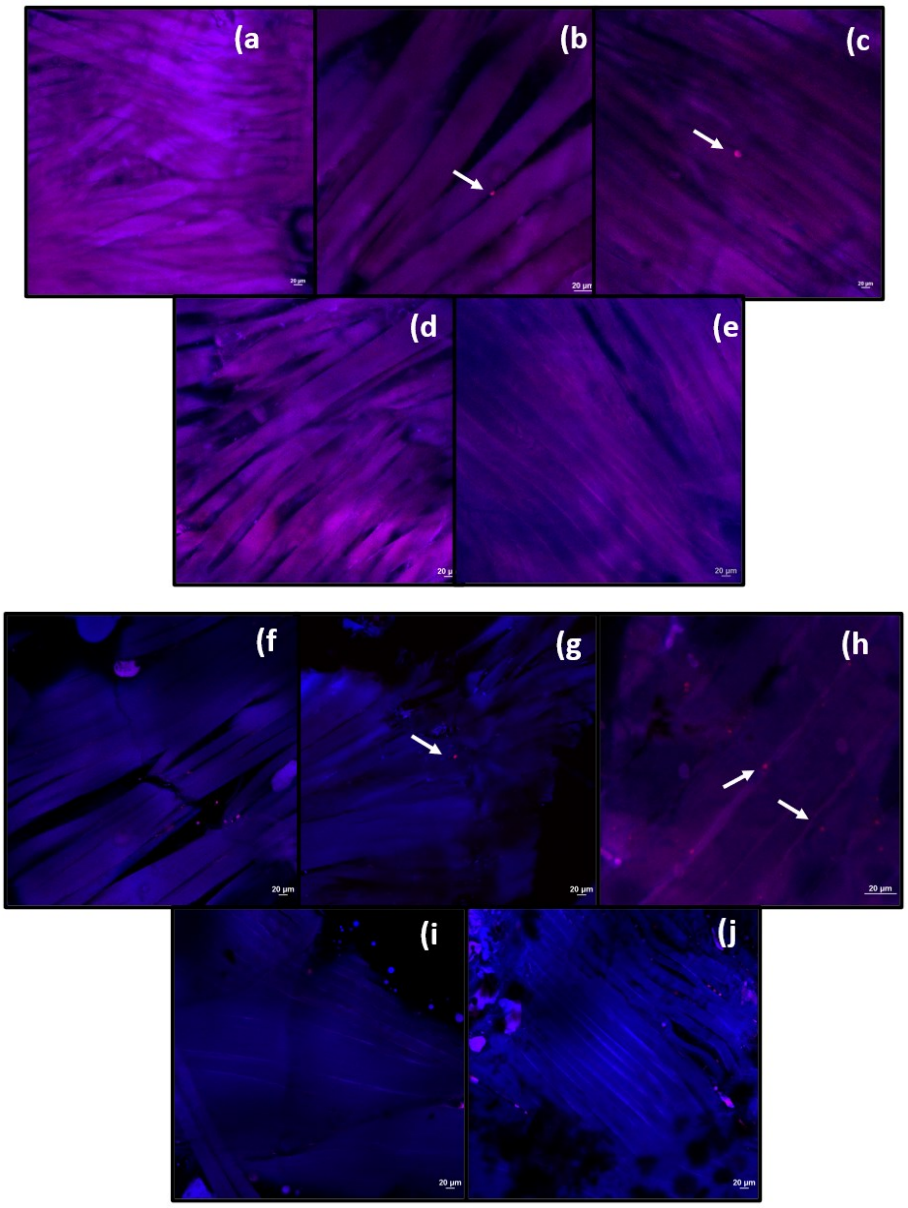

Fig. 9. Representative images of muscle samples of zebrafish fed on experimental diets: (a) CTRL, (b) A50, (c) A500, (d) B50, (e) B500, (f) CTRL-ASX, (g) A50-ASX, (h) A500-ASX, (i) B50-ASX, (j) B500-ASX. Scale bar = 20 µm. MPs are represented by the red dots (indicated with white indicators).

4.3 MPs quantification

Table 3 reports the MPs quantification in the intestine, liver, and muscle of adult zebrafish fed the experimental diets. No fluorescent MPs beads of both polymer A and B were found in the different tissues sampled from fish fed Control diets.

As regards fish fed the A-diets, a dose-dependent increase in polymer A microbeads accumulation was evident in all the tissues analysed. However, while no significant differences were evident between A50 and A50-ASX groups in all the tissues analysed, a significantly ($p < 0.05$) drastic reduction in polymer A abundance was observed in A500-ASX group compared to the A500 one, that was characterized by the highest level of polymer A accumulation in intestine, liver, and muscle.

Differently, the polymer B accumulation in the intestine was scarce in all the experimental groups and did not show significant differences among them, while no polymer B microbeads were found in liver and muscle samples of all the remaining experimental groups.

Table 3. MPs quantification (number of microbeads/mg) in the intestine, liver, and muscle of adult zebrafish fed experimental diets.

Polymer A (1-5 µm)						
	CTRL	**CTRL-ASX**	**A50**	**A50-ASX**	**A500**	**A500-ASX**
Intestine	0	0	0.9 ± 0.3 ^{a}	2.6 ± 1.9 ^{a}	170.9 ± 20.6 ^{b}	20.5 ± 12.0 ^{a}
Liver	0	0	5.5 ± 1.7 ^{a}	5.5 ± 5.1 ^{a}	821.1 ± 295.5 ^{b}	12.2 ± 5.0 ^{a}
Muscle	0	0	2.0 ± 0.2 ^{a}	1.9 ± 0.9 ^{a}	48.0 ± 4.3 ^{b}	5.2 ± 2.8 ^{a}
Polymer B (40-47 µm)						
	CTRL	**CTRL-ASX**	**B50**	**B50-ASX**	**B500**	**B500-ASX**
Intestine	0	0	0.6 ± 0.3 ^{a}	0.4 ± 0.2 ^{a}	0.8 ± 0.2 ^{a}	0.6 ± 0.4 ^{a}
Liver	0	0	0	0	0	0
Muscle	0	0	0	0	0	0

Data are reported as mean ± SD ($n = 9$). ^{a,b} Within each line, different letters denote statistically significant differences among the experimental group.

4.4 Histology

No pathological alterations or signs of inflammation were evident in both intestine architecture and hepatic parenchyma. Considering the intestinal mucosa, the folds height (Fig.10) and the relative abundance of Ab+ goblet cells (Fig. 11) were evaluated and reported in Table 4.

Table 4. Histological indexes measured in the intestine of adult zebrafish fed the experimental diets.

Polymer A (1-5 µm)						
	CTRL	**CTRL-ASX**	**A50**	**A50-ASX**	**A500**	**A500-ASX**
Mucosal fold height (µm)	127.8 ± 2.9 [a]	130.4 ± 4.5 [a]	125.2 ± 1.2 [a]	126.3 ± 2.3 [a]	124.0 ± 4.6 [a]	127.5 ± 2.9 [a]
Ab+ goblet cells' relative abundance	+	+	++	++	++	++
Polymer B (40-47 µm)						
	CTRL	**CTRL-ASX**	**B50**	**B50-ASX**	**B500**	**B500-ASX**
Mucosal fold height (µm)	127.8 ± 2.9 [a]	130.4 ± 4.5 [a]	91.2 ± 8.7 [b]	120.5 ± 24.1 [a]	90.9 ± 14.3 [b]	128.8 ± 31.4 [a]
Ab+ goblet cells' relative abundance	+	+	++	++	+++	++

Data of mucosal folds height are reported as mean ± SD (n = 15). [a,b] Within each line, different letters denote statistically significant differences among the experimental group. Ab+ goblet cells: + = 0 to 3 per fold; ++ = 4 to 6 per fold; + + + = more than 6 per fold.

No significant differences were detected among the experimental groups fed the A-diets and those fed the Control ones in terms of mucosal fold height. However, groups fed A-diets showed a slight increase in Ab+ goblet cells relative abundance compared to those fed the CTRL and CTRL-ASX diets.

Differently, considering the experimental groups fed B-diets, a significant ($p < 0.05$) reduction in mucosal folds height was evident in fish fed B50 and B500 diets compared to the Control group and the B50-ASX and B500-ASX ones which did not show significant differences among them. In addition, a slight increase in the relative abundance of Ab+ goblet cells was shown by all the groups fed B-diets compared to those fed the CTRL and CTRL-ASX ones, with the exception of B500 group that was

characterized by a more pronounced Ab+ goblet cells increase respect to all the other experimental groups analysed.

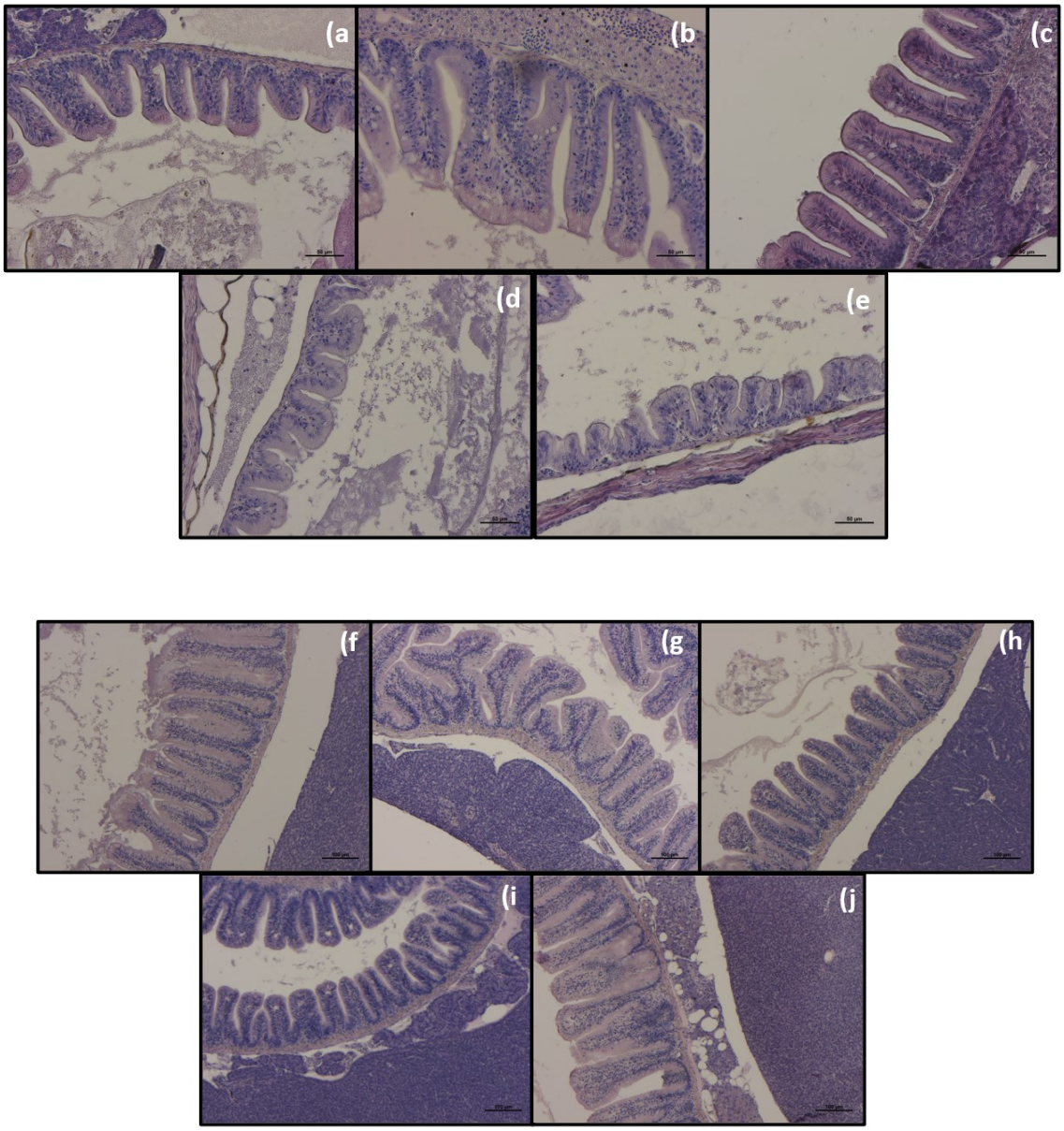

Fig. 10. Gut histomorphology of adult zebrafish fed experimental diets: (a) CTRL, (b) A50, (c) A500, (d) B50, (e) B500, (f) CTRL-ASX, (g) A50-ASX, (h) A500-ASX, (i) B50-ASX, (j) B500-ASX. Scale bar = 50 µm.

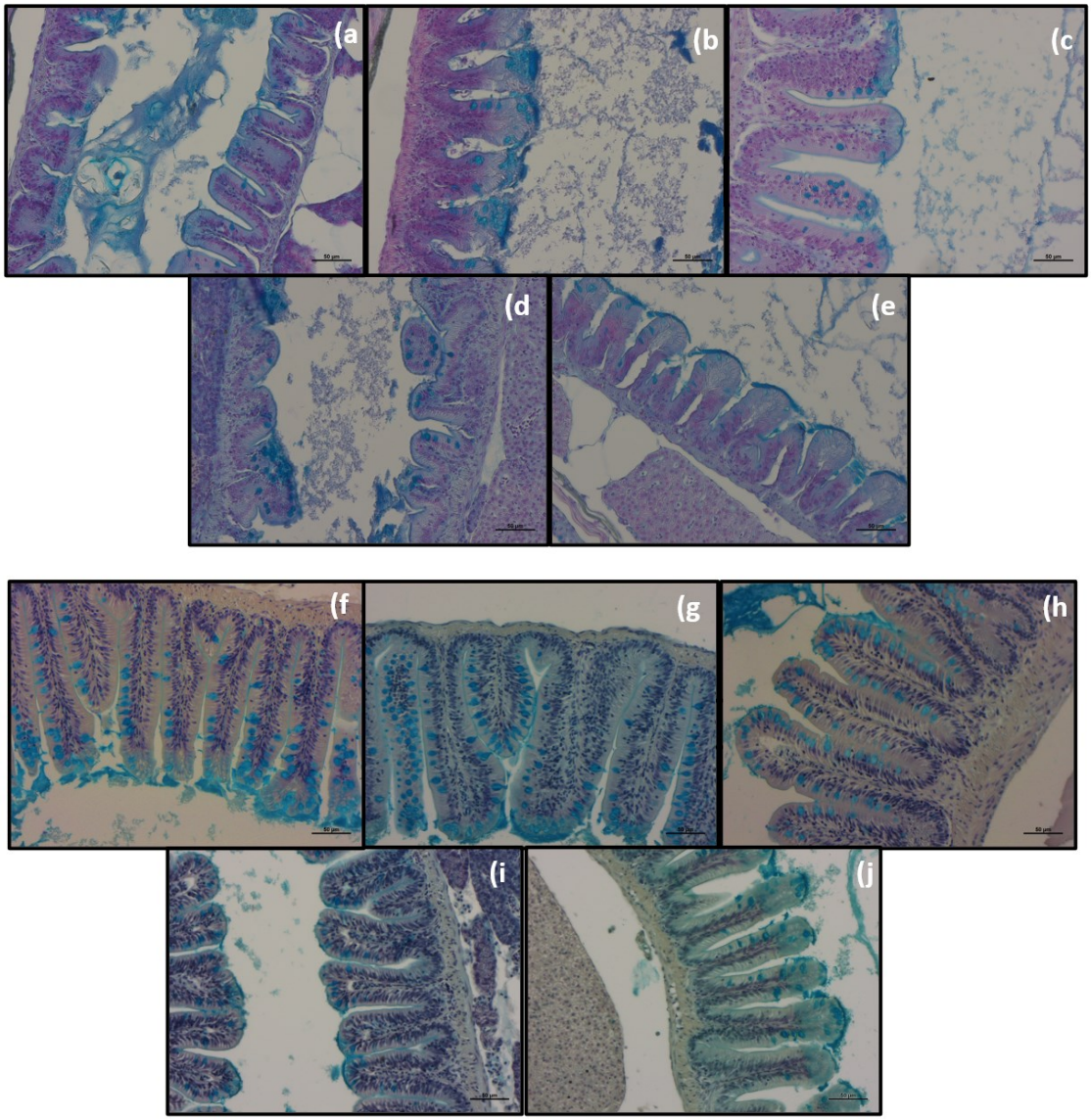

Fig. 11. Gut histomorphology of adult zebrafish fed experimental diets in which mucipar calyciform cells (Goblet cells) are highlighted: (a) CTRL, (b) A50, (c) A500, (d) B50, (e) B500, (f) CTRL-ASX, (g) A50-ASX, (h) A500-ASX, (i) B50-ASX, (j) B500-ASX. Scale bar = 50 µm.

The results obtained from histological analysis of the liver showed normal liver parenchyma structure and a low amount of lipid accumulation in all the experimental groups analysed (Fig. 12).

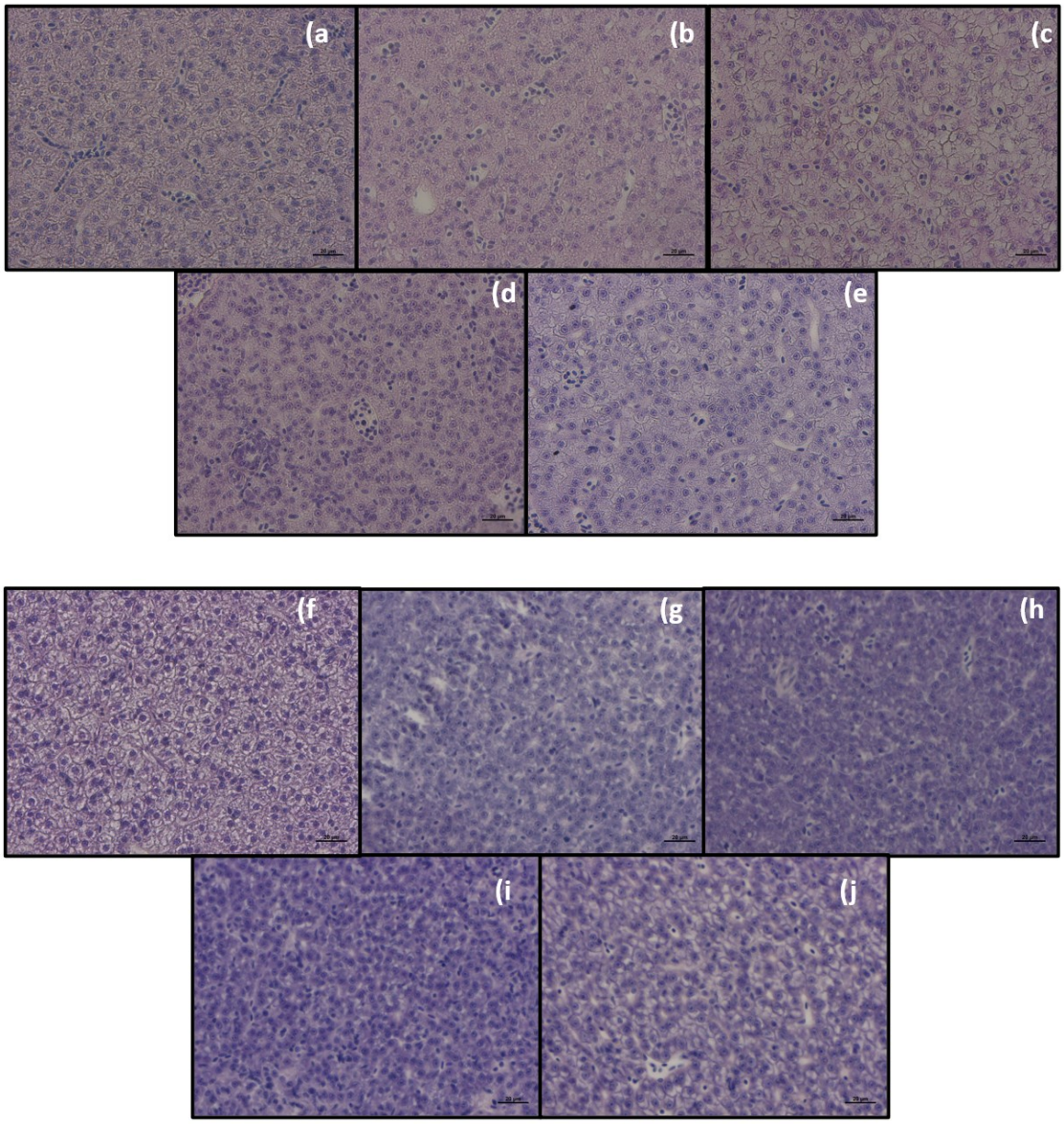

Fig. 12. Representative histologic images of the liver parenchyma of adult zebrafish fed experimental diets: (a) CTRL, (b) A50, (c) A500, (d) B50, (e) B500, (f) CTRL-ASX, (g) A50-ASX, (h) A500-ASX, (i) B50-ASX, (j) B500-ASX. Scale bar = 20 µm.

4.5 Real-time PCR

Intestine. Considering the relative expression of genes involved in the immune response (*il1b*, *il10*, *litaf*), no significant differences were evident among experimental groups fed

the A-diets and those fed the Control ones (Fig. 13 a-c) as well as among those fed B-diets and those fed the Control ones (Fig. 13 d-f).

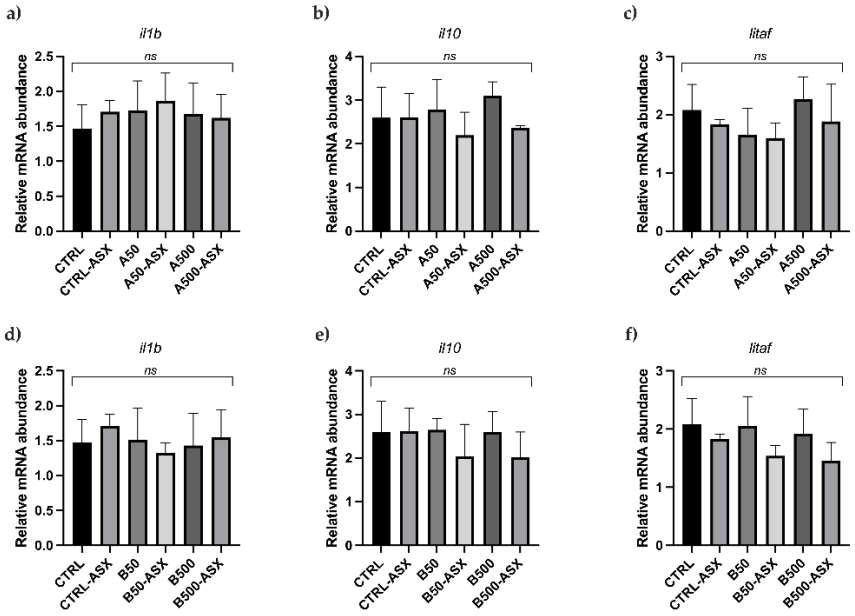

Figure 13. Relative mRNA abundance of genes involved in immune response (*il1b*, *il10*, *litaf*) analysed in the intestine of adult zebrafish fed the experimental diets. (**a-c**) groups fed A-diets (A50, A50-ASX, A500, A500-ASX) compared to those fed the Control ones (CTRL and CTRL-ASX); (**d-f**) groups fed B-diets (B50, B50-ASX, B500, B500-ASX) compared to those fed the Control ones. Data are reported as mean ± SD (n = 5). *ns*, no significant differences among the experimental groups ($p > 0.05$).

Liver. Figure 14 reports the relative expression of genes involved in the stress response. As regards fish fed the A-diets, a significant ($p < 0.05$) upregulation of both *nr3c1* (Fig. 14 a) and *hsp70.1* (Fig. 14 b) was detected in A50 and A500 groups compared to both the Control group and the A50-ASX and A500-ASX ones which did not show significant differences among them. Differently, no significant differences in terms of both *nr3c1* (Fig. 14 c) and *hsp70.1* (Fig. 14 d) relative gene expression were evident among groups fed B-diets and those fed the Control one.

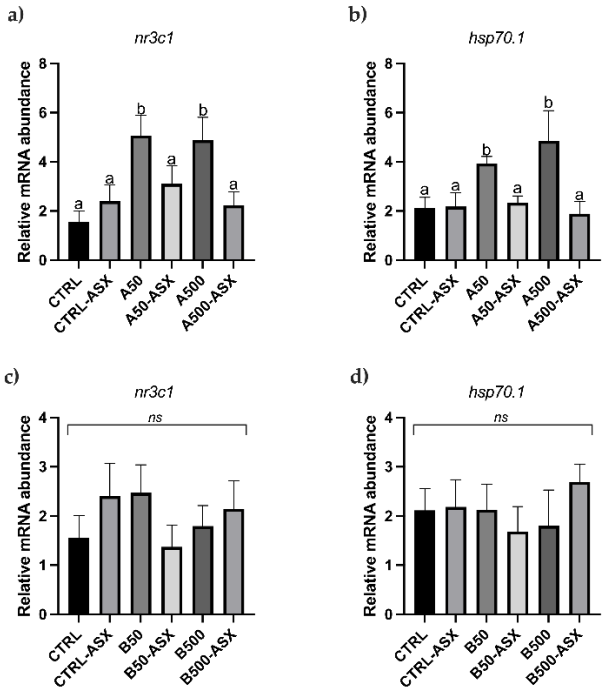

Figure 14. Relative mRNA abundance of genes involved in stress response (*nr3c1* and *hsp70.1*) analysed in the intestine of adult zebrafish fed the experimental diets. (**a**,**b**) groups fed A-diets (A50, A50-ASX, A500, A500-ASX) compared to those fed the Control ones (CTRL and CTRL-ASX); (**c**,**d**) groups fed B-diets (B50, B50-ASX, B500, B500-ASX) compared to those fed the Control ones. Data are reported as mean ± SD (n = 5). [a,b] Different letters denote statistically significant differences among the experimental group. *ns*, no significant differences among the experimental groups ($p > 0.05$).

Finally, Figure 15 shows the relative expression of genes involved in the oxidative stress response. Considering fish fed A-diets, a significant ($p < 0.05$) upregulation of *sod1*, *sod2,* and *cat* was observed in A50, A500, and A500-ASX groups compared to CTRL and CTRL-ASX groups (Fig. 15a-c). Differently, no significant differences were detected among both the Control groups and A50-ASX for *sod1*, *sod2,* and *cat* relative gene expression (Fig. 15a-c).

As regards fish fed B-diets, a significant ($p < 0.05$) upregulation of *sod1* was observed in B50 and B500 groups compared to the other experimental groups that did not show significant differences among them (Fig. 15 d). Finally, no significant differences were evident among the experimental groups fed the B-diets and those fed the Control ones in terms of both *sod2* (Fig. 15 e) and *cat* (Fig. 15 f) relative gene expression.

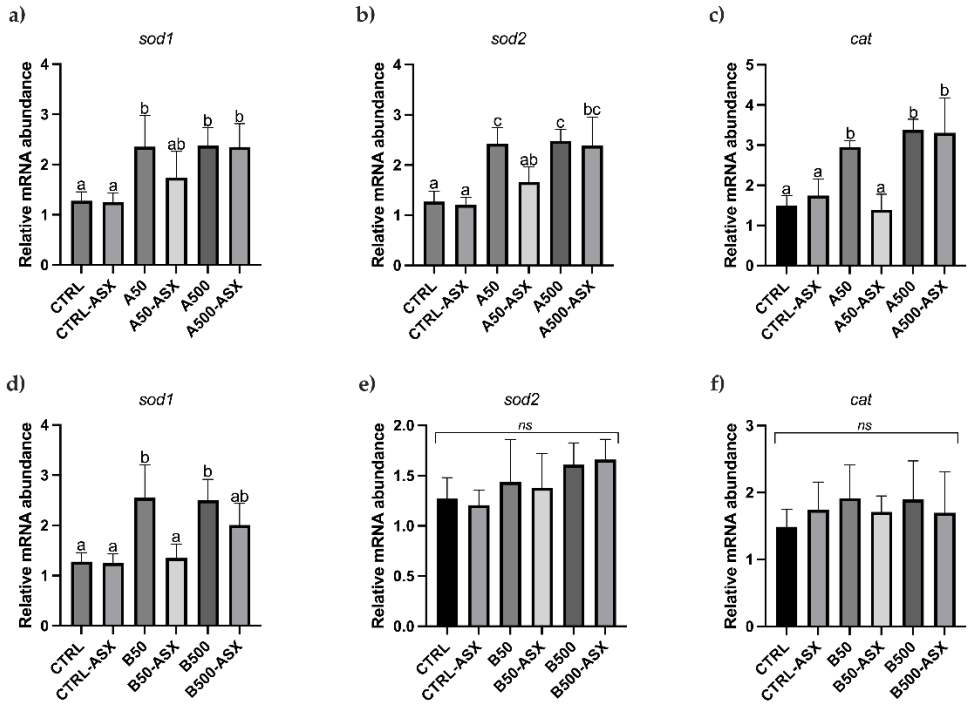

Figure 15. Relative mRNA abundance of genes involved in oxidative stress (*sod1, sod2 and cat*) analysed in the intestine of adult zebrafish fed the experimental diets. (**a-c**) groups fed A-diets (A50, A50-ASX, A500, A500-ASX) compared to those fed the Control ones (CTRL and CTRL-ASX); (**d-f**) groups fed B-diets (B50, B50-ASX, B500, B500-ASX) compared to those fed the Control ones. Data are reported as mean ± SD (n = 5). [a-c] Different letters denote statistically significant differences among the experimental group. *ns*, no significant differences among the experimental groups ($p > 0.05$).

5. Discussion

Microplastics pollution, nowadays, represents an ongoing and increasing problem with potential impact on marine ecosystems and organisms' health (Hale et al., 2020). This issue is also present in the aquaculture sector where MPs contamination can occur through wastewater, fishing gears, general equipment and aquafeed ingredients including fishmeal, directly derived from fish caught in a sea increasingly contaminated by the presence of these particles (Iheanacho et al., 2023). The quality and safety of the farmed

fish products are closely related to the health of the animal itself but also of the final human consumer (Vethaak and Leslie, 2016). Since no immediate remedies can be found to eliminate MPs in the sea and in the aquafeed ingredients, solutions to mitigate the MPs negative effects and to improve fish welfare and quality traits are needed.

Therefore, the purpose of the following study was to better understand the impact of different sized dietary microplastics on zebrafish (*Dario rerio*) individuals but also to test if astaxanthin can mitigate the negative side effects of dietary MPs exposure.

Survival rate and growth rate were not affected in all the experimental groups analysed and thus no significant differences were evidenced.

As regards MPs accumulation, the absence of these particles in control groups, CTRL and CTRL-ASX, was confirmed both by chemical digestion and confocal microscopy in all tissues investigated.

Considering the microbeads of the bigger size, polymer B (40-47 μm), they were not found in all the analysed tissues through the confocal microscopy and chemical digestion except for the intestine in which they were detected in a scattered way. This result agrees with the study carried out by Qiao et al., (2019) conducted on zebrafish, which higlighted that MPs characterized by a size > 20 μm simply transited through the gut lumen without being absorbed. The scattered presence of polymer B microbeads in the intestine can thus be related to the 24 hour-starving period prior the sampling, a sufficient time to have an empty gut.

Conversely, polymer A microbeads, with size of 1-5 μm, were found in intestine, liver and muscle using confocal microscopy demonstrating that these smaller microbeads are absorbed by the intestinal folds and, in accordance to a study conducted by De Sales-

Ribeiro et al., (2020) on zebrafish, using MPs of the same range, are secondly translocated to other tissues and organs. With the chemical digestion more robust results were obtained highlighting a higher number of polymer A microbeads in liver compared to intestine and muscle in all the experimental groups fed on polymer A-diet. This reflects the results obtained by Cattaneo et al., (2023) on zebrafish larvae and juveniles fed on the same diets, supporting one more time the concept that the liver acts as an accumulation organ for these MPs. In this sense the liver protects other tissues like the muscle from dietary MP contamination, resulting particularly interesting for the aquaculture production since the muscle represents the edible part of the fish for the final consumers.

Additionally, these results are in line with a previous study performed on European seabass which showed that fluorescent MPs beads of 1-5 μm in size were found in the fish fillet in a significant lower amount compared to the ingested quantity (Wan et al., 2019).

Quantitative analyses also showed a dose dependent accumulation of MPs in A-diets groups and evidenced a significant decrease of MPs quantity between A500 and A500-ASX groups of around 10 times in intestine and muscle, and even of 70 times in liver. This is a very interesting result which may be a possible strategy to reduce MPs accumulation in fish. However, the present study was not able to explain if the reason of this reduction was related to ASX administration or to the encapsulation process used. Nevertheless, since this difference was not detected in groups A50 and A50-ASX, the encapsulation process is probably the cause of this reduced MPs accumulation together with a higher dietary MPs content. The encapsulation process used the +POP technology which is made of a number of substances able to melt in the fish digestive tract after a certain time. These molecules are nontoxic and indigestible by fish and may have thus

occurred in clogging the smaller MPs beads in bigger ones that were not able to be absorbed at intestinal level. In this sense, further studies are necessary to better elucidate the role of +POP.

In each case, the delivery to fish of ASX showed some positive results. Groups fed on B-diets and ASX didn't show a relevant reduction of the mucosal folds' height, but only a small increase in the number of goblet cells. On the contrary, the experimental groups fed on the same diets but without ASX showed more severe signs of mucosal folds abrasion and a higher number of goblet cells. Since this polymer was not absorbed at intestinal level all the other analysis performed did not show significant differences.

Polymer A dietary administration showed more marked results. While no abrasive effect on mucosal folds was evidenced nor inflammatory events were detected at intestinal level, only a slight increase in the number of goblet cells was detected, suggesting that the intestine is simply a transit organ for these MPs.

On the contrary, at hepatic level, even if the histological analysis did not reveal any inflammation, molecular analyses evidenced an activation of stress and oxidative stress pathways, in a A50 and A500 groups, compared to CTRL group. These results are in accord to previous studies carried out on zebrafish (Lu et al., 2016) or on commercially important species such as sea bream (*Sparus aurata*) (Capó et al., 2021) that showed an increased oxidative stress in fish fed diets containing microplastics. On the other hand, other studies conducted on sea bass (*Dicentrarchus labrax*) and on striped mullet (*Mullus surmuletus*) (Alomar et al., 2017) showed opposite results. This inconsistency of the results must be related to the different MPs chemical features, size and shape as well as dietary concentration used in the different studies.

The inclusion of ASX in the diets showed promising results in counteracting the negative side effects of hepatic MPs accumulation and oxidative stress activation in fish. This was particularly evident in A50 groups which were exposed to a MPs dietary concentration similar to the natural one. The less promising results obtained in A500-ASXgroups can thus be related to a very high and unrealistic MPs concentration against which, the amount of ASX provided to the fish, was not able to mitigate the side effects.

Even if the quantity of MPs presents in intestine, liver and muscle of A500-ASX group decreased a lot compared to A500 group, the amount of ASX administrated is not enough to reach MPs quantity similar to A50-ASX group. Thus may be the reason of oxidative stress up regulation in A500-ASX group.

6. Conclusions

The present study showed that diets containing MPs of different sizes did not adversely affect the growth performance of zebrafish but depending on the size of the polymer associated with the feed, may have adverse effects on the exposed animals.

MPs microbeads of 40-47 μm in size only transited through the gut lumen without being absorbed causing an abrasive effect on intestinal mucosal folds and an increase in number of goblet cells. The administration of astaxanthin, as bioactive molecule, helped to reduce the mechanic damage caused by MP, preventing the reduction in mucosal fold's height.

The smaller polymer (1-5 μm) was absorbed at the mucosal folds level and was translocated mainly to the liver and only partially to the muscle. While the intestine acted as a transit organ, the liver acted as a trapping one, accumulating most of the MPS. As a consequence, no inflammation or alteration of the biomarkers analysed was observed at intestinal level while an increase in stress and oxidative stress was observed at hepatic level.

A beneficial effect against oxidative stress of ASX was evident in groups fed diets containing 50 mg of polymer A per kg of feed. Furthermore, the most interesting results was that the implementation of ASX in the diet is able to reduce the quantity of MPs accumulated by fish in intestine, liver and muscle.

All these are very applicative results in the aquaculture sector to maintain a welfare status of the reared animals and to safeguard the health of final consumers.

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