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Influence of RAS features on fish stress and welfare indicators

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Influence of RAS features on fish stress and welfare indicators

Safeguarding future production of fish in aquaculture systems with water recirculation (RASOPTA)

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Resum

El benestar dels peixos ha captat una atenció considerable durant l'última dècada, no només de la comunitat científica, sinó també del públic en general. Unes condicions adequades de cria dels peixos en captivitat, ja siguin destinats a la producció d'aliments per part de la indústria aqüícola o per a la investigació de laboratori, ofereix avantatges. A les instal·lacions d'aqüicultura modernes, com les que treballen en condicions de recirculació (sistemes d'aqüicultura de recirculació o RAS, segons les sigles en anglès), els peixos es poden protegir de les fluctuacions abruptes dels paràmetres ambientals (*e.g.* temperatura, concentracions de compostos nitrogenats, disponibilitat d'oxigen dissolt), però encara estan exposats a una varietat de factors estressants, inclosos els contaminants ambientals i altres estímuls (*e.g.* manipulació, transport, soroll). Investigar la resposta a l'estrès dels peixos de cultiu a aquests factors estressants i determinar el seu impacte en l'estat de salut dels animals és de gran importància per garantir el benestar dels peixos en RAS.

La present tesi s'ha centrat en investigar l'impacte de dos tipus d'estressors en la salut i el benestar dels peixos. D'una banda, es van avaluar els efectes de les exposicions prolongades a concentracions rellevants per al medi ambient i pics de contaminants emergents (CEC, segons les sigles en anglès), en particular, el gemfibrozil, un producte farmacèutic que regula els lípids, i els nanoplàstics de poliestirè, tots dos omnipresents als ecosistemes aquàtics, tant en espècies d'interès científic com comercial (*Sparus aurata* i *Carassius auratus*). D'altra banda, es van dur a terme dos estudis de cas, un investigant -i confirmant- la presència de nanoplàstics en RAS, i l'altre estudiant l'impacte de l'estrès sobre l'estat de salut del salmó (*Salmo salar*) després d'una operació de transferència rutinària de peixos en un RAS comercial. Es va utilitzar una combinació de tècniques tradicionals i -òmiques per intentar obtenir una comprensió integral de la resposta d'aquestes espècies als factors d'estrès esmentats.

Tant el gemfibrozil com els nanoplàstics van desencadenar lleus respostes antioxidants en el peix d'aigua dolça *C. auratus*. A més, els resultats indiquen que aquests CEC podrien alterar l'assignació de recursos energètics o el metabolisme energètic de les espècies estudiades, tot i que no es van observar efectes significatius sobre la mida, el pes o el creixement al llarg de l'exposició experimental. El més interessant és que el segon estudi de cas va aportar evidències rellevants sobre la inhibició de respostes associades a l'estrès en S. salar criats en SAR, suggerint que aquests peixos es troben sotmesos a un estrès crònic greu i llavors no generan una resposta completa.

Les investigacions posteriors haurien de centrar-se a confirmar aquesta darrera troballa i a investigar quins factors del RAS són potencialment responsables d'aquestes respostes. A més, s'haurien de destinar esforços a la reducció de la producció i alliberament de CEC, així com a la mitigació de la contaminació ja existent. També s'hauria de prestar especial atenció a l'eficàcia de les matrius biològiques no invasives per determinar la salut i el benestar dels peixos, per minimitzar el nombre de peixos manipulats i sacrificats amb aquesta finalitat.

Abstract

Fish welfare has gained considerable attention over the last decade, not only from the scientific community, but also from the general public. Adequate rearing and husbandry conditions for fish in captivity, whether destined for food production by the aquaculture industry or laboratory research, provides a series of advantages. In modern aquaculture facilities, such as those working under recirculating conditions (*i.e.* recirculating aquaculture systems, RAS), fish may be sheltered from abrupt fluctuations in environmental parameters (e.g. temperature, concentrations of nitrogenous compounds, dissolved oxygen availability), but are still exposed to a variety of stressors, including environmental pollutants and physical insults, as well as those inherently associated with RAS (*e.g.* handling, transportation, noise). Investigating the stress response of cultured fish to these stressors and determining their impact on the health status of the animals is of utmost importance to ensuring the welfare of fish in RAS.

The present thesis focused on investigating the impact of two types of stressors on the health and welfare of fish. On one side, the effects of prolonged exposures to environmentally relevant and spiked concentrations of contaminants of emerging concern (CECs), namely gemfibrozil, a lipid-regulating pharmaceutical, and polystyrene nanoplastics, both ubiquitous in aquatic ecosystems, were evaluated in species of particular scientific or commercial interest (i.e. *Carassius auratus* and *Sparus aurata*). On the other side, two case studies were conducted, one investigating – and confirming – the presence of nanoplastics in RAS, and the other studying the impact of a routine transfer operation on the health and stress status of *S. salar* in a commercial RAS. When possible, a combination of traditional and -omics techniques were employed in an attempt to obtain a comprehensive understanding of the response of these species to the aforementioned stressors.

Both gemfibrozil and nanoplastics triggered slight antioxidant responses in the freshwater *C. auratus*. Moreover, the results indicate that these CECs might alter the allocation of energetic resources, or the energy metabolism of the studies species, although no significant effects on size, weight, or growth were observed throughout the experimental exposure.

Most interestingly, the second case study hereby conducted provided insightful evidence on inhibited stress-related responses in *Salmo salar* reared in RAS, indicating that these fish do not mount a complete stress response under severe chronic stress.

Further research should focus on confirming this last finding, and on investigating which factor of RAS conditions are potentially responsible. Moreover, efforts should be allocated to the reduction of CECs production and release, as well as the mitigation of already existing contamination. In addition, particular attention should be given to the efficacy of non-invasive matrices to determine fish health and welfare to minimise the number of fish manipulated and sacrificed to this end.

Introduction

1

1. Introduction

Aquaculture, an ancient human activity, now plays a major role in ensuring global food supply. It is the fastest growing sector of the food production industry, and, with an increase of 6.6% between 2020 and 2022, it surpassed the fisheries sector for the first time, accounting for over 50% of all obtained seafood (FAO, 2024). Moreover, aquaculture emerges as a powerful bioremediation tool, through the rearing of organisms for the restocking of wild populations or the removal of pollutants in aquatic systems, for instance (Domingues et al., 2022; Loneragan et al., 2013; Nabhitabhata & Segawa, 2014). Sustainable aquaculture practices are essential to ensuring the environmental viability of this industry and are a keystone instrument in meeting many United Nation's Sustainable Development Goals (*e.g.* SDG2, SDG3, SDG12, SDG13, SDG14, SDG15, SDG17; Figure 1).

Figure 1: United Nation's Sustainable Development Goals (SDGs) providing guidelines shared amongst all member states in order to reach peace and prosperity for people and the planet. The content of this publication has not been approved by the United Nations and does not reflect the views of the United Nations or its officials or Member States.

1.1 Aquaculture and RAS

Although traditional aquaculture practices, such as flowthrough systems and ponds, still account for a major part of the facilities around the world, an increasing number of companies are turning to recirculated aquaculture systems (RAS). These systems offer culture facilities virtually isolated from

the external environment, decreasing interactions with surrounding ecosystems, reducing their environmental impact by minimising the occurrence of escapees, the horizontal transmission of disease to wild populations, and significantly increasing the quality of the water being released back into natural systems (Ahmed & Turchini, 2021; Bregnballe, 2022; Summerfelt & Vinci, 2008). However, the high associated costs, and the advanced technological knowledge and skills required to manage a RAS limits their implementation in areas where affordable fish protein is essential and resources scarce (Badiola et al., 2012, 2018).

1.2 Stressors in aquaculture

In aquaculture facilities, cultured organisms are subjected to a variety of stressors, including both biotic and abiotic factors. These can be classified as environmental and chemical stressors (*e.g.* temperature, dissolved oxygen, nitrogenous compounds, contaminants of emerging concern – CECs, pathogens), physical and mechanical stressors (*e.g.* handling, transportation, density, predation), and stressors related to the facility's structure (*e.g.* soundscape, photoperiod; Calabrese et al., 2017; Hang et al., 2021). In RAS, many of these, such as water quality, are controlled for, preventing significant fluctuations in these parameters (Bregnballe, 2022). In addition, RAS structures shelter the animals from climatic events and predators (Ahmed & Turchini, 2021). Thus, many stressors commonly encountered by fish in aquaculture facilities are absent in RAS. However, certain chemicals are poorly removed by RAS' filtration systems (*e.g.* pharmaceuticals) or originate from within the system's components (*e.g.* nanoplastics; NPs), and certain stressors are simply associated with common practices in such facilities (*e.g.* 24 h photoperiod, handling and transportation). Therefore, fish reared in RAS are still exposed to certain factors that might significantly influence their stress status and, thus, their welfare.

CECs are compounds that are not fully regulated, that are recognised as present in the environment, and whose effects on wildlife and humans, although poorly understood, are expected to be deleterious (*i.e.* NPs, pharmaceuticals). Often through a combination of high input rates into natural ecosystems, and slow degradation rates, many of these compounds are considered ubiquitous (Barreto et al., 2023; Gavrilescu et al., 2015). Therefore, understanding their presence in aquaculture systems, and their impact on the cultured organism is essential to determine their effect on the aquaculture sector and food security.

1.3 Fish stress and welfare

This thesis focuses on health and welfare of fish cultured in RAS and their response to stressors. Thus, it is important to tentatively clarify these concepts.

1.3.1 Fish stress

Among the many definitions of stress, the one considered for the present thesis was described by Schreck (2000), stating that "Stress is the physiological cascade of events that occurs when the organism is attempting to resist death or reestablish homeostatic norms in the face of an insult".

Stress can be classified in several ways, depending on its predictability, avoidability, intensity and duration. In most cases, stressors will be acute (*i.e.* short-term), avoidable, and predictable. Thus, the fish, having previously encountered similar stressors, has natural mechanisms to overcome the situation without further negative consequences. This stress is therefore adaptive, having positive

consequences on the individual, and denoted eustress (Schreck & Tort, 2016). However, if a stressor is severe, unavoidable or unpredictable, and lasts over prolonged periods (*i.e.* chronic), it can be maladaptive, having deleterious consequences on an individual's fitness and potentially affecting its survival. This type of stress is known as distress (Schreck, 2010; Yada & Tort, 2016).

1.3.2 Stress response in fishes

The stress response in fish, as in most vertebrates, can be classified in three categories; the primary stress response driven by the regulatory systems, which activates the secondary response of the coordinated physiological systems, which, in turn, triggers the tertiary response that shows the performance traits of the animal.

The primary stress response involves all regulatory systems (nervous, endocrine and immune) and is characterised by the activation of two main neuroendocrine stress axes; the brain-sympathetic-chromaffin (BSC) axis, and the hypothalamus-pituitary-interrenal (HPI) axis, later analogous to the mammalian hypothalamus-pituitary-adrenal (HPA) axis (Harris & Carr, 2016). The activation of the BSC axis consists of the stimulation of chromaffin cells in the head-kidney by neuronal signals leading to the release of catecholamines such as dopamine, adrenaline and noradrenaline in the bloodstream (Tort et al., 2024). The liberation of these neurotransmitters is almost instantaneous, making it difficult to evaluate in fish without interference of handling procedures in the measurements (Schreck & Tort, 2016). On the other hand, the activation of the HPI axis is slightly slower and more persistent. It is characterised by the release of corticotropin-releasing hormone/factor (CRH/CRF) from the brain into the pituitary, where it induces the release of adrenocorticotropin hormone (ACTH; Schreck & Tort, 2016). This hormone prompts the production and release of glucocorticoids (*e.g.* cortisol) from the head-kidney. In turn, cortisol inhibits the secretion of CRH in the hypothalamus and ACTH in a negative feedback loop (Mommsen et al., 1999). Finally, the increase in circulating levels of cortisol and catecholamines induces the next secondary stage of the stress response.

The secondary stress response is characterised by changes at the metabolic level reflecting the differential allocation of energetic resources in order to cope with the stressor (Schreck & Tort, 2016). These include alterations in the haematological profile (Caruso et al., 2005), changes in circulating lactate and glucose levels (Faught et al., 2016; Hermann et al., 2019; Wendelaar Bonga, 1997), cardiovascular responses, as well as changes in ion and acid/base balances, and immune function (Rodnick & Planas, 2016).

If homeostasis has not been returned to, following the primary and secondary responses, the tertiary response follows. This consists in medium-to-long-term alterations in the overall performance of an individual, with decreased growth rates, hindered reproductive ability, decreased disease resistance and altered cognition and behaviour (Noakes & Jones, 2016; Pankhurst, 2016; Sadoul & Vijayan, 2016).

1.3.3 Fish welfare

Welfare is a particularly difficult concept to define in animals. Although it has been suggested that it might simply be considered as the absence of stress, this absence of stress response indicators does not necessarily translate into a lack of stress (Schreck & Tort, 2016), making this extremely difficult

to assess. Moreover, it is likely that in the complete absence of stress, including eustress, the performance of an individual would be suboptimal (Schreck, 2010), potentially affecting its welfare status. In general, the notion of welfare, particularly on a legislation point of view, lies on the "Five Freedoms" principle, which are considered the optimal ethical requirements for animals held in captivity.

The five freedoms are the following:

- Freedom from thirst and hunger
- Freedom from fear and distress
- Freedom from pain, injury and disease
- Freedom from discomfort
- Freedom to exhibit normal behaviour, including reproduction

Although these might appear as unattainable standards, seldom aligning for either wild or farmed animals, they offer guidelines for the husbandry of animals in captivity. The "Five Freedoms" have been questioned over the years, and the idea of welfare has evolved to include the "Five Domains", and the concept of "A Life Worth Living". Strengths and limitations of each of these concepts are discussed by Webster (2016). Thus, welfare rests on the concept of quality of life and wellbeing as experienced by the animals (Stien et al., 2013).

1.3.4 Importance of fish welfare (Aquaculture production, public perception, scientific robustness)

The importance of maximising welfare for captive animals can be appreciated from various perspectives.

Firstly, significant disturbances in fishes' performance, including immunosuppression, as well as increased aggression and reduced growth rates, as a result of poor welfare, might have serious deleterious effects on the production of the aquaculture sector, affecting the economic viability of this industry in the short term, and impacting global food security and human health, on the long run.

Second, the public perception of the seafood production industry, including both freshwater and marine species, has evolved over the last decade, with an increasing number of consumers placing greater importance on the origin of the product, and the conditions under which the fish was cultivated and slaughtered (Röcklinsberg, 2015). Given the potential increase in product price arising from responsible production, it is of greatest relevance to ensure optimal rearing conditions for cultured species, not only from an ethical point of view, but also from an economical perspective (Seibel et al., 2020).

Lastly, animal welfare is of utmost importance to science. Indeed, ensuring adequate husbandry protocols, good laboratory practices and ethical animal handling, both before and during experimental procedures, minimises unwanted stress on experimental subjects and any subsequent bias, maximising the robustness of the design, the replicability of the experiment, and the reliability of the obtained results (Loss et al., 2021; Moritz et al., 2024).

1.3.5 Stress and welfare indicators in fish

Understanding and selecting the appropriate stress indicators is essential for the early detection of welfare and immune compromising parameters, and for the prevention of mass mortality events. Although stress indicators in fish are generally shared amongst species, their sensitivity and critical values are mostly species-specific. An important factor to take into consideration when selecting indicators for stress or welfare is the degree of invasiveness. Some, denoted as non-invasive indicators, might be assessed through simple visual observation (*e.g.* behaviour) or water analyses, whereas others require handling (*i.e.* little-invasive), biopsy (*i.e.* invasive) or even euthanising individuals (*i.e.* lethal), which will inevitably result in stress for the animals or in production loss for farm managers. In addition, the representativity and relevance of indicators must be evaluated in relation to the aspects of stress or welfare to be investigated. In many cases, non-invasive methods can be employed to gain an interesting insight on the health and welfare status of individuals, or groups of fish in aquaculture systems (e.g. water parameters, behavioural indicators). Below follows a table listing different indicators that might be able to assess the welfare status of fish in aquaculture farms (Table 1). This list is non-exhaustive, and indicators should be selected carefully on a case-by-case basis, considering all factors, such as invasiveness, as well as required equipment, technical knowledge and other associated costs. Baseline values for indicators vary between species and might display further variation depending on sex and life-stage. Thus, establishing reference values – which are often hard to determine or not readily available - for these indicators is essential for their effective implementation. Additionally, it must be noted that single-indicator approaches are likely to provide incomplete information leading stakeholders to draw inaccurate conclusions, and that a combination of indicators should be employed to obtain a comprehensive understanding of the stress and welfare status of farmed animals. Regarding welfare indicators specifically, those are related to performance and to relevant markers of environmental quality, together with the absence of stress indicators. On the other hand, it is much more difficult to assess the “good-welfare indicators”, as the ones showing that the fish is able to develop normal behaviour

Matrix	Invasiveness	Indicates	Indicator/Assay	High Cost	Special Equipment	Comments	Reference
Blood	Little invasive	Oxygen carrying capacity	Erythrocyte cell count	No	No	Prompt analysis.	Barreto et al., 2017
			Haematocrit	-	-	Anticoagulants	Braham et al., 2017
			Haemoglobin concentration	-	-	(*e.g.* heparin)	de Andrade et al., 2004
		Immune response	Leukocyte cell count	-	-	must be added	Ruiz et al., 2024
		Presence of wounds	Thrombocyte count	-	-	to the blood and	Parrino et al., 2018
		Genotoxicity (Intracellular damage)	Erythrocyte nuclear abnormalities (ENA)	-	-	analysed promptly.	
			Micronuclei	-	-	Cannot be	
			Comet assay	-	-	frozen.	
Plasma/Serum	Little invasive	Physiological stress	Catecholamines	No	No	Minimal	Bertotto et al., 2009
			Cortisol	-	-	manipulation	Franco-Martinez et al., 2022
			Lactate	-	-	needed to	Sadoul & Geffroy, 2019
		Hepatic health	Glucose	-	-	prevent	Teles et al., 2019
			Alanine transaminase	-	-	interference with	Lulijwa et al., 2022
			Alkaline phosphatase	-	-	the actual	
		Oxidative stress/Antioxidant responses	Aspartate transaminase	-	-	measured	
			Total antioxidant capacity	-	-	values.	
			Total oxidative status	-	-		
		Immune activity					
			Esterase activity	-	-		
			Bacteriolytic capacity	-	-		
		Metabolic status	Lysozyme activity	-	-		
			Metabolomic profiling (*e.g.* through nuclear magnetic resonance)	Yes	Yes		
Mucosal surfaces (*e.g.* gills, gut, skin)	Lethal	Metabolic status	Metabolomics	Yes	Yes		Lulijwa et al., 2022 Merrifield & Rodiles, 2015
		Composition microbiota/ Dysbiosis	Microbiome profiling	-	-	Difficult to assess as microbial ecology assessment still in progress.	Li et al., 2012 Liang et al., 2021 Zhang et al., 2018 Sadoul & Geffroy, 2019
		Transcriptome status	RNA-Sequencing (RNASeq)	-	-		
		Gene expression:	Real-time quantitative PCR (RT-qPCR)	No	-		

Mucosal surfaces (*e.g.* gills, gut, skin)	Lethal	Immune function Oxidative stress Physiological stress Lipid metabolism Osmolality	Interleukins, *mhc,* *sod, cat* *gr, mucins, mr* *ppara, lpl*			Targeted analysis. Investigating single endpoints might lead to inaccurate conclusions.	
		Physiological stress, immune activity… (as in plasma/serum)	In mucus samples, biochemical indicators can be measured similarly to plasma/serum *e.g.* cortisol, glucose	No	No		
		Tissue damage	Histopathology	-	-	Potential use of carcinogenic compounds (*e.g.* formaldehyde). Requires training.	
Internal organs	Lethal	Metabolic status Transcriptome Immune function Lipid metabolism Gene expression	Metabolomics RNAseq *e.g.* Esterase activity *e.g.* Cholesterol RT-qPCR (*e.g. gr, crh, acth, star*)	Yes - No - -	Yes - No - Yes	Target genes are tissue-dependent	Lulijwa et al., 2022 Lama et al., 2020
		Tissue damage	Histopathology	-	No		
Faeces	Non-invasive (Lethal if collected from gastrointestinal tract)	Physiological stress Intestinal health Microbiome	Cortisol	No	No	No information at the individual level or at specific time point. Depends on sludge collection.	Sadoul & Geffroy, 2019 Silva et al., 2020
				Yes	Yes		
Behaviour	Non-invasive	Feeding activity	Feed anticipatory activity Foraging behaviour	No -	No -	Difficult to assess with high stocking density	Noble et al., 2018
		Social behaviour	Shoaling Aggressive behaviour	- No	- No		

Behaviour	Non-invasive	Swimming performance and health status	Position in tank	-	-	Difficult to assess with high stocking density	Noble et al., 2018
			Balance/reflexes	-	-		
			Speed	-	-		
		Cardiovascular health, strenuous physical activity	Ventilation rate (opercular movement)	-	-		
			Cardiac activity	-	Yes		
		Poor welfare	Erratic swimming	-	No		
		Neural impairment	Lethargy	-	-		
			Stereotypical behaviour			Needs scoring	
Scales, fin clips	Little-invasive	Physiological stress	Cortisol	No	No	Requires manipulation	Sadoul & Geffroy, 2019
Whole body	Non-invasive	Welfare (can be related to water quality, aggression or presence of pathogens)	Deformities	No	No	In some cases, evident signs of disease appear too late for treatment.	Noble et al., 2018
			Wounds	-	-		
			Presence of parasites	-	-		
			Clinical signs	-	-		
	Lethal	Physiological stress	Cortisol	-	-	Clinical signs might not be pathogen specific.	
		Hepatic health					
		Gonadal health	Hepatosomatic Index	-	-		
			Gonadosomatic Index	-	-		
Performance	Little to non-invasive	Overall performance	Growth	No	No	Requires some degree of manipulation in some cases	Ramírez-Coronel et al., 2024 Sopinka et al., 2016
			Size at maturity	-	-		
			Feed Conversion Ratio	-	-		
			Specific Growth Rates	-	-		
			Survival	-	-		
		Reproductive capacity	Egg and sperm viability	-	-		
			Number of eggs	-	-		
Product quality	Lethal	Filet quality	Rigor mortis onset time	No	No	Measurements performed at end of production line.	Noble et al., 2018 Poli et al., 2005 Sánchez-Muros et al., 2013
			Colour	-	Yes		
		Nutritional quality	Fat content	-	No		
			Protein content	-	-		
Biofilter	Non-invasive	Nitrification efficiency	Microbiome	Yes	Yes	Directly affects water quality.	Ridha & Cruz, 2001

						Indirect welfare indicator.	
Water	Non-invasive	Pathogen load	eDNA (pathogen genetic material)	No	Yes	Pathogen-specific.	Bohara et al., 2022 Benedicenti et al., 2024
		Pathogen load/host gene expression	eRNA (host/virus genetic material)	-	-	Difficult to assess link between environmental host pathogenic load, and outbreaks. Link between water and host RNA abundance in progress. High interference rates do - full sequencing not recommended.	Hiki et al., 2023 Miyata et al., 2025 Sadoul & Geffroy, 2019 Noble et al., 2018
		Physiological stress (Population level)	Cortisol	-	No	Values must be compared with in-house controls and measured periodically. No information at individual level or at specific time point.	
		Water quality	Temperature	-	-		
			Dissolved oxygen	-	-		
			Current speed	-	-		
			Nitrogenous compounds	-	-		

Table 1: Commonly used stress and welfare indicators for fish in laboratory and industrial facilities. Special equipment refers to any necessary tools that are not commonly found in laboratories or commercial farms (*c.f.* microscope, spectrophotometer, grader, commercially available kits such as ELISA, water quality probes). "-" indicates same value as above. The list hereby displayed is non-exhaustive but indicative, and should be considered with care, particularly when selecting target genes for RT-qPCR. Ideally, using a combination of these indicators is recommended to avoid drawing inaccurate conclusions.

1.4 Studied species

1.4.1 *Carassius auratus* (Goldfish)

The goldfish (*Carassius auratus*; Linnaeus, 1758) is a teleost fish from the *Cyprinidae* family native to east Asia. Being a potamodromous species, it thrives in freshwater systems although it has limited tolerance to salinity (Peterson & Meador, 1994; Riede, 2004). It is an extremely resistant species, being able to cope with significant changes in temperature (0 – 41 ºC; Beitinger & Bennett, 2000), high turbidity and eutrophication levels (Blanco et al., 2018; Page, 2008), and low concentrations of dissolved oxygen (Walker & Johansen, 1977). This, in combination with its genetical closeness to other cyprinid fishes, important to both the scientific community (*i.e. Danio rerio*) and the aquaculture industry (*Carassius carassius*), its commercial availability, ease of handling and low maintenance costs make of this species an ideal model organism (Blanco et al., 2018). Indeed, *C. auratus* has been widely employed in ecotoxicological studies (Gandar et al., 2016; Kim et al., 2024; Sudagar et al., 2024). Moreover, given its robustness, *C. auratus* is a recognised invasive species all over the world (Beatty et al., 2017).

1.4.2 *Oreochromis niloticus* (Nile Tilapia)

The Nile tilapia (*Oreochromis niloticus*; Linnaeus, 1758) is a diurnal teleost fish from the *Cichlidae* family, native to the Nile basin, and a variety of other African freshwater systems (Trewavas, 1983). This is a preferentially freshwater species, but it displays a certain degree of tolerance to brackish water. This species thrives at temperatures ranging from 14 to 33ºC, where it may grow to 60cm total length, and over 4Kg in total weight (Fishbase, 2024). Similarly to the goldfish, *O. niloticus* is a successful invader, with established populations throughout the globe, partly arising from accidental releases from aquaculture facilities (Champneys et al., 2021). Indeed, Nile tilapia is now the third most produced fish by the aquaculture industry, with over 5 million metric tonnes produced yearly, representing a 400% increase over the last two decades (FAO, 2024). Due to its robustness and versatility in terms of environmental conditions, as well as its low production and market costs, *O. niloticus* is an ideal candidate for aquaculture, being particularly suitable for RAS (Dalsgaard et al., 2013), and representing a valuable asset in preserving food supply in regions where low-cost protein is essential.

1.4.3 *Salmo salar* (Atlantic salmon)

Atlantic salmon (*Salmo salar*; Linnaeus, 1758) is a teleost fish from the *Salmonidae* family, naturally occurring through the temperate and arctic zones of the northern hemisphere. Additionally, it has been introduced to the southern hemisphere, particularly in Chile and Argentina, in South America and Oceania. Being an anadromous species, *S. salar* inhabits freshwater systems during the early life stages and migrates towards the ocean following smoltification (Gilbey et al., 2021). This is the most produced marine fish species, representing over 70% of total salmonid production and 30% of the total fish production from the marine aquaculture industry, equalling over 2.7 million metric tonnes per year (FAO, 2022; Pandey et al., 2023). Although only a small proportion of countries are responsible for over 90% of this species' production (FAO, 2022; Iversen et al., 2020), *S. salar* is one of the most economically important fish. The farming of *S. salar* requires both a freshwater and marine phase, and the former is increasingly done in RAS (Lazado & Good, 2021).

1.4.4 *Sparus aurata* (Gilthead seabream)

The gilthead seabream (*Sparus aurata*; Linnaeus, 1758) is a teleost fish form the *Sparidae* family native to the Eastern Atlantic and the Mediterranean Sea. It is euryhaline and eurythermal, being able to tolerate significant fluctuations in temperature and salinity, allowing it to thrive both in marine and brackish environments (Craig et al., 2008). It is a protandrous hermaphrodite, with all individuals being born male and becoming females after reaching sexual maturity (Chaves-Pozo et al., 2008). This species is the third most economically important fish produced by the mariculture industry, with particular importance in the Mediterranean area. It is extensively cultured in both sea pens and RAS, with an exponentially growing production, rising from less than 90 thousand tonnes in 2000, to over 280 thousand tonnes in 2020 (FAO, 2022). In addition, this species has been widely employed as an experimental subject in ecotoxicology (Barbosa et al., 2019; Ribecco et al., 2011; Rodrigues et al., 2018).

The species considered in the present work were selected taking into consideration commercial availability and proximity to the AQUAB facilities, economic, scientific and environmental relevance, as well as ease of handling and maintenance.

1.5 Context of this Thesis. Overview of the project in which the Thesis is included

The present thesis is part of a series of Ph.D. projects funded by the *European Union's Horizon 2020 research and innovation programme* under the Marie Skłodowska-Curie grant agreement No 956481. This consortium, called **"Safeguarding Future Production Of Fish In Aquaculture Systems With Water Recirculation (RASOPTA)"**, is composed of 12 Early-Stage Researchers (ESRs) working at different universities and research centres in Europe. The primary objective of RASOPTA is to identify knowledge gaps in the production of fish species in European RAS, and bridge important findings between the academic and industrial worlds. The 12 projects are distributed throughout three work packages (WPs), which share the common goal of designing and developing a novel nucleic acid chip for the early detection of stress-inducing, immune-compromising, and flavour-spoiling microorganisms.

The WPs are the following.

- WP1: Improving water quality by optimizing waste removal and biofilter efficiency (ESRs 1-3).
- WP2: Improving the management of off-flavours in RAS (ESRs 4-7)
- **WP3: Improving fish health and welfare in RAS (ESRs 8-12)**

The author of this thesis is RASOPTA's ESR8, working in WP3 and focusing on fish health and welfare in RAS.

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Aim, Objectives and Hypotheses

2

2. Aim and objectives

The aim of the present work is to examine fish health and welfare indicators by -omics and other conventional methodologies, in order to pinpoint stress and immune-indicative responses in fish in RAS.

To this end, five distinct objectives were defined:

1- To assess the impact of different RAS settings on fish stress and welfare
2- To evaluate the stress response of different fish species to emerging contaminants
3- To evaluate the stress response of fish reared in commercial settings and exposed to industrial stressors
4- To achieve a comprehensive understanding of the immune, endocrine, and nervous responses of different fish species to different stressors
5- To evaluate the suitability of little-to-non-invasive assays to assess stress status, health, and welfare in fish

2.1 Hypotheses

The general hypothesis of this thesis was that when fish are cultured in RAS they are subjected to a myriad of specific stressors, both endogenous and exogenous to the system, and that these will impact fish welfare, as well as their ability to respond to, and cope with, subsequent stressors. It was further hypothesised that NPs are already present in RAS, and that chronic exposures to these contaminants would trigger a series of stress responses in fish, similar to what is observed with other CECs, with marked differences between freshwater and marine species.

2.2 Experimental designs

All experiments were designed as to maximise the representativeness of the obtained samples, minimising the number of animals used, and avoiding all unnecessary suffering, as per the 3R principle of ethical animal research. Laboratory experiments involving CECs were designed as to employ environmentally relevant concentrations of the respective compounds, and case studies in commercial facilities were undertaken during real-life standard operations, without any previous indications from the researchers to the farm operators, preventing any unwanted interferences.

2.3 Rationale

Other than testing the main hypothesis, this thesis aimed to detail what are the consequences of RAS stressors on the response of fish, either physiological, immune, metabolic or in performance. Thus, the thesis is structured in 5 studies, with Studies 1, 2, and 3 each detailing single controlled laboratory experiments where fish are subjected to environmentally relevant concentrations of CECs, and the remaining studies describing study cases undertaken in commercial farms under industrial settings. Amongst these, Study 4 is centred on CECs, and Study 5 investigates the impact of common factors in RAS on fish health, and welfare.

In the first study, an automated biochemical analyser is validated with a model organism (*i.e. C. auratus*) in order to guarantee the repeatability and reliability of the obtained results in subsequent experiments. Adult goldfish were subjected to an environmentally relevant concentration of a CEC

(*i.e.* waterborne polystyrene nanoplastics; PSNPs), a compound of major interest for the present thesis which is employed again in later studies. The second study focuses on investigating the impact of another CEC, this time being a lipid regulating pharmaceutical manufactured for human use which is ubiquitous in aquatic environments (*i.e.* gemfibrozil; GEM), in the same model organism, employing the previously validated biochemical analyser. The third study describes the first prolonged waterborne exposure of *S. aurata* juveniles to environmentally relevant concentrations of PSNPs under laboratory conditions. The fourth study is a case study was performed in an industrial Spanish RAS, with the aim of confirming the presence of different NPs within the system, both in the water and the cultured *O. niloticus*, making it a first of its kind. The fifth and final study presents a case study undertaken at a commercial Faroese RAS, where a standard procedure of transferring fish in-site to reduce density is investigated for its impact on the stress status of *S. salar* smolts.

3

Evaluation of a chronic exposure to nanoplastics in goldfish (Carassius auratus): Analytical validation of automated assays for the measurement of biochemical markers

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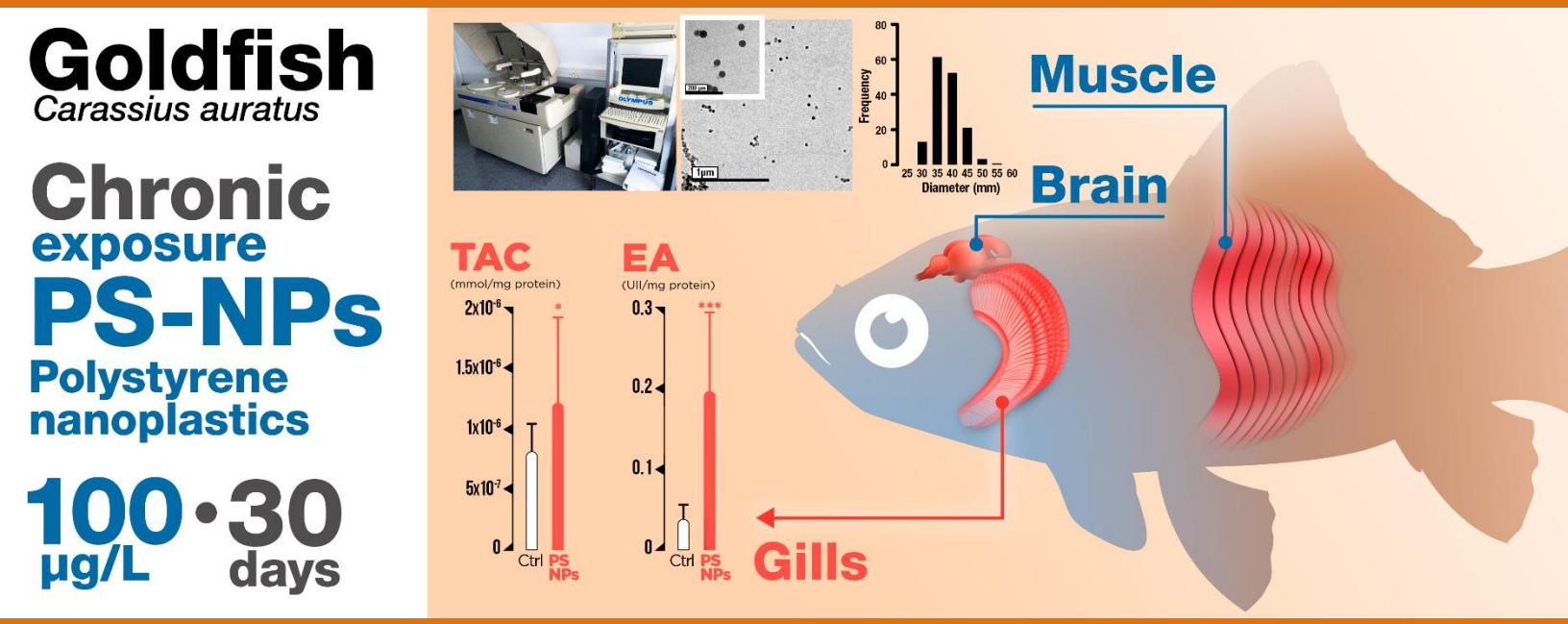

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Evaluation of a chronic exposure to nanoplastics in goldfish (*Carassius auratus*): Analytical validation of automated assays for the measurement of biochemical markers

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ABSTRACT

The bulk of plastic pollution is mainly composed of small fragments, micro- and nanoplastics (NPs). Although many studies are currently published on NPs, research on the effects of NPs in fish after a chronic exposure is still scarce. The present study aimed to validate a series of automated assays to be used in the monitoring of fish challenged with a chronic exposure to NPs, using *Carassius auratus* (goldfish) as model species. For this purpose, adult *C. auratus* were exposed to 100 μg/L polystyrene (PS)-NPs for a 30-day period. Total oxidative status (TOS), total antioxidant capacity (TAC), esterase activity (EA) and adenosine deaminase activity (ADA) were measured in the gills, brain and muscle of fish. In addition, acetylcholinesterase activity (AChE) and creatinine kinase (CK) were measured in the muscle. All biomarkers were successfully validated in goldfish tissues and consequently used to assess the effects of NPs following a chronic exposure. Results showed that EA and TAC significantly increased in gills, while EA decreased significantly in the brain, and no effects were observed in any of these parameters in muscle. These results indicate that both immune and antioxidant responses were triggered by NPs in gills, but not in the brain nor muscle. This suggests that gills may be a primary target for NPs, potentially leading to a cascading effect on gas exchange, or osmo- and ionic regulation that should be further investigated.

1. Introduction

Plastic pollution in aquatic environments is a worldwide problem and has raised increasing concern amongst the scientific community over the last decades (da Costa et al., 2016). As a result of both biotic (e.g., digestion; Dawson et al., 2018) and abiotic factors (e.g., UV radiation, Lambert and Wagner, 2016), discarded plastics are fragmented into microplastics (MPs, <5 mm, Browne et al., 2007), and eventually broken down into nanoplastics (NPs < 1000 nm, Hartmann et al., 2019). In addition, the presence of NPs in both industrial cleaning and personal care products represents an additional pathway for this emerging contaminant to enter aquatic systems (Hernandez et al., 2017; Saroglia and Terova, 2020). NPs appear as emerging contaminants in both natural and urbanised environments (Cai et al., 2021; Materić et al., 2022), and conventional wastewater treatments have proven unable to effectively remove them from influents (Zhou et al., 2018). One of the most reported plastic polymers in aquatic environments is polystyrene (PS; Cai et al., 2021; Llorca et al., 2021). An increasing number of scientific studies have focused on the effects of PS-NPs on the health and performance of aquatic organisms, such as fishes (Barría et al., 2020; Brandts et al., 2021a; Pitt et al., 2018). Moreover, the increasing usage of Recirculation Aquaculture Systems (RAS) for aquaculture production raise interest on the assessment of NPs in these systems and the impact on fish reared in such systems.

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Most of the published research regarding PS-NPs has explored their effects on the model species zebrafish (*Danio rerio)*, leaving only a small fraction of the literature investigating the response in other fish species (reviewed by Barría et al., 2020). However, when considering relevant factors such as rearing and maintenance costs, commercial availability, genetic distance with farmed species, and facility to handle, the goldfish (*Carassius auratus*) emerges as an ideal model organism to investigate the effects of NPs (Blanco et al., 2018; Filice et al., 2021). On the other hand, when reviewing NPs' ecotoxicology, most of the available literature deals with the response of fish to short-term exposures of no longer than 7 days (Barria et *al.*, 2020). In real-life scenarios, it is more likely that organisms are subjected to long-term exposures, with low but persistent concentrations of NPs present in the ecosystems (de Ruijter et al., 2020; Weis and Palmquist, 2021). Nevertheless, the effects of chronic exposures to NPs in fish are still poorly understood, although ontogenetic, intestinal, metabolic, or behavioural anomalies, amongst others, have already been reported in some species (Gu et al., 2020; Guimarães et al., 2021; Marana et al., 2022; Mattsson et al., 2015). To the best of the authors' knowledge only one previous study has explored the long-term effects of PS-NPs in goldfish, carried out by this research group (Brandts et al. 2022). In this recent study, the presence of NPs in goldfish liver and muscle after a chronic exposure was confirmed, and genotoxic damage in red blood cells was found. Therefore, further investigating the response to a chronic exposure to PS-NPs in *C. auratus* as a model species is of great interest, as it can provide tools key answers that could be relevant to other fish species.

NPs have been observed to enter fish organs mainly through the gills and the gastrointestinal track, under laboratory conditions, (Clark et al., 2022; van Pomeren et al., 2017), being eventually translocated through blood transport into other organs, where they can accumulate (Ma et al., 2022). In fish, the exposure to PS-NPs has been found to elicit immune responses, and to have deleterious effects on the digestive, reproductive, and endocrine systems, as well as to negatively impact behaviour and significantly alter gut microbiota (Brandts et al., 2021a; Marana et al., 2022; Pitt et al., 2018; Yin et al., 2021). Therefore, investigating biological endpoints related to oxidative status, and immune and metabolic activities may provide interesting insights on the toxicity of NPs (Franco-Martinez et al., 2016; Yin et al., 2021). In addition, nanoplastics have been demonstrated to enter organisms through trophic transfer (Chae et al., 2018), indicating an additional exposure source for humans through the ingestion of contaminated food. Furthermore, previous studies have shown the ability of these nanoparticles to cross the blood–brain barrier, not only in fish (Ma et al., 2022), but also in mammals (Shan et al., 2022). Thus, investigating the effects of PS-NPs in fish, particularly their brains may allow for a better understanding of the potential risks that this contaminant may pose to human health and welfare.

Biomarkers, defined as biochemical, physiological, or histological indicators of either exposure to, or effects of, anthropogenic stress (Huggett, 2018), have been applied in ecotoxicology and ecological risk assessment for>20 years (van der Oost et al., 2003). One of their primary strengths is that they can provide early signs of response to exposure, allowing us to determine damage and defence processes at a sub-cellular level (Roméo et al., 2013), measurable before effects on individual performance and population/community dynamics occur (Forbes et al., 2006). Thus, developing biomarkers to monitor NPs' pollution can be extremely valuable to indicate that organisms have been or are being exposed to these pollutants, and could suffer future impairments of ecological relevance. Generally, biomarkers are determined using manual techniques and often without being validated for the species under study (Franco-Martinez et al., 2016; Oliveira et al., 2018). Determining biomarkers via automated analysers and assays presents significant advantages compared to manual techniques, being more time- and cost-effective, eliminating operator-tied errors and, consequently, increasing the repeatability and reliability of the obtained results (Franco-Martinez et al., 2018; Oliveira et al., 2018). However, these must be previously validated in the appropriate species and matrix, to ensure their reliability, accuracy, and precision as indicators for a specific factor (Franco-Martínez et al., 2021). To this end, the specific objectives of the present study are twofold: 1) to achieve the analytical validation of biochemical markers in goldfish tissues; and 2) to assess the effects of a chronic exposure to PS-NPs in the gills, muscle, and brain of *C. auratus*, using the validated biomarkers. We studied a panel of biomarkers, namely, total oxidative status (TOS), total antioxidant capacity (TAC), oxidative status index (OSI), esterase activity (EA), adenosine deaminase (ADA), acetylcholinesterase (AChE) levels, and creatine kinase (CK), with the final aim of providing a set of adequate tools for giving a first assessment of general fish health status after a chronic exposure to NPs.

2. Materials and methods

2.1. Fish husbandry, bioassay and sampling

A total of 32 adult *C. auratus* (11.16 ± 3.23 cm total length and 7.07 ± 0.64 g weight) were selected for the present study. Individuals were randomly allocated to experimental aquaria and subjected to either of two experimental conditions: "Control" (0 μg/L PS-NPs) and "Exposed" (100 μg/L PS-NPs). To this date, no substantial environmental quantification of PS-NPs has been established, therefore the exposure concentration selected for this study took into consideration estimated concentrations in the environment (Lenz et al., 2016), in conjunction with those utilised in previous studies (Brandts et al., 2018; Brandts et al., 2020; Brandts et al., 2021b). Regarding the chosen polymer, PS has been found in real NPs environmental samples (Llorca et al., 2021; Ter Halle et al., 2017) and it has also been documented that this polymer contributed to a significant fraction of plastic waste in monitoring studies (de Haan et al., 2019). For the experiment, we used commercial PS-NPs (44 nm size) obtained from Bangs Laboratories. Dynamic light scattering (DLS) was employed to characterize the NPs' hydrodynamic size and zeta potential (Zetasizer Nano ZS, Malvern) in ultrapure water, and results were as shown in Brandts et al. (2022). Four experimental aquaria were prepared for each treatment, each containing 20 L of water and 4 individuals (n = 16; N = 32). Fish were exposed to the experimental conditions for a 30-day period, as per guideline 215 of the Organization for Economic Co-operation and Development (OECD; Fish, 2000). Throughout the experimental period, fish were fed *ad-libitum* at the same hour every day with a commercial diet (TROPICAL Goldfish Colour Pellet; crude protein 45.0 %, crude fat 7.0 %, crude fibre 3.0 %, moisture 10 %; fish and fish derivatives, derivatives of vegetable origin, oils, fats, and minerals), and the bottom of the aquaria was cleared of impurities which could negatively affect water quality. Fish mortality, as well as behavioural parameters (e.g., aggressiveness, activity rate:

hyperactivity/lethargy/erratic swimming, feeding behaviour/appetite) were recorded daily. The physicochemical parameters of the water were kept at optimal levels for the studied species (Table 1).

Approximately 75 % of the medium was replaced once every fifth day and PS-NPs were added, aiming to maintain the contaminant's level as close to the starting concentration as possible. At the end of the 30-day experimental period, all individuals were over-anesthetized in a tricaine methane-sulfonate bath (MS-222; 1 g/L). Each fish was subsequently measured and weighted, and blood was successively extracted through caudal puncture using heparinized syringes. Brain, gills, and muscle samples were collected from 7 randomly selected individuals from each group (n = 7; N = 14), snap-frozen with liquid nitrogen, and stored at −80 °C until analysis. The remaining fish were either used to test the reliability of the employed techniques or stored at −80 °C until further analysis. The entirety of the experiment was carried out following the 3 Rs of Animal Experimentation (Replacement, Reduction, and Refinement), under Spanish legislation (law 32/2007 and RD53/2013), and in agreement with the International Guiding Principles for Biomedical Research Involving Animals (EU 2010/63).

Table 1
Parameters of water quality maintained throughout the acclimatisation and experimental period.

Temperature (T°)	Alkalinity	Dissolved Oxygen	pH	Nitrate (NO_3^-)	Nitrite (NO_2^-)	Ammonia (NH_3)	Ammonium (NH_{4+})	Hardness
16 °C	6.7 dkH	4 mg/L	8.5	<10 mg/L	<0.5 mg/L	<0.15 mg/L	<0.25 mg/L	6–7 dGH

2.2. Sample processing prior to analysis

Samples of gill, brain and muscle were homogenized in potassium phosphate buffer 0.1 M (pH 7.2), at a weight:volume ratio of 1:4. Following a 30-min incubation at room temperature, the samples were centrifuged at 15,000 × g for 90 min, at 4 °C, and the obtained supernatant translocated to a clean tube, and placed at −80 °C until further use.

2.3. Validation of the biochemical parameters in goldfish tissues

Precision and accuracy were evaluated for all biomarkers in tissue homogenates. Precision was assessed by the determination of intra-assay coefficients of variation. For this, biomarkers were measured in one sample with high and one sample with low values three times in the same run. Coefficient of variation (CV) was calculated using the following equation (1):

$$CV(\%) = \frac{\sigma}{\bar{x}} * 100 \quad (1)$$

where: σ = standard deviation; $\bar{x}$ = average.

Accuracy was evaluated indirectly by performing linearity and recovery tests. To assess linearity under dilution, samples with high concentrations were serially diluted with a phosphate buffer. To evaluate the ability of the assays to recover the amount of added analyte, spiking recovery was performed by mixing two samples (one with high and one with low concentrations) at different rates. Test recovery (percentage) was calculated for each dilution for comparison of expected versus measured concentrations of each analyte in each tissue homogenate.

2.4. Measurement of biochemical biomarkers

TOS was measured as previously described (Erel, 2005). TAC was assessed by evaluating ferric reducing ability of the samples as described by Benzie and Strain (1996). EA was measured using p-nitrophenyl acetate as substrate (Haagen and Brock, 1992) adapted to automatic analyser (Tvarijonaviciute et al., 2012a). AChE was measured following the methodology described by Tecles et al. (2000). CK and ADA activity were determined using commercially available kits (Creatine Kinase, Olympus Systems Reagents; Olympus life and Material Science Europe GmbH, Hamburg, Germany; Adenosine Deaminase assay kit, Diazyme Laboratories, Poway, CA, USA) following manufacturer's indications. All parameters were determined with an automatic analyser (Olympus Diagnostica, GmbH).

2.5. Oxidative stress index

The oxidative stress index (OSI) was determined through the following equation:

$$OSI = TOS/TAC$$

2.6. Data processing and analysis

Data manipulation and analysis were carried out through GraphPad Prism 8.0.1 (GraphPad Software, Inc.). Homogeneity of variance was tested with an F-test, and the normality of the data was tested with the Shapiro-Wilk test. Data complying with normal distribution and homoscedasticity were analysed with a student's *t*-test. The non-parametric Mann-Whitney *U* test was employed to analyse data that did not follow a normal distribution. The results are presented as bar graphs, displaying the mean ± standard deviation (sd; n = 7), considering the threshold for significance at $p < 0.05$. Ordinary linear regression analysis, comparing measured and expected concentrations of analytes in different samples, was used to evaluate the linearity under dilution, and Runs-test was performed to determine whether data deviated significantly from the linearity.

3. Results

3.1. Parameter validation

The precision and accuracy data of the methods are presented in Table 2. All methods showed a coefficient of variation below 15 %. Serial dilutions of samples resulted in linear regression equations in which correlation coefficients did not differ from 1, the slope and intercept were close to 1 and 0, respectively, and the Runs test revealed no deviation from linearity ($P > 0.05$). Recovery between observed and expected concentrations ranged from 81 to 114 %, in all cases.

3.2. Biochemical parameters in gills, brain and muscle

The analysed biochemical parameters for all three studied tissues are shown in Fig. 1. No significant differences were found in TOS nor OSI

Table 2
Precision and accuracy of employed techniques. Intra-assay coefficient of variation (CV), linearity under dilution and spiking recovery rates of total oxidant status (TOS), total antioxidant capacity (TAC), esterase activity (EA), adenosine deaminase (ADA), acetylcholinesterase activity (AChE), and creatinine kinase (CK) in homogenates from the gills, brain and muscle of the teleost fish Carassius auratus.

Tissue	Analyte	CV, % (Range)	Linearity under dilution*		Recovery, % (Range)
			Linear Regression Equations	R^2	
Gills	TOS	3.6–7.3	y = 0.566x + 0.0177	0.999	90.3–108.7
	TAC	2.2–7.2	y = 1.1713x − 0.0109	0.979	100.0–103.5
	EA	1.9–5.4	y = 0.6892x − 0.0289	0.995	81.5–100.0
	ADA	0.6–2.5	y = 0.6684x + 3.3182	0.999	98.4–100.0
Brain	TOS	7.1–10.3	y = 0.8569x − 0.4469	0.999	90.8–100.0
	TAC	0.5–2.8	y = 1.0883x + 0.0066	0.990	96.2–108.9
	EA	8.0–14.1	y = 0.9623x − 0.0102	0.983	89.8–100.0
	ADA	0.9–1.9	y = 1.0136x − 0.9661	0.998	95.9–100.0
Muscle	TOS	9.1–13.0	y = 1.9064x − 7.4016	0.989	100.0 – 106.5
	TAC	6.5–9.4	y = 0.9191x + 0.0206	0.968	94.9–100.0
	EA	7.7–12.4	y = 0.8232x + 0.0105	0.998	96.3–101.1
	ADA	6.2–11.8	y = 0.9993x − 0.0085	0.996	95.7–106.4
	AChE	4.4–9.1	y = 0.9567x + 1.4078	0.994	87.4–113.3
	CK	1.5–5.3	y = 0.7845x + 12.435	0.998	96.8–101.9

*, Runs-tests were not significant ($P > 0.05$) in all cases.

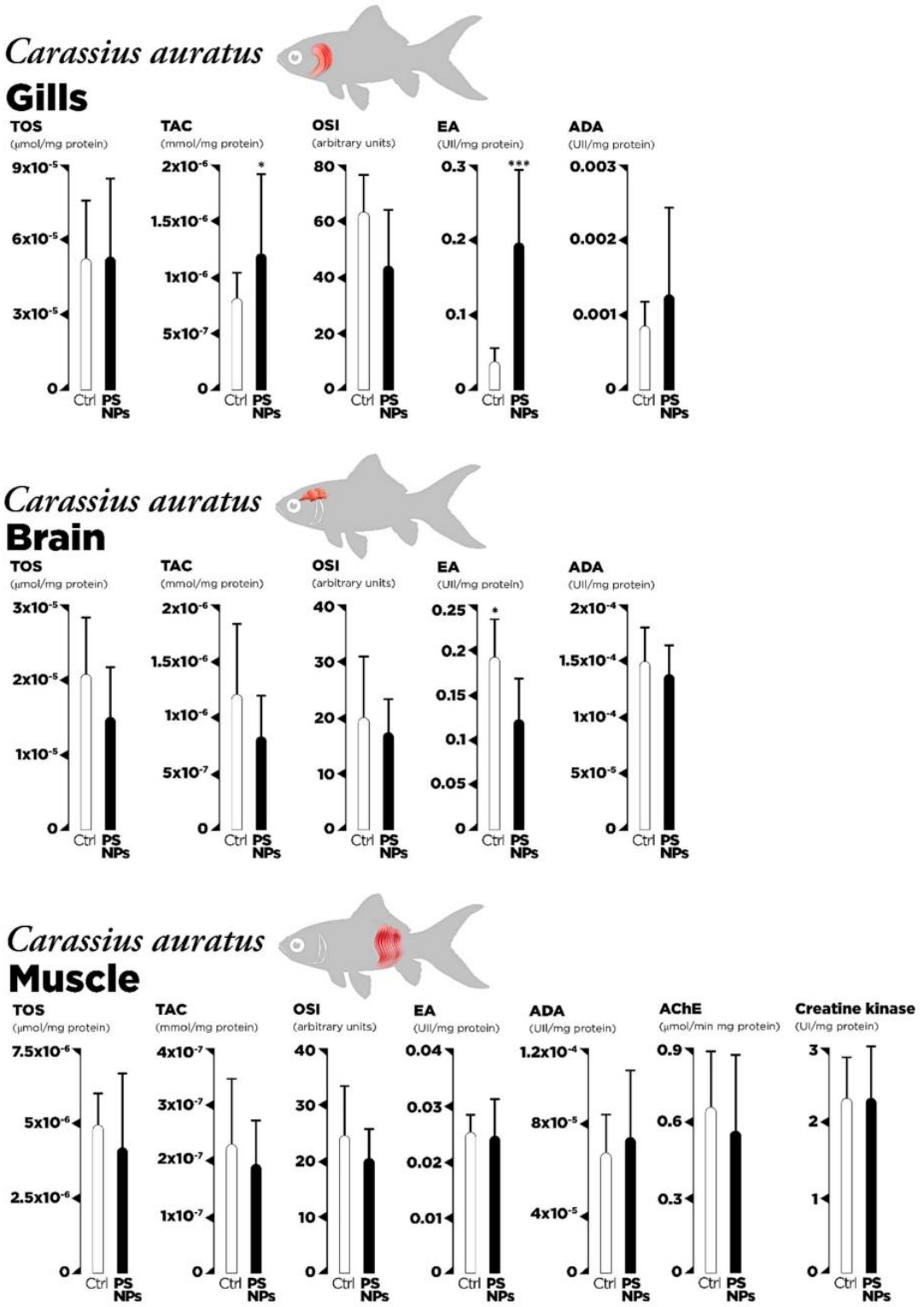

Fig. 1. Effects of PS-NPs on the gills, brain, and muscle of *Carassius auratus* (goldfish). Control conditions (ctrl = 0 µg/L PS NPs; n = 7) are compared to the treatment group (PS NPs = 100 µg/L PS NPs; n = 7). Variations in Total Oxidative Status (TOS), Total Antioxidant Capacity (TAC), Oxidative Status Index (OSI), Esterase Activity (EA), Adenosine Deaminase (ADA), Acetylcholinesterase activity (AChE), and Creatine Kinase (CK) are depicted in the corresponding organs. Significant differences, as well as degree of significance between control and treatment groups are indicated (* = P-value < 0.05, P-value < 0.01 ***).

between the control and the fish exposed to NPs in any of the studied organs. However, gill samples from the individuals exposed to 100 μg/L of PS-NPs displayed significantly higher TAC when compared to the control group (p-value = 0.016). Moreover, EA was also significantly increased in the gills of fish exposed to PS-NPs (p-value < 0.001). In contrast, the results indicated a significant decrease of EA in the brain (p-value = 0.01) of fish exposed to NPs compared to the control group. In muscle, no significant variations were observed in any of the biomarkers assessed.

4. Discussion

Analytical validation of given biomarkers is essential to ensure that test results reflect the condition of an animal, rather than variations caused by the laboratory itself due to method imprecision and inaccuracy, among others (Jensen and Kjelgaard-Hansen, 2010; Tvarijonaviciute et al., 2012b). Therefore, validation studies must ensure that analytical methods can detect the corresponding analyte in a precise and accurate way in a target sample. This practice is of particular importance in novel fields like the effect of exposure to NPs in living organisms, in order to avoid under- or over-estimating the extent of a problematic, and to enable for standardisation of techniques, allowing for the correct comparison between results of different studies (Dor et al., 1999). Furthermore, validating biomarkers for a particular condition is of utmost importance to ensure the accuracy of ecotoxicological studies monitoring nanoplastics pollution and its effect on both wild and cultured populations. In this study, automated methods for oxidative stress markers (TAC, TOS, EA), biomarkers of immune response (EA, ADA), neurotoxicity (AChE), and a biomarker of muscle damage (CK), were validated in gills, brains and muscle homogenates of *C. auratus*. All the evaluated methods presented adequate precision in tissue homogenates with intra-assay CVs lower than 20 %, the limit for an objective analytic performance standard for precision (US Department of Health and Human Services, FDA, CDER, CVM, 2018). In the same line, the accuracy of the methods has fulfilled the established criteria consisting of (1) obtaining a regression equation approximating R2 to 1.0 in the regression analysis of the relationship of the measured and expected analyte values in the linearity under dilution test; and (2) obtaining percentages between 80 % and 120 % in the spiking recovery test (Jensen and Kjelgaard-Hansen, 2010). These findings confirm all the methods to be precise and accurate when quantifying their respective analytes in *C. auratus* tissue homogenates.

This study showed that, in *C. auratus*, immune and antioxidant responses occur in the gills following a chronic waterborne exposure to PS-NPs, but not in brain nor muscle. To the best of the authors' knowledge, no study has investigated the effects of PS-NPs on the considered biomarkers in the gills, muscle, and brain of *C. auratus*, after a chronic exposure. For these reasons, the results hereby presented are mostly compared with previous findings with different species or polymers, with some exceptions.

The increase in TAC detected in gills of *C. auratus* in the present experiment indicates a boosted production of antioxidant compounds aiming to inhibit the effects of reactive oxygen species (ROS), which have been documented in fish exposed to different types of NPs (Jacob et al., 2020). Similarly, a recent study indicates that waterborne, chronic (28-day) exposure to PS-NPs (250 nm, 0.05–5 mg/L) induces oxidative stress in *C. auratus*, although different biomarkers as those hereby considered were investigated (Abarghouei et al., 2021). Indeed, the authors reported significant increases in the levels of superoxide dismutase (SOD) and catalase (CAT) in serum. In addition, PS-NPs appeared to have size-, and concentration-dependent effects on the health of *C. auratus*, with smaller particles at higher concentrations causing severe histopathological changes in gills, liver, and gut. Moreover, a number of previous studies have reported evidence that exposure to PS-NPs, and other NPs, cause oxidative stress in fish (Reviewed by Han et al., 2021). Similarly, Brandts et al. (2021c) detected a significant increase of TAC in gills of *Sparus aurata* exposed to polymethylmethacrylate (PMMA) NPs concentrations ranging from 0.001 to 0.1 mg/L. This suggests a similar effect of PMMA-NPs and PS-NPs, both causing oxidative stress in gills. Nonetheless, in yellow croaker (*Larimichthys crocea*), dietary exposure to PS-NPs did not cause any significant variations in TAC in liver, but did on muscle (Lai et al., 2021), and waterborne exposure led to oxidative stress in the liver, with evident rises in levels of CAT, SOD, and glutathione peroxidase (GPx; Gu et al., 2021). This points towards variations in the effects of NPs linked to species, organ, and exposure type. Furthermore, a significant increase was found in TOS in the muscle and liver of *S. aurata* exposed to similar concentrations of PMMA-NPs (Balasch et al., 2021; Brandts et al., 2021b). Nevertheless, no significant differences in TOS were found in gills nor skin under the same experimental conditions (Brandts et al., 2021c). In addition, Brandts et al. (2021a) reported no significant differences in TAC or OSI when examining skin mucus and plasma of *Dicentrarchus labrax* exposed to PS-NPs. This further indicates inter-organ variations in the response to NPs, although these differences could also be explained by the specificities of each polymer, such as size, composition, and shape, and the way these factors affect the organ-NPs interaction (Guerrera et al., 2021). These results suggest an additional inter-specific component that could explain the observed differences, by which different organs in different species display varying responses to similar NPs polymers and concentrations. The present results also suggest that gills are one of the main pathways for NPs to be internalized in freshwater fish, as shown previously for other xenobiotics (Bhagat et al., 2020). It should be considered that the ingestion of food during chronic bioassays might modify the nutritional status of fish and help to counteract the effects induced by NPs, notably oxidative stress (Sinha et al., 2015), on internal organs; but not in gills, which would serve as a portal of entry and internalization. In addition, the differences in findings regarding the potential of oxidative stress or oxidative damage caused by NPs exposure between the current study and the available literature could be attributed to differences in targeted biomarkers (Han et al., 2021).

ADA has been widely investigated as an indicator for immune response and oxidative stress management (Baldissera et al., 2018; Capiotti et al., 2016; Zhang et al., 2022). This enzyme, involved in purine metabolism (Cristalli et al., 2001), has been reported to play a major role in neutrophil regulation, and in controlling the immune response to bacterial stressors (Kälvegren et al., 2010). The lack of response of this parameter to long-term exposure to PS-NPs in any of the studied organs suggests that the specific mechanisms regulated by this enzyme were not significantly affected. Similarly, Brandts et al. (2021b) and Balasch et al. (2021) did not report significant alterations in ADA in gilthead seabream subjected to short-term exposure to PMMA-NPs. Moreover, Brandts et al. (2021a) did not detect significant differences in ADA in skin mucus nor plasma of *D. labrax* exposed to PS-NPs, further corroborating the present findings. Moreover, the esterase activity (EA) was significantly increased in the gills of fish exposed to PS-NPs (p-value < 0.001), which suggests that the mechanism to counteract the oxidative stress caused by an anti-inflammatory response due to PS-NPs was successfully triggered. EA is also often employed as a proxy for immune activity (Marcos-López et al., 2017), and the observed response could indicate that the gills were developing an anti-inflammatory response to the contact and internalization of NPs. The present results are in contradiction with the findings published by Brandts et al. (2021c), describing a significant decrease of EA in both gills and liver of *S. aurata* exposed to PMMA-NPs, but, once again, this could point not only to inter-specific variations, but also differences linked to the nature of the polymer, the exposure period, and the absence of food throughout the acute tests. The significant decrease in EA observed in brain, could indicate an alteration in the immune capacity of this organ (Oliveira et al., 2018), and could be attributed to the direct effect of the PS-NPs on this enzyme, or to its relocation as a response to oxidative stress in gills (Brandts et al., 2021c). Interestingly, besides the immune and antioxidant responses observed in gills,

previous studies reported a general genotoxic response in blood cells (Brandts et al., 2021b), which might mean that NPs are entering blood circulation through the gills, inducing oxidative stress in blood cells, reaching preferentially the liver due to a fenestrated endothelium, compared to muscle or brain. In accordance with previous experiments carried out in a different species (*S. aurata*) and NPs polymer (PMMA) (Balasch et al., 2021), EA did not result in significant differences in muscles between treatment groups. This absence in variations of EA in muscle indicates that esterase did not take part in counteracting the oxidative stress caused by PS-NPs in this organ.

AChE activity is widely used as a biomarker for neurotoxicity of environmental pollutants (Fulton and Key, 2001), and alterations in this factor are known to lead to motor problems, potentially causing severe paralysis and, eventually, death (Modesto and Martinez, 2010). No abnormal behaviour of any kind was reported in goldfish in the present study, and the lack of alterations in AChE activity seems to match these observations. Previous studies have reported either an upregulation or downregulation in AChE activity following acute exposure of *S. aurata* to PMMA-NPs, and *D. rerio* and *Oreochromis niloticus* to PS-NPs (Balasch et al., 2021; Chen et al., 2017a; Chen et al., 2017b; Ding et al., 2018). However, the differences in previously reported results might be linked to the characteristics of each particular polymer (Guerrera et al., 2021), the exposure period (Atli and Canli, 2011; Kögel et al., 2020), or the species of interest and their developmental stage when subjected to the stressor (Barton, 2002; Fowler et al., 2009).

CK is a reliable indicator for muscle damage (Wallimann et al., 2011; Yousaf and Powell, 2012), and high levels of this protein could cause changes in the swimming behaviour. Therefore, the lack of alterations in the activity of this enzyme supports the fact that the exposed fish did not display any sort of abnormal behaviour throughout the experimental period. This result is in accordance with what Balasch et al. (2021) recorded, stating that no significant effect of PMMA-NPs on CK was observed. The main difference between the present study and the literature described above, other than the exact type of contaminant used and the species of interest, is the exposure period. In a recent study from the authors' research team, it was observed that NPs were preferentially accumulated in muscle of *C. auratus* (Brandts et al., 2022), but the complete absence of response observed in this organ during the present experiment indicates that the number of particles was below the threshold to trigger an immune or antioxidant response.

Altogether, it appears as if the gills sustained greater oxidative damage compared with the brain or muscle, which could be justified by gills, as one of the major entry portals, being subjected to direct exposure to xenobiotics (Arellano et al., 2001). Thus, gills are generally more susceptible to external contaminants than brain or muscle, as it could have been expected. Furthermore, differences regarding the main organs accumulating nanoplastics, and therefore displaying responses to this contaminant, could be expected between freshwater and marine species. Indeed, marine fishes tend to drink large quantities of seawater to compensate for the important osmotic loss through the gills, due to their euryhaline environment (Takei, 2021), making the intestinal tract a greater portal of entry than gills. In contrast, freshwater fishes are subjected to osmotic gain of water through the gills due to their oligohaline surroundings (Edwards and Marshall, 2012), therefore, gills play a major role as a portal of entry for xenobiotics. This should be taken into consideration when selecting organs of interest for investigating both the accumulation potential, and the effects of NPs in fish.

Research concerning NPs still has multiple knowledge gaps, which limits the assessment of risk of exposure and potential effects of NPs in biota and humans (European Commission and Directorate-General for Research and Innovation, 2019). The accurate quantification of NPs in the environment in order both to establish environmental concentrations and to be able to quantify the bioaccumulation of NPs in organism and studies evaluating the sub-lethal effects of chronic exposures to NPs are crucial stepping-stone in the field. Moreover, species-specific effects and potential sex-specific effects within each species should also be considered and further investigated in these chronic scenarios, as both oxidative-stress and general stress related responses have been shown to differ between sexes and could condition the response to NPs (Balasch and Tort, 2019; Niksirat et al., 2021).

Although estimates on the environmental concentrations of NPs don't have consensus among researchers on the field, it has been suggested that the environmental concentration range varying between ca.1 pg/L and ca. 20 μg/L (Lenz et al., 2016). Moreover, to the best of the author's knowledge, the only existing studies that report actual environmental NPs quantifications, report concentration ranging from 7 to 52 μg/L (Llorca et al. 2021; Materić et al. 2022). On the other hand, reports giving information on MPs concentrations found in industrial effluent discharges account for concentrations of $\leq$ 30 mg/L (Lechner and Ramler, 2015). Considering that secondary NPs are constantly released by the fragmentation and degradation of macro and micro plastic debris, concentrations are bound to be increasing and some authors point to NPs concentrations 10^{14} times higher than those measured for MPs (Besseling et al., 2019). Furthermore, future predictions visualize increases of at least 50-fold from present-day concentrations during this 21st century (Everaert et al., 2018). Considering this, the dose used in this study, (100 μg/L) would fall close within the range of estimated concentrations of NPs in the environment and only one order of magnitude greater than real environmental quantifications. Future studies should further explore more environmentally realistic chronic-exposure scenarios, leaning towards lower doses of NPs and plastic nanoparticles resulting from breakdown of larger plastic objects instead of model spherical particles, to better understand potential ecosystem effects. More data is essential for risk assessment, risk management, and risk communication.

5. Concluding remarks

New automated methods for biomarkers of oxidative stress (TAC, TOS, EA), immune response (EA, ADA), neurotoxicity (AChE), and muscle damage (CK), were successfully validated in gills, brain, and muscle homogenates of *C. auratus*, showing their adequate analytical performance. These methods enabled the assessment of the impact of PS-NPs on gills, where it possibly caused oxidative stress and subsequently triggered antioxidant mechanisms. It appears as if PS-NPs had a heavier impact in gills when compared to brain or muscle, where they had little to no evident effect. The obtained results, compared with those available in the published literature, confirm strong inter-specific and inter-organ variations. Even though some responses seem generalised, specific organs react differently to the presence of PS-NPs, although some of these differences might be due to the specificities of the particles used (e.g., size, shape). Further research is needed to fully understand the effect of chronic waterborne exposure of fish to PS-NPs. However, although the effects observed in brains are relatively mild, the results could have implications for human health, as humans are in constant exposure to these contaminants, and might suffer from the NPs ability to cross the blood–brain barrier.

CRediT authorship contribution statement

M. Blonç: Writing – original draft, Writing – review & editing. **I. Brandts:** Conceptualization, Investigation, Writing – original draft, Writing – review & editing. **M. Cánovas:** Investigation. **L. Franco-Martínez:** Methodology. **C.P. Rubio:** Methodology. **L. Tort:** Funding acquisition, Supervision, Writing – review & editing. **A. Tvarijonaviciute:** Funding acquisition, Methodology, Writing – review & editing. **C. Gravato:** Writing – review & editing. **M. Teles:** Conceptualization, Funding acquisition, Supervision, Writing – review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial

interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Effects of a chronic exposure to gemfibrozil in Carassius auratus

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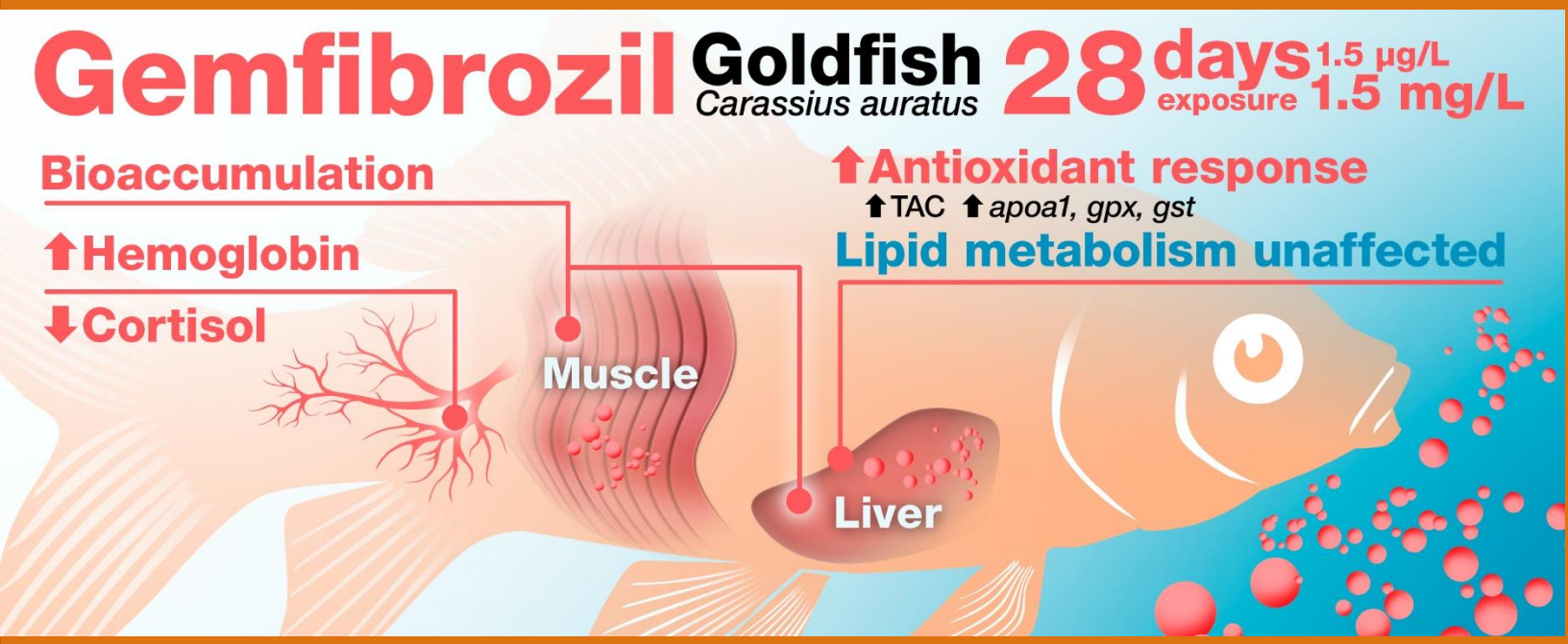

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Effects of a chronic exposure to gemfibrozil in *Carassius auratus*

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ABSTRACT

Lipid regulators, such as fibrates, are pharmaceuticals manufactured to treat dyslipidaemias in humans. Their constant use and discard combined to their environmental persistence and poor removal rates from wastewater makes of these emergent contaminants ubiquitous in aquatic systems, with gemfibrozil (GEM) being the most commonly detected fibrate in water. The present study aimed to describe the effects of a 28day waterborne exposure to both an environmentally relevant concentration (1.5 μg/L) and a spiked concentration (15 mg/L) of GEM, in adult individuals of the model organism *Carassius auratus* (goldfish). To this end, bioaccumulation of this compound in liver and muscle, as well as variations on haematological parameters, plasma biochemistry endpoints, and the expression of relevant target genes in the liver were investigated. The results indicated that, following exposure to the highest concentration of GEM, this compound accumulated in both liver and muscle. Similarly, significant increases were observed in haemoglobin levels, and mean corpuscular haemoglobin concentrations in individuals exposed to 15 mg/L of GEM. The biochemical profiling of plasma revealed significant decreases of cortisol and glucose levels, as well as a significant increase in the total antioxidant capacity (TAC) together with significant upregulations of gene transcripts related to antioxidant defences (*gpx, gst*) and lipid metabolism (*apoa1*). However, no changes were observed between treatments in the relative expression of key pro-inflammatory markers (*il1β, il6* and *il8*) nor genetic markers of lipid metabolism (*pparα pparβ* and *pparγ*) or plasma levels of cholesterol and triglycerides. Overall, the results suggest that bioaccumulation of GEM in *C. auratus* triggers mild antioxidant responses at high doses but not at environmentally relevant ones.

Introduction

Lipid regulators, such as fibrates or statins, are pharmaceuticals commonly prescribed to treat dyslipidaemias (Pahan, 2006), and their production, use and discard rates are in accelerating growth (Fang et al., 2012). The constant input of these compounds in wastewater influents (Xia et al., 2005), combined with the common inefficacy of treatment plants to remove these type of compounds from effluents (Khalil et al., 2022; Martínez et al., 2012), and their environmental persistence (Fang et al., 2012), makes of lipid regulators ubiquitous in natural environments (Pahan, 2006; Zhang et al., 2020). Fibrates decrease blood cholesterol levels in humans by activating β-oxidation pathways (Pahan, 2006), acting primarily as agonists to the peroxisome proliferator system alpha (PPARα; Zhang et al., 2020), one of a class of transcription factors. Together with the β and γ PPAR isotypes, PPARα has a major role in mammalian lipid and carbohydrate metabolism, cell differentiation and growth, inflammatory onset and antioxidant responses during whole-body homoeostasis and neurogenesis (Di Giacomo et al., 2017). It has been reported that these lipid regulators act in a similar manner in aquatic vertebrates (Al-Habsi et al., 2016), and that prolonged exposure to these compounds might influence lipid metabolism, reproduction, development, and immunity in fishes (Blonç et al., 2023b; Skolness et al., 2012; Teles et al., 2016).

Concentrations of these pharmaceuticals in a series of aquatic systems have been quantified around Europe, with the most commonly detected being gemfibrozil (GEM), a fibrate derivative widely prescribed to regulate hypertriglyceridemia and cholesterol levels (Grenni et al., 2013; Loos et al., 2009). GEM is a persistent emergent contaminant with

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a half-life between 119 and 288 days in surface waters (Araujo et al., 2011). Measured values of GEM reach 1.7 ng/L in Italian tap water (Loos et al., 2007), 10.34 ng/L in Portuguese surface waters (Pereira et al., 2017), 758 ng/L in coastal waters (Gaw et al., 2014), up to 1 μg/L in Spanish rivers (Martínez et al., 2010), an average of 16.9 μg/L in commercial crop-derived irrigation waters (García-Valverde et al., 2023) and 5.315 μg/L in wastewaters derived from hospital practices in the Mediterranean region (Castaño-Trias et al., 2023). Insufficient or unspecific wastewater management and climate-derived water scarcities may alter the amount of pharmaceutically active compounds in water bodies, aggravating the effects on the normal physiology in fish, even in cultured ones, potentially having significant impacts on the fish production sector. A major part of the literature investigating the effects of fibrates in fish focuses precisely on GEM, its presence in the environment, and its short-term effects (Blonç et al., 2023b). However, more long-term studies are needed to ascertain the impact of persistent xenobiotics on marine organisms. Studies on the effects of prolonged exposures to GEM in fish have focused mainly on zebrafish (*Danio rerio*), a classical model species, with fewer studies dedicated to the effects of these lipid regulators in commercial species (Al-Habsi et al., 2016; Blonç et al., 2023b; Fraz et al., 2018; Galus et al., M. 2014; Hammill et al., 2018; Henriques et al., 2016).

The goldfish, *Carassius auratus*, also widely acknowledged as a model organism (Blanco et al., 2018; Filice et al., 2022), presents some advantages compared to zebrafish, such as larger size, and greater resistance to environmental extremes, facilitating handling and blood extraction for haematological studies (Blanco et al., 2018). In goldfish, chronic waterborne exposures to GEM may cause bioaccumulation of GEM in plasma (Mimeault et al., 2005, 2006) leading to decreased levels of plasmatic testosterone, and the activation of antioxidant defences, as well as alterations in the lipid metabolism. Exposure to GEM in several fish species seems to elicit alterations in swimming and social behaviour (Barreto et al., 2018; Galus et al., M. 2014). However, in goldfish, long-term co-exposure to GEM and serotonin uptake inhibitors not always correlate with neurotransmitter alterations and may not influence swimming activity (Simmons et al., 2017). In other fish species, GEM exposure tampers with the bioavailability of energetic resources (Al-Habsi et al., 2016; Fraz et al., 2018), and have implications on reproduction, transgenerational performance, development, and homoeostasis, although often with evident differences between male and female individuals (Fraz et al., 2019; Lee et al., 2019; Prindiville et al., 2011; Skolness et al., 2012).

The present study aims to expand the knowledge available on the effects of chronic exposures to GEM in fish, by challenging adult *C. auratus* to a waterborne exposure to GEM over a period of 28 days. To this end, the potential for bioaccumulation of GEM in muscle and liver, the possible haematological alterations as well as differences in plasma biochemical biomarkers linked to antioxidant defences and lipid metabolism were investigated. Additionally, relative expression of transcripts related to oxidative stress (catalase, *cat*; glutathione Peroxidase 1, *gpx*, and glutathione S-transferase, *gst*), inflammatory responses (interleukins 1β, 6 and 8, *il1β, il6, il8*) and lipid metabolism (apolipoprotein A, *apoa1*; lipoprotein lipase, *lpl*; peroxisome proliferator-activated receptors α, β and γ, *pparα, pparβ, pparγ*), were also assessed.

Materials & methods

Housing and husbandry

60 adult goldfish (14.03 ± 1.47 cm total length, 26.8 ± 5.45 g total weight) were acquired from Acuario Plantado, Pamplona, Spain. The fish were randomly allocated into 12 aerated experimental tanks, each containing 20 L of water and 5 individuals, and left to acclimate for 1 week prior to the start of the challenge. Throughout the acclimation period, fish were fed to satiation (Goldfish colour pellet, Tropical). Water parameters (Temperature, pH, Ammonium, nitrites, and nitrates) were monitored daily, and 75% of the medium was changed every 3 days in order to avoid the accumulation of metabolites, and to maintain water quality at optimal levels.

Exposure to gemfibrozil and sampling

Following the acclimation period, 75% of the water was replaced in all tanks, guaranteeing that water quality parameters were at optimal levels for the start of the experiment. The twelve experimental tanks were randomly assigned to the different experimental treatments (5 fish per tank, 4 replicates per treatment), namely "Control" (Ctrl), "Low concentration" (LC), and "High concentration" (HC). Individuals belonging to the Ctrl group were exposed solely to dimethyl sulfoxide (DMSO, CAS: 67–68–5, MERCK) at a concentration of 0.5 mL DMSO/L of water. The LC group was exposed to 1.5 μg/L GEM (CAS: 25,812–30–0, MERCK) dissolved in DMSO, an environmentally relevant concentration usually found on surface waters (Fang et al., 2012), and the HC group was exposed to 15 mg/L GEM (Barreto et al., 2018). The exposure lasted 28 days, during which fish were fed daily to satiation at 10 am. Water quality parameters, mortality, and abrupt changes in behaviour were monitored throughout the experimental period, and the tanks were maintained clean. Furthermore, 75% water changes were performed every 3 days to avoid the accumulation of metabolites, and the test compounds were added again to maintain experimental concentrations in each tank throughout the test period.

Once the 28-day exposure period elapsed, fish were individually euthanised by immersion in lethal doses (300 mg/L) of MS-222 (CAS 886–86–2) buffered with sodium bicarbonate (NaHCO3, CAS 144–55–8) at a 1:2 ratio. Once stage 4 of anaesthesia (medullar collapse) was reached, the fish were collected from the anaesthesia tank and blood was extracted through caudal puncture employing 1 mL heparinised syringes equipped with 25 G needles. 1 mL of blood was immediately transferred into two Eppendorf (500 μL each) containing heparin. One aliquot was destined for haematology, and therefore stored at 4° C until analysis, whereas the remaining of the blood was centrifuged at 2000 G for 10 min in order to obtain plasma for biochemical analyses. Following blood extraction, death of the fish was confirmed through spinal rupture and necropsy was performed. Liver was collected from each fish and immediately snap frozen in liquid nitrogen and stored at −80° C until further analysis.

Gemfibrozil extraction

GEM was extracted from fish tissue samples according to Chen et al. (2015) with little modifications. The whole liver or muscle (between 0.08 and 0.5 g) was homogenised and introduced in an Eppendorf tube. Then, the matrix was spiked with 5 μl of Gemfibrozil D-16 (GEM-d16, MERCK), here used as an internal standard, at 10 mg/L and left to equilibrium for 20 min. Then, 1 ml of acetone (Chromasolv, USA) was added, homogenized in a vortex for 1 min and for 5 min in an ultrasonic bath. Then, the mixture was centrifuged at 12,000 rpm for 5 min, and 0.5 ml of the supernatant was collected and introduced in a LC-vial. This procedure was repeated twice, and the acetone collected (1.5 ml) was evaporated till dryness under nitrogen stream. Finally, the extracts were reconstituted in 0.5 ml of methanol:water (1:9; Chromasolv, USA) and preserved at −20 °C until instrumental analysis. In parallel to samples extraction, a blank consisting on acetone was used to monitor any possible cross contamination during the extraction process.

Gemfibrozil analysis

The analysis was carried out by means of liquid chromatography coupled to high resolution mass spectrometry (LC—HRMS, QExactive). The Acquity LC system was equipped with Purospher® STAR RP-18 end capped analytical column (5 μm, 2 × 125 mm, MERCK). The injection volume was 20 μL and the chromatographic separation was carried out

using gradient conditions with (A) methanol and (B) water. The starting gradient was 10% (A) for 30 s and then increased, reaching 90% (A) at 18.5 min. The gradient was maintained for 5 min in isocratic conditions, re-equilibrated until the initial gradient during 1 min and maintained for 30 s, resulting in a total run time of 25 min. The chromatographic system was coupled to the QExactive, equipped with an electrospray ionization source (ESI), operating in negative ionization mode for GEM and GEM-d16. Data acquisition was performed in Full MS (m/z 100 – 500) at a resolution of 70,000 FWHM and data dependant scan MS2 (FS-ddMS2) with a mass resolution of 17,500 FWHM for the most intense ion transition in both cases ($m/z = 121$).

The whole system was controlled by Xcalibur 3.0. The data was processed by means of Xcalibur 3.0 QuanBrowser and the quantification by means of calibration curve normalized by internal standard built in methanol:water (1:9) from 0.1 to 500 ng/ml.

Control and quality parameters

Quality and control parameters including recoveries for each matrix, reproducibility, expressed in relative standard deviation (%RSD), and method limit of detection were experimentally calculated by spiking real samples with labelled gemfibrozil pure standard (GEM-d6). Real samples were spiked at 50 ng/mL in water and with 5 µl of GEM-d6 at 1 mg/L in muscle and liver. The recoveries for matrices were 77 % for liver and 104 % for muscle, both extracted with acetone, and 90 % for water experiments extracted by SPE. The reproducibility expressed as%RSD was adequate, being 23 % for liver ($n = 10$), 6 % for muscle ($n = 10$) and 32 % for water ($n = 5$). In addition, the method's limits of quantification were settled at 0.22 ng/g for liver and muscle as well as 0.2 µg/L for water samples.

Haematology

Haematological analyses were performed less than 12 h after sampling employing the automated flow cytometer blood cell analyser Sysmex XN-1000 V, manufactured for veterinary purposes (Sysmex Corp., Kobe, Japan). The system undergoes a daily internal quality control using commercially available material specifically manufactured for this purpose: Sysmex XN Check level 1 (low range), level 2 (normal range), level 3 (abnormally high range).

Biochemistry

Total oxidative status (TOS) was determined as per Erel (2005), whereas total antioxidant capacity (TAC) was measured through analysis of the ferric reducing ability of the plasma (Benzie and Strain, 1996). Adenosine Deaminase (ADA) activity was determined with commercially available kits (Adenosine Deaminase assay kit, Diazyme Laboratories, Poway, CA, USA). Cortisol levels were determined with a commercially available enzyme immunoassay involving solid-phase chemiluminescence (COR Cortisol, LKC01, Siemens Health Diagnostics, Deerfield, IL) in an automated analyser (Immulite 1000, Immulite System, Siemens Health Diagnostics; Franco-Martinez et al., 2019). Plasma glucose, cholesterol and triglyceride levels were measured using the automated analyser Olympus Diagnostica (GmbH, Freiburg, Germany) following the manufacturer's indications (Blonç et al., 2023a; Brandts et al., 2021).

Gene expression

Total RNA extraction was performed using TRI REAGENT (Sigma) as per manufacturer's recommended protocols. RNA concentration and purity was determined employing a NanoDropND-1000 Spectrophotometer (Thermo Fisher Scientific). cDNA was generated through reverse transcription with a commercially available kit (iScript cDNA Synthesis Kit, Bio-Rad), following the manufacturer's indications. The selected primers were tested for efficiencies through RT-qPCR using serial dilutions of pooled cDNA (Pfaffl, 2001), and primers with efficiencies between 90% and 110% were selected (Table 1). RT-qPCRs were run in 384-well plates each containing 10 µL of SsoAdvanced™ Universal SYBR® Green Supermix (Bio-Rad), 200 nM primers and 2 µL of cDNA, for a total volume of 20 µL, through the BIO-RADCFX384 Real-Time PCR Detection System (Bio-Rad). The selected housekeeping genes, namely *βactin, rps5* and *rpl7*, were assessed for suitability using the GENorm algorithm integrated into the Bio-Rad CFX Maestro software (Bio-Rad). The results indicated that the most stable genes were *rps5* and *rpl7*, which were therefore employed as reference genes. The gene expression was calculated relative to 0 as per the Bio-Rad CFX Maestro Used Guide's recommendations.

Statistical analyses

All collected data was analysed using the open-source software RStudio. Data was tested for homoscedasticity and normality employing the *Bartlett's test* and the *Shapiro-Wilk* test, respectively. As the data met the assumptions for parametric tests, one-way Analyses of Variance (ANOVA) were run to determine significant differences amongst groups (P-values < 0.05). For the post-hoc test, the authors employed a pairwise comparison with Bonferroni adjustment.

Results

Bioaccumulation of gemfibrozil in liver and muscle

The results from the quantification of GEM in both liver and muscle indicate that bioaccumulation occurred at significantly higher rates in the HC group, compared to both the Ctrl and the LC groups (Fig. 1). GEM was also detected in both organs of Ctrl and LC, but levels remained below the threshold for quantification. Therefore, no statistically significant differences were found between these two groups. Furthermore, no significant differences were detected in the bioaccumulation rates between liver and muscle.

Haematology

The results obtained from the haematological analyses indicate a significant increase of haemoglobin (HGB) in HC compared to Ctrl (Fig. 2). Mean corpuscular haemoglobin concentration (MCHC) resulted significantly lower in LC compared to HC. However, no significant differences were found between Ctrl and LC nor between Ctrl and HC. None of the other investigated parameters, including white and red blood cell counts (WBC; RBC), haematocrit (HCT), and platelet count (PT), displayed significant variations between experimental groups.

Biochemical markers in plasma

The results from the biochemical analyses of blood plasma (Fig. 3) indicate significant reductions in cortisol and glucose levels between the Ctrl and both exposed groups. No significant differences in these parameters were detected between LC and HC. Whereas no differences between treatments were found concerning TOS, TAC showed a significant increase in the HC group when compared to Ctrl and LC. No significant differences were found between treatment groups in ADA activity, triglycerides and cholesterol plasma levels between treatments.

Gene expression in liver

The results obtained through real time qPCR analysis (Fig. 4) revealed that *apoa1* transcripts were significantly upregulated in the HC group compared to the Ctrl and LC groups but the expression of the rest of transcripts associated to lipid metabolism (*lpl*, and *pparα, pparβ* and *pparγ*) remained unaltered at the end of the 28-day exposure to GEM.

Table 1
Primer sequences used in the real time qPCR analysis.

Gene	GenBank Accession number	Sequence (5′->3′)		Product size
rps5	XM_026226870.1	Fw	5′ CACGCCTTTGAGATCATCCAC	128
		Rv	5′ TGTCTCCTCACGGTTCCAG	
rlp7	XM_026286641.1	Fw	5′ ATGGTGTCAGCCCTAAAGTCC	190
		Rv	5′ ATCTTGCCAAATCCACGCTTG	
pparα	XM_026238527.1	Fw	5′ CCCCAGAGTCTCCAAATGACC	269
		Rv	5′ TGACTGGCTTTGTGAGGAGG	
pparβ	XM_026270284.1	Fw	5′ TGGCTTTGTGGATCTCTTCC	178
		Rv	5′ GATCTCGCTGAAAGGTTTGC	
pparυ	XM_026240742.1	Fw	5‘ TTCCACAGCTGTCAGTCTCG	201
		Rv	5′ CATGAAGATCTGTCCGTAGG	
lpl	XM_026198452.1	Fw	5′ GGCCAAGTTTGTCAACTGG	166
		Rv	5′ CTCAAAACTAGGGCCAGCA	
apoa 1	XM_026226007.1	Fw	5′ TCAACTGGGAGGAGACCAAG	201
		Rv	5′ TGCCCAACTCTTCCATCTTC	
il1β	XM_026220385.1	Fw	5′ GATGCGCTGCTCAGCTTCT	66
		Rv	5′ AGTGGGTGCTACATTAACCATACG	
il6	XM_026289280.1	Fw	5′ CTGGATGCAGTGCCTGTCTA	214
		Rv	5′ TCACGTCTTCAAACGCAGTC	
il8	XM_026204952	Fw	5′ CTGAGAGTCGACGCATTGGAA	75
		Rv	5′ TGGTGTCTTTACAGTGTGAGTTTGG	
cat	XM_026203265.1	Fw	5′ TACACCGATGAGGGCAACT	170
		Rv	5′ AACGATTCAGGACGCAAAC	
gpx	XR_003293223.1	Fw	5′ GAAGTGAACGGTGTGAACGC	94
		Rv	5′ GATCCCCCATCAAGGACACG	
gst	XM_026224781.1	Fw	5′ GTGGATTACTTCAATGGCAGAG	211
		Rv	5′ CAGCGATGTAGTTCAGGATGG	

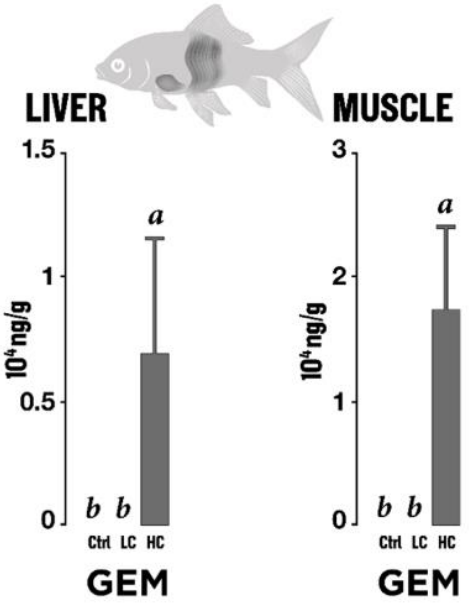

Fig. 1. Bioaccumulation of gemfibrozil (GEM; mean ± SD) in liver and muscle of adult *Carassius auratus* following a 28-day waterborne exposure ($n = 10$; Ctrl: 0 mg GEM/L; LC: 1.5 μg GEM/L; HC: 15 mg GEM/L). Significant differences ($P<0.05$) are depicted by different letters.

Similarly, the expression of transcripts related to inflammatory responses (*il1β, il6* and *il8*) did not change the expression levels between treatments. Concerning the anti-oxidative mediators, *gpx* and *gst*, but not *cat*, transcripts appeared significantly upregulated in LC compared to Ctrl and in HC compared to LC.

Discussion

Once absorbed by the gastrointestinal tract, GEM exerts its multi-tissue hypolipidemic effects in mammals by activating the PPAR receptors, inducing an up-regulation of *apoa1* and *lpl* mainly in muscle and adipose tissues. In turn, this decreases the levels of plasma triglycerides, inhibits peripheral lipolysis, and reduces the hepatic removal of fatty acids by mechanisms still not entirely understood (Roy and Pahan, 2009). The present results showed that GEM bioaccumulates at quantifiable concentrations in the liver and muscle of fish at high (15 mg/L) exposure concentrations (Fig. 1). However, GEM was also detected in the control group and in fish challenged with LC (1.5 μg/L) concentrations of this compound, although below the threshold for quantification. The bioaccumulation of GEM observed in goldfish tissues is in accordance with the described bioaccumulation of GEM in muscular and fatty tissues of fish(X. Zhang et al., 2010), plasma (Mimeault et al., 2005), and liver (Ramirez et al., 2009; Skolness et al., 2012), at environmentally relevant, and spiked waterborne concentrations. In addition, a previous study described bioretention of GEM in whole-fish samples collected from wild populations, with lower concentrations being quantified in fish tissues than in the surrounding water (Meador et al., 2016). The detection of GEM in the tissues of the control group could be explained by the possible current presence of this compound in Spanish tap water, as described in other European countries (Loos et al., 2007). Furthermore, it is likely that, as in humans, GEM undergoes hepatic metabolism, being transformed into inactive metabolites in the liver (Quintanilla Rodriguez and Correa, 2019). Therefore, the evident differences in accumulation rates between the analysed organs might be directly linked to the pharmacokinetic properties of this compound, and the enzymatic differences between liver and muscle. In fact, the bioaccumulation properties of pharmaceuticals and other xenobiotics in fish seem to differ between organs and tissues, due to species-specific metabolic needs (Chen et al., 2017), and usually the liver seem to be more efficient (due to a more complex enzymatic pool of xenobiotic degradative components) than muscle in the short-term clearance of xenobiotics and unwanted pharmaceuticals, lessening the outcome of oxidative stress (Beghin et al., 2021). This may explain the observed accumulation of GEM in the muscle vs. liver in goldfish (the former being a bona fide indicator of chronic contamination, as suggested by Orias et al. (2015), and may also relate to the appearance on myopathologies and muscle disorders related to an excess of Gemfibrozil in mammals, mainly due to inhibition of the glucuronidation pathway and cytochrome P4502C8 (CYP2C8) activity (Dubińska-Magiera et al.,

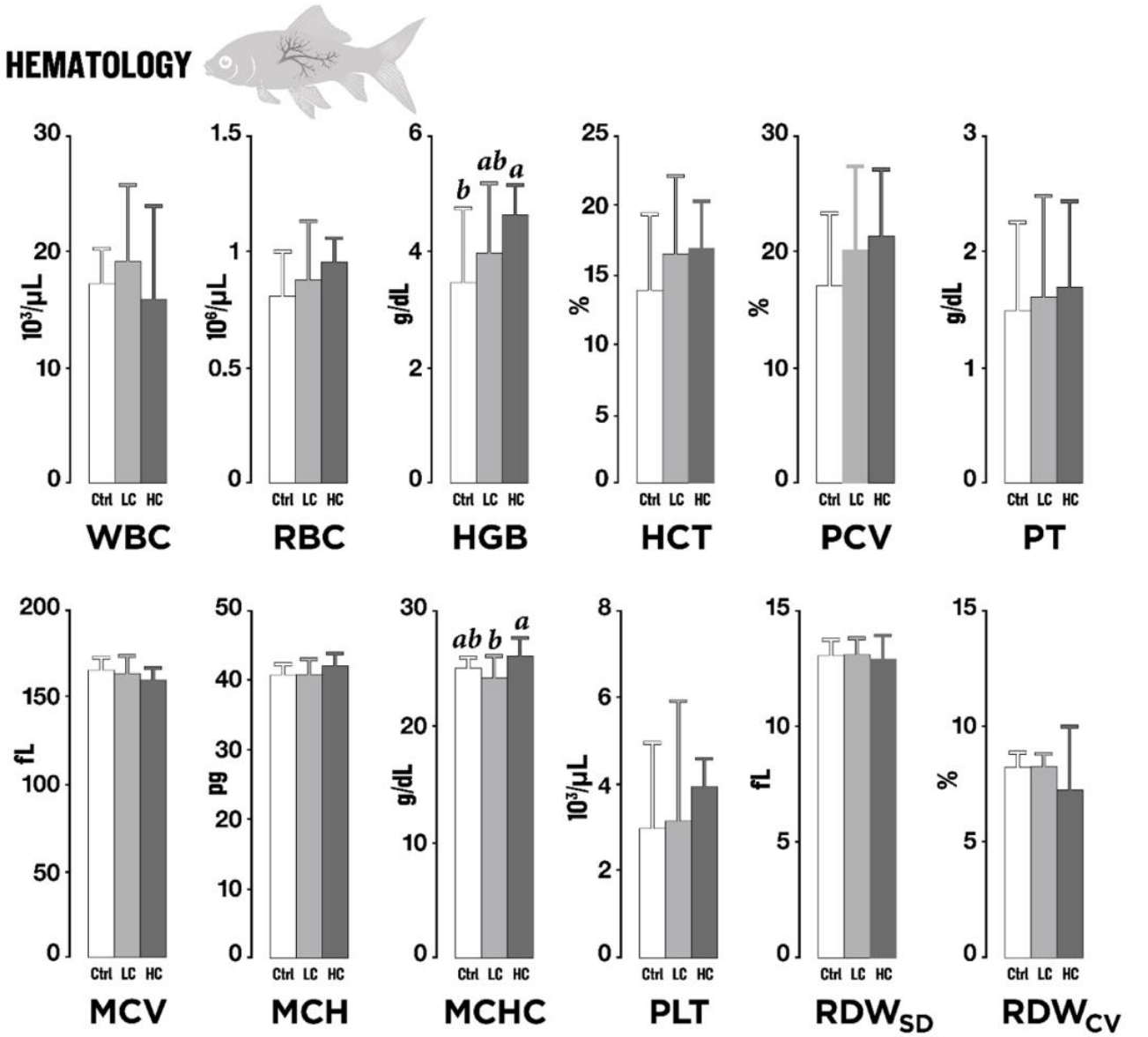

Fig. 2. Haematological parameters of adult *Carassius auratus* following a 28-day exposure to gemfibrozil (GEM; $n = 10$; Ctrl: 0 mg GEM /L; LC: 1.5 µg GEM/L; HC: 15 mg GEM/L; mean ± SD). Significant differences ($P<0.05$) are depicted by different letters. The investigated parameters were: White Blood Cell count (WBC), Red Blood Cell count (RBC), Haemoglobin (HGB), Haematocrit (HCT), Packed Cell Volume (PCV), Prothrombin Time test (PT), Mean Corpuscular Volume (MCV), Mean Corpuscular Haemoglobin (MCH), Mean Corpuscular Haemoglobin Concentration (MCHC), Platelet count (PLT), Red Cell Distribution Width (RDW).

2021) Present results, however, contrast with a previous report of bioaccumulation in *C. auratus* plasma following a 14-day waterborne exposure to 1.5 µg/L and 1.5 mg/L of GEM (Mimeault et al., 2005). Altogether this suggests a variable pattern of bioaccumulation during chronic exposures to the drug that, once taken up through the gills (Mimeault et al., 2005), is then transported by blood and accumulated within the plasma at first, accumulating in organs (e.g. muscle) only after longer exposures, or at higher doses.

A common side effect of GEM in humans is the development of anaemia (Strauss et al., 2012). Present results indicate that chronic exposure to GEM did not induce significant changes in the number of erythrocytes, leukocytes nor haematocrit in goldfish after the 28-day treatment. Nonetheless, total haemoglobin (HGB), as well as mean corpuscular haemoglobin concentration (MCHC) resulted higher in the HC group. This could indicate that, as opposed as what has been reported in humans (Scatena et al., 1995), haemoconcentration occurred due to a reduced affinity of red blood cells to haemoglobin. Therefore, the observed response is similar to that observed in fish subjected to hypoxic conditions (Tervonen et al., 2006), or to an acidic medium (Kulkarni and Barad, 2015). However, it cannot be ruled out that the present results reflected the remains of an over-compensatory erythropoietin (EPO)-mediated effect regulated by the activation of hypoxia-inducible factor (HIF) as described for PPARα-knockout mice exposed to GEM (Estrela et al., 2020). The authors reported a transient (14 days) PPARα-dependant anaemia, leukopenia and changes in haemoglobin and haematocrit levels that were reverted on day 21 after GEM treatment, concurrent with elevated EPO plasma levels and increased expression of *HIF-2α* and EPO in renal tissue of treated mice. The mechanisms of GEM-induced anaemia are still far from being resolved, and the EPO-dependant regulation of erythropoiesis in fish differs from

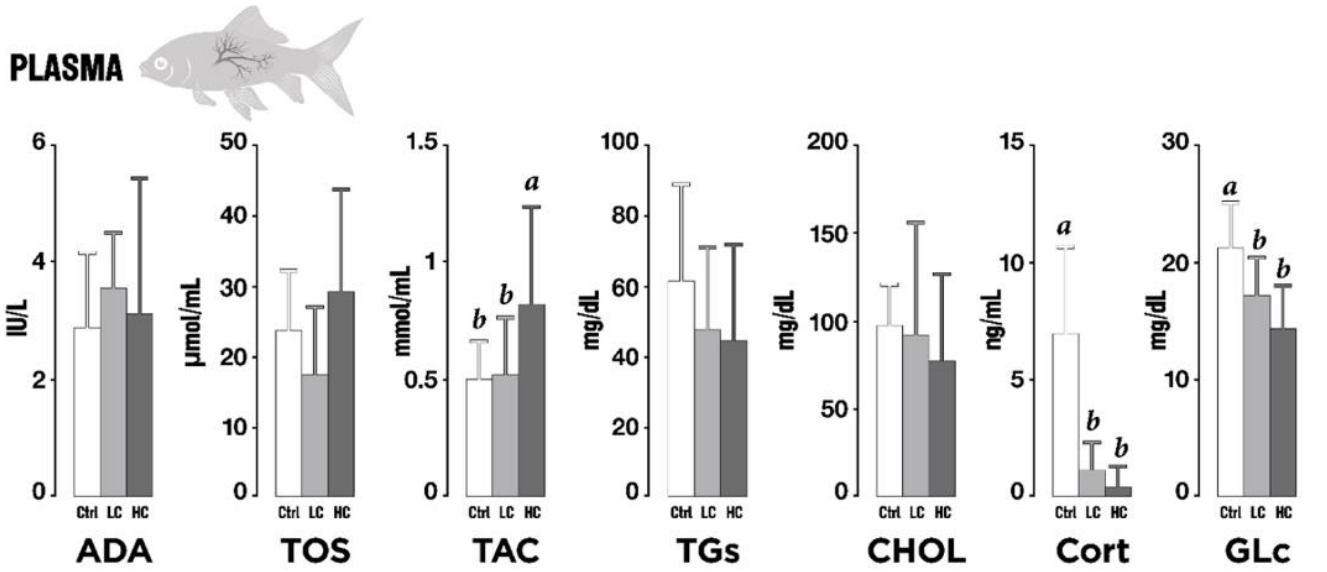

Fig. 3. Biochemical parameters in *Carassius auratus* blood plasma following a 28-day waterborne exposure to gemfibrozil (GEM; $n = 10$; Ctrl: 0 mg GEM/L; LC: 1.5 µg GEM/L; HC: 15 mg GEM/L; mean ± SD). Statistical differences ($P<0.05$) are represented by different letters above the corresponding columns. The investigated endpoints were Adenosine Deaminase (ADA), Total Antioxidant Capacity (TAC), Total Oxidative Status (TOS), Triglycerides (TGs), Cholesterol (CHOL), Cortisol (Cort), and Glucose (GLc).

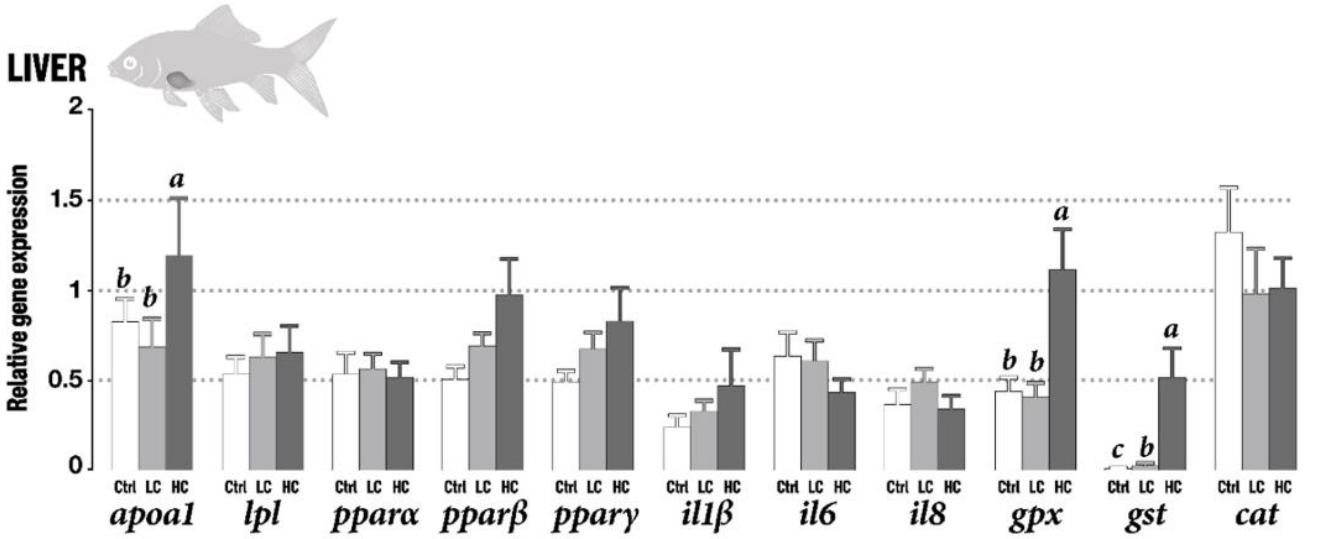

Fig. 4. Relative gene expression of *Carassius auratus* liver homogenates following a 28-day waterborne exposure to gemfibrozil (GEM; $n = 10$; Ctrl: 0 mg GEM/L; LC: 1.5 µg GEM/L; HC: 15 mg GEM/L; mean ± SD). Statistical differences ($P<0.05$) are represented by different letters above the corresponding columns. The investigated endpoints were Catalase, *cat*; Glutathione Peroxidase 1, *gpx*, glutathione *S*-transferase, *gst*, interleukins 6, 8 and 1β, *il6, il8, il1β;* apolipoprotein A, *apoa1*; lipoprotein lipase, *lpl*; peroxisome proliferator-*act*ivated receptors α, β, and ϒ, *pparα, pparβ, pparγ*.

that of mammals (Kulkeaw and Sugiyama, 2012), but present results seem to agree with the timing of haematological variations described for short-term/chronic GEM exposure in mice.

Absence of major changes in lipid metabolism and immune biomarkers in goldfish after chronic exposure to gemfibrozil

The 28-day exposure to GEM did not lead to significant changes in plasma levels of neither cholesterol nor triglycerides. These results agree with previously published literature, stating that no significant changes in plasma cholesterol and triglycerides occurred following a waterborne exposure of *Pimephales promelas* (fathead minnow) to GEM (Skolness et al., 2012). In contrast, it has been reported that *Oncorhynchus mykiss* (rainbow trout) exposed to this emergent contaminant through injection did display significant alterations in these parameters (Prindiville et al., 2011). This suggests a species-specific scenario of lipid physiology in fish that also involves the regulatory adipogenesis networks. The management of lipids in fish results from a balance between hypertrophy and hyperplasia of adipocytes in visceral adipose tissues (Salmerón, 2018). This is orchestrated by *pparγ* and assisted by adipogenic gene markers such as *lpl*, involved in the hydrolysis of circulating triglycerides to produce the free fatty acids (FFA) required for growth and metabolism (Rakhshandehroo et al., 2010), or *apoa1*, implicated in the transport of cholesterol (Xie et al., 2020). *Pparα* and *pparβ* complete the minimal

genetic repertoire to adequately process lipids, with varied tissue representation but similar ligands in fish species (Páscoa et al., 2022). However, the pleiotropic effects of the PPAR family extend beyond the management of lipids and affect general homoeostasis. For instance, the activation of PPARα upon feeding on high-fat content diets enhances the expression of antioxidant regulators, such as *cat* and *gpx*. Furthermore, it inhibits the expression of pro-inflammatory cytokines such as *il1β* or *il6* in an effort to control the upsurge of reactive oxygen species (ROS) derived from the oxidative metabolism of lipids and FFA absorption (Ahmadian et al., 2013). *Pparγ*, in turn, may be inhibited by an excess of ROS, adding layers of complexity to the regulation of lipid metabolism in presence of xenobiotics (Li et al., 2022). In the present experiment, a significant increase in the expression of *apoa1* mRNA in goldfish liver exposed to GEM was observed, being consistent with previously published literature. It has been reported that after a short-term exposure to 150 µg/L of GEM in water, *Sparus aurata* (gilthead seabream) displayed a significant upregulation of this gene in the liver (Teles et al., 2016). Concerning PPARs, the significant differences in the expression of *pparα* transcripts were somewhat expected, as it has been demonstrated that PPARs may be affected, to some extent, by GEM. In addition, they may regulate the expression of other genes involved in lipid metabolism (e.g. *apoa1, lpl*) in mammals (Singh et al., 2011; Staels et al., 1998),. However, previous studies on fishes have stated that GEM does not necessarily induce the activation of *ppar* pathways in the liver, although it may alter the abundance of other genes related to lipid metabolism, such as *lpl* and *apoa1* (Teles et al., 2016). This was reported, for instance, in rainbow trout exposed to GEM through injections over a period of 15 days, where an increase in *lpl*, was observed, although not accompanied by up- or down-regulations of *pparα* (Prindiville et al., 2011). This is consistent with the results obtained through the present experiment. Additionally, *C. auratus* exposed to waterborne GEM at concentrations reaching 1500 µg/L failed to display increased levels of *pparα* and *pparγ*. However, the expression of *pparβ* mRNA resulted significantly downregulated (Mimeault et al., 2006). On the other hand, an experiment challenging *D. rerio* with dietary exposure to GEM over a 30-day period resulted in significant upregulations of *pparα,* only in females, and of *pparγ*, regardless of sex (Al-Habsi et al., 2016). In addition to *pparα, pparβ* and *pparγ* transcripts remained unresponsive to GEM that, together with the lack of differences in plasma cholesterol and triglyceride levels between treatments, indicates a lack of altered overall lipid metabolism in response to long-term exposure to GEM in goldfish.

The absence of changes in the expression of *pparα* transcripts may explain the absence of inflammation at day 28, as indicated by the lack of changes in the expression of key pro-inflammatory immune transcripts, namely *il1β, il6*, and *il8*. Previously published studies have reported a significant increase in the expression of *il1β* following a short-term waterborne exposure to GEM, both in the liver (Teles et al., 2016), and in the gills and head kidney of *S. aurata* (Oliveira et al., 2018). However, the authors described this effect as time- and dose-dependant. Similarly, the expression of *il6* did not change between groups in the present study. Oliveira et al. (2018) described a significant increase in the abundance of this gene in the head kidney of *S. aurata*. However, this was only observed at 1500 µg/L, and not at lower nor higher concentrations in this organ. In addition, the levels of this gene remained unchanged in the gills of gilthead seabream, regardless of the exposure concentration (Oliveira et al., 2018). This further corroborates that the response to this emergent contaminant is dependant on the dose and the organ investigated. Lastly, the expression of *il8* did not display any significant differences amongst groups in the present study. In humans, GEM has been demonstrated to significantly reduce the expression levels of this pro-inflammatory cytokine, either as a direct effect (Wägner et al., 2011), or through the inhibition of *il1β*-induced expression of *il8*, as observed with other fibrates (Stolarz et al., 2015). However, as the present results did not indicate any significant decrease in either *il1β* or *il8*, it can be hypothesized that the mechanism of actions of GEM, as other fibrates, may differ from mammals to fish. Therefore, further studies are required to fully understand the effects GEM may have on the immune response amongst these taxa.

Impact of long-term exposure to gemfibrozil on cortisol levels in goldfish

More intriguing is the downfall of plasma cortisol levels at day 28 in goldfish exposed to low and high concentrations of GEM (see Fig. 3). The expected physiological response to stressors is an increase of cortisol levels in plasma, resulting from an activation of the hypothalamic-pituitary-interrenal (HPI) axis (Aluru and Vijayan, 2009). Enduring a chronic exposure to GEM, which also acts as an endocrine disruptor, could alter the availability of energetic resources, such as steroids, and affect the HPI axis as observed with other endocrine disruptors (Teles et al., 2006), impairing the cortisol-mediated response. Alterations of stress neuroendocrine axis in fish exposed to pharmaceutical xenobiotics have been described previously in zebrafish (Al-Habsi et al., 2016). Indeed, low levels of cortisol were also demonstrated in females, but not in males, treated chronically (30 days) with GEM and other anti-dyslipidaemics. The authors argued that this could be due to altered signalling in reproductive-related triglyceride and cholesterol balance and abundance (Al-Habsi et al., 2016), which were unaltered in the present study. Instead, present results seem to correlate with the effects described in zebrafish exposed to antipsychotic drugs such as diazepam and fluoxetine, that diminish cortisol levels at short- and long-term challenges, effectively blocking the cortisol-mediated behavioural responses to acute stress and anxiety (Egan et al., 2009), whereas risperidone and aripiprazole increase the plasma cortisol levels in the same species (reviewed by Mauro et al., 2021). In the present research study, glucose follows the same pattern described for cortisol, but, considering the lack of expression of *pparα* transcripts, that once active are known to decrease glucose levels (Chou et al., 2002), the diminishing pattern probably reflects the decrease in the cortisol-dependant hepatic gluconeogenesis (Jentoft et al., 2005). Reduced plasma glucose levels may also reflect the depletion of an energetic resource due to a higher energetic demand of exposed individuals undergoing a series of highly energetically demanding processes. These may include antioxidant mechanisms to counter the effects of ROS associated with xenobiotic exposure (Liang et al., 2020). Clearly, the effects of pharmaceutically active compounds, such as GEM, on the stress physiology of fish are species-, sex-, drug-, dose-, and developmental stage-dependant and may be influenced by the combined interaction between several xenobiotics. The underlying physiological mechanisms that lower plasma cortisol levels in presence of pharmaceutical xenobiotics are still unresolved in fish but may correlate with the affectation of serotonergic signalling in the central nervous system (Porretti et al., 2022), that partially determines behavioural patterns. In this sense, male zebrafish early exposed to fluoxetine maintained hypocortisolism and exploratory behaviour in the offspring (Vera-Chang et al., 2018). This suggests that, if persistent, the potential anxiolytic effects induced by chronic exposure to both low and high levels of GEM in goldfish may be of special concern. Furthermore, these may be difficult to predict in terms of behavioural coping with stressful environments prone to biomagnification and unpredictable changes in physicochemical variables. It has been suggested that, in fish, plasmatic cortisol levels are tightly linked to the hierarchical position of individuals (Sloman et al., 2004), and that, consequently, cortisol-linked social interactions might be hampered by prolonged exposure to GEM. Similarly, a wide range of cortisol-related processes, such as anti-predatory behaviour, responses to environmental stressors, or growth, might be impaired, having strong deleterious effects on the fitness of individuals, and significantly affecting their survival. (Gagnon et al., 2006; Teles et al., 2006). Therefore, the presence of GEM in aquatic systems may have effects not only at an individual level, but also at a population or species scale.

Oxidative management after long-term exposure to gemfibrozil in goldfish

GEM has been considered an agonist of PPARα and thus been added to the formulation of fish diets with high content of carbohydrates, in an attempt to control for the undesirable oxidative and immune effects of such diets in such dysregulated metabolism scenarios (Luo et al., 2020). However, the present results indicate that the agonistic effects of GEM on goldfish were only partially fulfilled: the apparent immune quiescence contrasts with the upregulated expression of oxidative-stress-related transcripts at high doses, together with an enhanced TAC. TAC and TOS reflect the combined action of different antioxidants, and the presence of different oxidant species, respectively, and have been suggested as valuable endpoints to assess the possible oxidative damage in fish following an exposure to chemicals (Teles et al., 2016). In the present study, TAC, but not TOS nor ADA, a biomarker for both oxidative stress and immune response (Blonç et al., 2023a), increased in the HC group, compared to both the LC and Ctrl groups. The observed TAC values may reflect, in part, the upregulation of transcripts related to antioxidant defences. The relative expression of hepatic *gpx* mRNAs was significantly higher in the HC group when compared to the Ctrl and LC groups, agreeing with the previous findings in the same species (Mimeault et al., 2006). However, in contradiction with the results of the present experiment, the authors described a significant increase of *gpx* related enzymes at the lowest tested concentrations, equivalent to the one hereby imposed on the LC group, compared to the control, but not between exposed groups. In *S. aurata*, previous findings have reported an upregulation of this antioxidant defence-related transcript, as well as superoxide dismutase (*sod2*), in gills and head kidney after a 96-h exposure to 15 mg/L GEM (Oliveira et al., 2018). However, the authors stated that no other antioxidant indicator resulted significantly altered by this exposure. In contrast, Teles et al. (2016), following a similar experimental design, described no significant changes in the activity of any antioxidant genes in the liver of the same species, under the same exposure conditions. On the other hand, a study replicating the experiment found significant increases in the hepatic expression of *gpx* at concentrations ranging from 15 µg/L to 15 mg/L GEM (Barreto et al., 2018). On the contrary, intraperitoneal (IP) injection of GEM in *Solea senegalensis* resulted in significantly lower *gpx* activity compared to the control group (Solé et al., 2014). The upregulation of hepatic *gst* transcripts observed in the present study with increasing GEM concentration agrees partly with previously published results stating significant increases in this gene's activity only at 1.5 µg/L, but not at higher concentrations (Mimeault et al., 2006). Nonetheless, no changes in the expression of this antioxidant indicator were reported in either liver, gills nor head kidney of *S. aurata* and *Anguilla anguilla* (European eel; Barreto et al., 2018; Lyssimachou et al., 2014; Oliveira et al., 2018; Teles et al., 2016). As with *gpx, gst* activity in the liver of *S. senegalensis* was significantly lowered by IP injection of GEM (Solé et al., 2014). Lastly, no significant differences were found in abundance of *cat* mRNA in the present study. These results agree with previous findings on *S. aurata* challenged with an acute waterborne exposure to GEM, where no significant alterations in catalase activity were described (Oliveira et al., 2018; Teles et al., 2016). Nonetheless, a study on *C. auratus* with similar experimental design as the present, resulted in a significant increase in the expression of hepatic *cat* with increasing GEM concentration. Similarly, Barreto et al. (2018) observed an increase in *cat* at 15 mg/L GEM in *S. aurata*. In addition, Lyssimachou et al. (2014) reported an upregulation of *cat*, although only in *A. anguilla* injected with 0.1 µg/L, and not at higher concentrations. In contrast, in *S. senegalensis* injected with GEM, a significant decrease in catalase activity was reported (Solé et al., 2014).

Overall, the anti-oxidative response in goldfish enduring 28 days of GEM seems restricted to high doses and limited in intensity and recruitment of antioxidant markers. This may indicate a decaying defence against ROS during the initial exposure to GEM that trends towards normal homoeostasis at day 28. Alternatively, this could indicate a mildly oxidative stress elicited by continuous exposure to GEM. However, as discussed above, *pparγ* and pro-inflammatory transcripts remained unaltered, which suggests an absence of a strong immune-related ROS production that may eventually inhibit *pparγ* (Li et al., 2022). On the other hand, this could result from the activation of macrophages and other cellular components on the defence response against a xenobiotic. Therefore, the anti-oxidative response to GEM may obey the complex effects of the breakdown of GEM at high but sublethal doses following the bioaccumulation in liver and muscle of goldfish. In mammals, drug and xenobiotic biotransformation involves the cytochrome P450 (CYP)-dependant phase I and the conjugation-dependant phase II enzymatic reactions. The former includes hydrolysis and oxidation of molecules and the later the presence of endogenous polar components, such as glutathione, that links to the xenobiotic due to transferases such as glutathione S-transferases (Xu et al., 2005). Both enzymatic complexes oxidize GEM to highly reactive phenolic and glucuronide metabolites and induce oxidative stress that, in turn, requires the activation of antioxidant biomarkers (Stading et al., 2020). Activity of CYP enzymes was not hereby evaluated. However, considering the upregulation of *gst* transcripts observed in goldfish exposed to high doses of GEM, and the quiescence of the pro-inflammatory mRNAs analysed, the overrepresentation of reactive metabolites derived from the biotransformation of chronic exposure to high doses of GEM may account for the mild anti-oxidative responses observed. However, it is worth mentioning that the biotransformation reactions are complex, still poorly studied in fish and strongly organ-, species-, sex-, and xenobiotic type-dependant.

Concluding remarks

To summarize, the present results show that a chronic (28-day) exposure to environmentally relevant and high sublethal doses of GEM induced bioaccumulation of this pharmaceutical in the goldfish liver and muscle. The latter is of particular importance to the aquaculture industry, as it is the most relevant in terms of fish consumption. Therefore, it represents an additional exposure and biomagnification pathway for humans through the consumption of contaminated seafood products. Both prolonged exposure and bioconcentration may have played a role in the observed differences in biochemical plasma endpoints between treatments, suggesting (1) a mild anti-oxidative response to high but not low doses of GEM that maybe related to the biotransformation enzymatic reactions to GEM, (2) the possibility of transient anaemia during the treatment, (3) an unaltered expression of lipid metabolism biomarkers, including key regulators such as *pparα* and *pparγ*, and pro-inflammatory cytokine transcripts, notably *il1β*, a canonical telltale of inflammatory outbursts, and (4) an intriguing reduction of plasma cortisol levels. This indicates that GEM may act, to some extent, as a stress-inducing agent, and, as described in other fish species, may tamper with reproduction, development, stress responses, and overall fitness of both wild and cultured populations, affecting the bioavailability of energetic resource in a strongly species-specific manner. The effects of GEM depend upon the developmental stage, sex, exposure concentration and duration. Therefore, further studies are needed to describe the in-deep mechanisms of physiological disturbance that high doses of GEM. These disturbances may originate from inappropriate wastewater management or unregulated bioaccumulation in natural and cultured systems. The present findings should serve as a foundation for future research, which is needed to understand their potential effects at different life stages of different species, both wild and cultured, and may help shape future legislations concerning pharmaceutical waste management and sewage treatment practices. Furthermore, the relevance of long-term exposure in terms of adaptive phenotypes in the population and persistent transgenerational physiological changes must be looked into. Moreover, investigating the synergistic effect of GEM in combination with other contaminants (Andrzejczyk et al., 2020), or environmental factors, may give a more comprehensive insight on the

mechanism of action of this compound, and their implications, not only from an environmental point of view, but for the aquaculture industry, and ultimately global food security.

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Author contribution

Conceptualization: MB, NR, MT, LT; Funding acquisition: LT, MT, MF; Investigation: MB, NR, JCB, LT, MF, ML, AT, MT; Writing – original draft: MB, NR, JCB, ML, AT; Writing – review & editing: All authors, Supervision: LT, MT.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Further reading

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5

Impact of a chronic waterborne exposure to polystyrene nanoplastics on the gilthead seabream (Sparus aurata): combining traditional and multi-omics approaches

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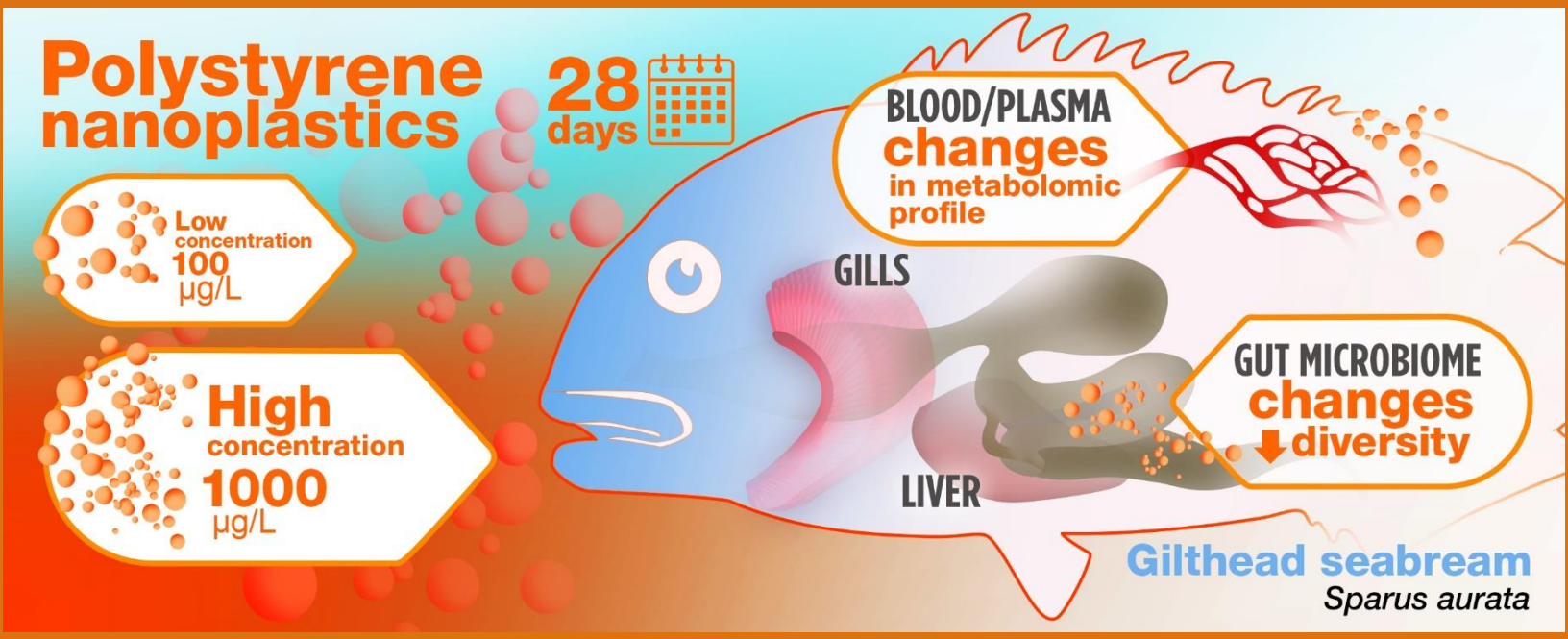

Impact of a chronic waterborne exposure to polystyrene nanoplastics on the gilthead seabream (*Sparus aurata*): combining traditional and multi-omics approaches.

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Abstract

Recirculated aquaculture systems (RAS) represent a valuable asset to meet a growing seafood demand in a sustainable way. However, micro- and nanoplastic (MP <5 µm, NP <1000 nm, respectively) contamination still poses a risk in such systems. The present study aimed to elucidate the effect of prolonged waterborne exposures to polystyrene nanoplastics (PSNPs) on one of the most commercially important fish species of Mediterranean aquaculture, the gilthead seabream (*Sparus aurata*). Juvenile fish were exposed to two concentrations of PSNPs (100 µg/L and 1000 µg/L) over 28 days, and a series of traditional (*i.e.* morphometrics, haematology, histology) and omics (*i.e.* metabolomics, microbiome sequencing) approaches were employed to obtain a comprehensive understanding of the toxicity of PSNPs in *S. aurata*. The present results indicate that the studied experimental conditions did not impact morphometrics or haematological profile, nor did it cause histopathological lesions. However, waterborne PSNPs induced changes in the microbiome and the metabolomic profile in fish in a dose-specific manner, having potential repercussions on the energy metabolism and overall performance.

Environmental Implication

Polystyrene nanoplastics (PSNPs) are one of the most common polymers in aquatic systems, whereas *Sparus aurata* is one of the most economically important fish species in the Mediterranean. The results indicate that PSNPs accumulate in the different organs of this species at different rates. Analysed muscle samples contained PSNPs, representing additional exposure routes to humans through the consumption of contaminated fish. In addition, the exposure led to alterations in the microbiome and metabolome of the gilthead seabream, which may have repercussions on its health status, affecting its growth, and having deleterious consequences on the performance of the aquaculture industry.

Keywords: Fish welfare; metabolomics; microbiome; haematology; aquaculture

Introduction:

Global food security, an essential component to ensuring human health worldwide, faces a plethora of challenges. These include a variety of interconnected anthropogenic threats, such as climate change, overexploitation of natural resources and biodiversity loss, and environmental pollution, otherwise referred to as the triple planetary crisis. Today, plastic pollution has become a major concern to the scientific community due to the ever-increasing production and use of these materials and the poor management of plastic waste. Particularly, micro (<5 mm) and nano (<1000 nm; Galgani et al., 2013; Gigault et al., 2018) plastics (MPs and NPs) have gained attention and are now widely recognised as contaminants of emerging concern, being ubiquitous in both terrestrial and aquatic environments (Kumar et al., 2020; Llorca et al., 2021; Rosso et al., 2023; Ter Halle et al., 2017). Nanoplastics (NPs) can be classified into two categories. On one hand, primary NPs are directly manufactured nano-sized and are commonly used in personal care products and flame retardants (Gonçalves & Bebianno, 2021). On the other hand, secondary NPs are the result of the biotic and abiotic degradation of larger plastic objects (El Hadri et al., 2020). Therefore, with an increase in plastic production and discard (Yan et al., 2024), a rapid increase in the quantity of NPs in the environment is expected. These compounds have been detected and quantified in a large variety of environmental matrices (*e.g.* drinking water, throughout the oceanic water column, alpine snow), with polystyrene (PS) and polyethylene (PE) being the most commonly found polymers (Reviewed by Cai et al., 2021; Llorca et al., 2021).

Aquaculture, consisting of farming aquatic organisms, if done sustainably, represents an invaluable asset in protecting biodiversity and safeguarding global food security (Yadav et al., 2024) in the current scenario of increasing world population (Gu et al., 2021). In particular, the use of recirculated aquaculture systems (RAS) offers the possibility to virtually isolate the farmed organisms, both sheltering them from environmental variables and minimising the impact of the practice on surrounding ecosystems. RAS protect the cultured species from climatic events, predators and fluctuations in environmental parameters (e.g. temperature, nitrogenous compounds, pathogens, pH; Ahmed & Turchini, 2021; Bregnballe, 2022; Summerfelt & Vinci, 2008). However, it has been demonstrated that NPs are still present in such systems, suggesting that they originate both from external (i.e. inlet water) and internal (e.g. bio-beads) sources (Blonç et al., 2023). Therefore, it is clear that aquaculture species, whether farmed in open or closed systems, are continuously exposed to these emergent contaminants. The gilthead seabream (*Sparus aurata*) is one of the most economically important cultured fish species in the Mediterranean area (FAO, 2021, 2022; Mhalhel et al., 2023), and the presence of contaminants in the water, such as NPs, might have negative repercussions on the viability of the production by affecting reproduction and growth, amongst others, potentially affecting millions of people. Thus, it is of utmost importance to thoroughly investigate the effect that NPs might have on this species to better understand the implications of NP pollution to the aquaculture sector and global food security.

Current research on the impact of NPs on fish has mainly focused on short-term, or acute, exposures (Barría et al., 2020), leaving only a few to investigate the effects of prolonged, or chronic, exposures (Brandts et al., 2021; Marana et al., 2022). Most of these chronic studies have researched the impact of NPs in model organisms, such as medaka (*Oryzias latipes*; Zhou et al., 2022; Zhou et al., 2023), zebrafish (*Danio rerio*; Sarasamma et al., 2020), and goldfish (*Carassius auratus;* Brandts et al., 2022). The results indicate that NPs may alter the lipid metabolism of fish, hindering their ability to

efficiently allocate their energetic resources, affecting their reproduction, development (Brandts et al., 2022; Lin et al., 2023; Zhou, Jin, et al., 2023), and behaviour (Sarasamma et al., 2020). Furthermore, it has been reported that NPs are internalised by aquatic organisms, accumulate in different tissues at differing rates, and travel through the food chain, reaching top-consumers by trophic transfer (He et al., 2022; Mattsson et al., 2017). Moreover, NPs have been described as significantly disturbing the metabolome of *C. auratus* and *Carassius carassius* (Mattsson et al., 2015; Shi et al., 2021) and the gut microbiome of *D. rerio, O, latipes,* and *Sebastes schlegelii*, amongst others (Sun et al., 2023; Yu et al., 2022; Zhou, Gui, et al., 2023).

However, the effect of PSNPs in aquatic organisms appears as highly dependent on the organisms' characteristics (e.g. species, life stage, sex), as well as the physio-chemical properties of the contaminant (e.g. polymer type, size, shape, purity), and the type of exposure (e.g. duration, administration route, co-contaminants; reviewed by Barría et al., 2020 and Gong et al., 2023).

Given the inherent intricacies of NPs toxicity, it is essential to combine a variety of analytical methods when investigating the impacts of NP pollution. Indeed, single-omics approaches, for instance, are not sufficient to understand the effect of NPs in living organisms (Bhagat et al., 2022), and must be coupled with additional assays in order to avoid drawing inaccurate conclusions.

To the best of the authors knowledge, the present study is the first to investigate the impact of a chronic waterborne exposure to PSNPs in a commercially important marine fish, in this case *S. aurata*. To this end, a multidisciplinary approach was employed, integrating traditional assays (*e.g.* less energy-, technologically, and economically demanding) and omics technologies (*e.g.* higher throughput and result yield) to acquire a comprehensive understanding of how this compound may affect the overall health and welfare status of this species, and its potential impact on the aquaculture sector and, ultimately, global food security. This study analysed retention rates of PSNPs, and the potential alterations in the morphometrics, histology, haematological profile, metabolome, and microbiome of the gilthead seabream induced by a 28-day exposure to waterborne PSNPs. These contaminants were selected given their omnipresence in the environment, and the frequency of studies investigating this specific polymer, therefore providing a substantial amount of data and scientific publications to build upon and compare the results of the present study.

Materials and methods:

Experimental animals housing and husbandry:

Juvenile *S. aurata* (N = 60; 10.35 ± 11.53 cm total length, and 13.69 ± 5.73 g total weight) were purchased from AVRAMAR (Castellón, Spain). The fish were held for three weeks in the AQUAB facilities at the Autonomous University of Barcelona, in 1000L tank containing aerated artificial sea water (ASW) at 35 ppt (Sea salt, Aquaforest) working under recirculation settings, and fed twice a day 2 % of the total biomass as a maintenance diet. Fish were then transferred into 20 L glass tanks of ASW, each containing 5 fish, for acclimation to the experimental conditions. Water parameters were checked daily to ensure water quality was optimal for the tested organism, and water changes were performed when necessary.

Experimental conditions:

Following the acclimation period, the fish were subjected to a 28-day waterborne exposure to PSNPs under three distinct experimental groups, namely, the control group (Ctrl: 0 µg/L), the low concentration group (LC: 100 µg/L PSNPs) and the high concentration group (HC: 1000 µg/L PSNPs). Each of the three experimental groups was represented by quadruplicated tanks.
To reach the required PSNPs concentration in the experimental tanks, a small volume of a stock PSNPs solution was dissolved in 1L of tank water, which was then added back to the tank. The water quality parameters were controlled on a daily basis, and 75% of the water was changed every 48h to prevent the accumulation of metabolites, ensuring optimal conditions for the experimental subjects during the trial. Following the water changes, PSNPs were added again to maintain the desired concentration throughout the experimental period. Any individual displaying evident signs of distress was humanely euthanized through an overdose of tricaine methane sulfonate (MS-222) to prevent the unnecessary suffering of the animals.

Sampling:
Following the 28-day exposure to PSNPs, fish were individually euthanised by immersion in an MS-222 bath (300 mg/L) buffered with sodium bicarbonate ($NaHCO_3$; 600 mg/L), after which, death was confirmed through spinal rupture. Once the fish had reached stage 4 of anaesthesia (i.e. surgical anaesthesia), individuals were measured and weighed, and blood was immediately extracted using heparinised syringes (25G needles), mixed with lithium heparin (Deltalab, Spain) at a 25:1000 heparin to blood ratio, and preserved in dark conditions at 4 ºC until further processing and analysis. Blood samples were divided into three aliquots. One aliquot was used to obtain plasma through centrifugation (2000 *g*, 4 ºC, 10 min), another for semi-quantification of nanoplastics and the last one for a genotoxicity assay. Fish organs, namely, liver, gut, brain, muscle, and gills were collected under sterile conditions, and immediately snap frozen in liquid nitrogen. The samples destined for NPs semi-quantification were stored in crystal vials (Agilent Technologies). All tissue samples were then stored at -80 ºC until further processing and analysis.

Fulton's condition factor:
Total length (L, cm) and total weight (W, g) were used to determine the Fulton's condition factor (K_c) through the following equation: $K_c = 100 * \frac{W}{L^3}$.

Polystyrene nanoplastics characterisation:
Polystyrene nanoplastics of 40 nm (Bangs Laboratories) were characterized in different media (*i.e.* Ultrapure-MiliQ water and ASW) by means of dynamic light scattering (DLS; Zetasizer Pro, Malvern Instruments) to obtain particle size and polydispersity index (PDI). To this end, serial dilutions of the stock solution (100 mg/mL) were performed to obtain the lowest experimental concentration (*i.e.* 100 µg/L) and measurements were run 5 times at 25ºC. A PDI value over 0.2 was considered an indicator of particle aggregation.

Polystyrene nanoplastics extraction and semi-quantification:
PSNPs were extracted from tissue samples and purified. In summary, the samples were digested with KOH (10 %) at 60 ºC, for 6 h, and then rested at room temperature overnight. Subsequently, the samples were filtered through 0.7 µm fiberglass filters where the residual organic material present was further digested at room temperature with HNO_3 at 20 % over 1 h. Following this, the filters were cleaned with water and dried overnight at 60 ºC. PSNPs were extracted using ultrasonic-assisted extraction with 10 mL of toluene for 10 min and the supernatant was transferred to crystal vials. This procedure was repeated twice again. The final extract was then evaporated with a nitrogen stream

in order to concentrate the samples around 1.5 mL. The extracts were then vortexed and transferred to Liquid Chromatography vials and evaporated again to 1.5 mL. NPs were analysed by Liquid Chromatography coupled to High-Resolution Mass Spectrometry (HRMS) in an Acquity Liquid Chromatography (Waters, Milford) chromatographic system equipped with an advanced polymer chromatography column (Acquity APC XT45 1.7 µm) in isocratic conditions with toluene. The system was coupled to a Q-Exactive (Thermo Fisher Scientific) hybrid quadrupole-Orbitrap mass spectrometer, equipped with an atmospheric pressure photoionization source working in negative conditions. Data acquisition was performed in full scan mode (m/z 500-3000) with a resolution of 17,500 FWHM.

NMR Metabolomics in plasma:

Macromolecular components (>3KDa) were filtered from plasma using Amicon Ultra-0.5 Centrifugal FilterTubes. First, the filters were thoroughly washed with Mili-Q water and centrifuged for 5 minutes at 13793 *g* a total of four times to remove any potential traces of glycerol preservative. Once the filters were properly rinsed, the plasma samples were immediately centrifuged in the filter tubes at 13000 *g* for 30 minutes at 4 ºC. 200 µL of the obtained filtrate were then transferred to individual Nuclear Magnetic Resonance (NMR) tubes where 300 µL of the internal standard solution (i.e. 0.2 mM DSS in D_2O) was added, for a total volume of 500 µL and a final DSS concentration of 0.12 mM in each tube. ^{1}H NMR spectra were then measured in a Bruker 600-MHz AVANCE III NMR spectrometer equipped with a triple channel probe TXI 600 S3 H-C/N-D 05-Z-BTO (Bruker Biospin, Rheinstetten, Germany), operating at a ^{1}H frequency of 600.13 MHz and 298 K with a 5 min temperature equilibration time for each sample. The standard 1D ^{1}H-nuclear Overhauser effect spectroscopy (1D-NOESY) pulse sequence from the Bruker library was used (noesygppr1d). The relevant parameters applied were: mixing time: 100 ms (d8), recovery delay: 2 s (d1), 90◦ pulse: 8.95 µs (p1), spectral width: 7211.539 Hz, spectral size: 32 k, number of scans 512 and acquisition time: 2.27 s. For metabolite identification and quantification data processing and analysis Spectra were processed and analysed through the Chenomx 8.0 profiler software (Chenomx Inc., Edmonton, Canada). This software contains tools for automatic phase, baseline correction (spline), automatic shim correction, reference calibration and a library of metabolites for spectral profiling. The concentration of each metabolite identified was determined based on the standard internal reference DSS (concentration 0.12 mmol/L). The obtained data were processed using SAS v9.4 (SAS Institute Inc., Cary, NC, USA.). Two sets of statistical analyses were performed. For identified metabolites, non-parametric Kruskal-Wallis tests were undertaken, followed by a Wilcoxon test with Bonferroni correction. Furthermore, a principal component analysis (PCA) was performed to determine how many factors could separate the different metabolites, and a discriminant analysis was run to evaluate the components' capacity to differentiate the experimental groups. For non-identified metabolites, a qualitative assessment was performed by applying Chi-Squared tests or likelihood-ratio tests, as suitable. The *p*-value to reject null hypothesis was set at 0.05.

Blood analysis:

The haematological analyses were performed by inserting 90 µL of fresh heparinised blood in the automated haematological analyser XN-1000V (SYSMEX) working with an adapted version of the bird blood analysis software. Leukocyte and erythrocyte counts, haemoglobin, haematocrit, mean corpuscular volume, mean corpuscular haemoglobin, mean corpuscular haemoglobin concentration and thrombocyte count were determined.

To evaluate Erythrocyte Nuclear Abnormalities (ENA) blood smears were conducted with a drop of fresh blood on a microscope slide. These were dyed with a modified Romanowsky dye and observed

under an optical microscope (100X magnification) for erythrocyte differentiation. 1000 erythrocytes in each sample were classified into two main classes, namely normal and abnormal red blood cells, with the latter being further subclassified according to the anomalies they presented (i.e. micronucleus, lobed nucleus, segmented nucleus and reniform nucleus), as previously described (Pacheco & Santos, 1996). Erythrocytes with intact membranes were exclusively selected for the analysis, and the results are expressed as a frequency of ENAs per 1000 cells.

Histological analyses:

Gut, liver and gill samples were fixed in phosphate-buffered saline at pH 7.2 containing formalin (10 %). Subsequently, the preparations were washed in distilled water, dehydrated in alcohol, cleared in xylene, and embedded in paraffin wax. Sections (6 µm) were cut and mounted on gelatinized slides using a rotary microtome. Sections were then rehydrated in distilled water and stained with hematoxylin/eosin (H&E) Giemsa or periodic acid-Schiff (PAS). Prepared slides were examined and photographed (Jenoptik ProgRes CT5) under a light microscope (Nikon eclipse Ci-L).

Microbiome analyses:

Genomic DNA was extracted from whole gut samples using the Power Soil Pro kit (Qiagen). Libraries were prepared for the sequencing of the v3-v4 region of the 16S gene. 12.5 ng of extracted DNA was amplified with 16S primers (Forward 5'-CCTACGGGNGGCWGCAG-3' and Reverse 5'-GACTACHVGGGTATCTAATCC-3) equipped with the Illumina adapters. The PCR consisted of a 3-min hold at 95ºC, followed by a 30 s at 95 ºC – 30 s at 55 ºC – 30 s at 72 ºC cycle, repeated 25 times, and a final 5-min hold at 72 ºC. Following a magnetic bead-assisted-clean-up the corresponding indices were added to the Nextera® XT DNA Index (96 indices, 384 samples) FC-131-1002 (Illumina), and purified with magnetic beads. After each PCR the quality of the process was verified with a LabChip Bioanalyser (Agilent Technologies Inc.). Once all samples were obtained, these were multiplexed by mixing equimolar concentrations of each sample. All samples were sequenced in the MiSeq employing the MiSeq® Reagent Micro (500 cycles).

The sequencing reads were processed using USEARCH v.11 (R. C. Edgar, 2010) following the pipeline recommended by the author. In brief, the reads were truncated at the first sequence with a quality score (Q) below 30; the reads were then merged, and the primer binding sequences were stripped. The merged reads were quality-filtered and dereplicated to obtain unique sequences using the default setting. Denoising (i.e., predicting the true biological sequence (Amplicon Sequencing Variant - ASVs) from these unique sequences) was performed. The chimeric and singletons (i.e. sequences that appear below eight reads in all samples) were removed in the denoising process. Merged reads were mapped to ASVs to obtain the read counts for each sample. The taxonomic classification for the obtained ASVs was predicted using the SINTAX classifier (R. Edgar, 2016) with the RDP training set v19 as the reference database. The ASVs representing non-bacteria taxa (e.g. chloroplasts, eukaryotes, and taxa of known kit contaminants) were removed.

From the sequencing of 24 samples, a total raw pair reads of 2.28 million read pairs were obtained. Of these raw pairs, 20.41 M pairs (89.41%) were successfully merged, with the mean length of the merged reads being 461 bp. Most non-mergeable raw reads were discarded (106.4K or 4.65%) because no alignment was found. The reads were rarefied to 54,502 reads (minimum reads in all samples). No samples were discarded. Finally, 24 samples and a count table with 1,154 ASVs and median reads of 82,654 reads per sample were used in the downstream analysis. The rarefaction analyses are plotted in Suppl. 1.

Hill's number 0 (observed ASV richness (S), 1 (exponential Shannon's entropy, exp. H'), and 2 (inverse Simpson ($1/D_S$) were selected to express community alpha diversity. Rarefying reads before calculating the diversity metrics could bias the estimate, and using a measurement error model is

encouraged (Willis, 2019). The diversity values after extrapolation (i.e., error modelling) were used as implemented in the R package (iNEXT; Hsieh et al., 2016). Beta diversity was assessed using various distance metrics, namely, Bray-Curtis, Sørensen-Dice, Unifrac, and weighted Unifrac dissimilarity. Difference in alpha diversity of gut microbiome between treatment group was tested using pair-wise Wilcoxon test, while the difference in microbiome composition was investigated using PERMANOVA on the distance matrix based on Bray-Curti's dissimilarity metric. The differentially abundant families in the gut microbiome between fish in the control and high groups were investigated using the linear discriminant analysis of effect size (LEfSe) method (Segata et al., 2011). The p-value to reject null hypotheses was set at 0.05.

Statistical analysis:
Unless otherwise described in specific subsections of the Materials and Methods section, all data was tested for normality and homoscedasticity and analysed with one-way ANOVA or its non-parametric equivalent, the Kruskal-Wallis test, when suitable. All these statistical analyses were performed with GraphPad Prism v8.0.1, and the threshold for significance was considered at p-value <0.05.

Results:

Characterisation of polystyrene nanoplastics and semiquantification in fish tissues:
DLS characterisation of PSNPs revealed medium-dependent particle size (Table 1). PSNPs in ultrapure-MiliQ water showed less dispersion in size than those in ASW. A PDI value over 0.2, as observed in ASW, indicates that the particles in this medium aggregated, as reflected by the increased in size. Nonetheless, the characterised particles remain, in all cases, in the nanoscale.

Table 1: Characterisation of polystyrene nanoplastics (100 µg/L) in ultrapure-MiliQ water and artificial salt water (ASW). Measured parameters were size (nm) and polydispersity index (PDI). Expressed as mean ± standard deviation

Water	Size (nm)	PDI (IU)
Ultrapure-MiliQ	39.91 ±0.638	0.062 ± 0.007
Artificial saltwater (ASW, 35 ppt)	561.8 ± 69.38	0.98 ± 0.115

Quantification of PSNPs in fish organs revealed the presence of this compound in all brain and gills samples. PSNPs were only detected in one muscle sample obtained from the LC group, whereas none were detected in any of the Ctrl nor HC muscle samples. The analysis determined that none of the analysed blood samples contained PSNPs. In the liver, the analysis indicated the presence of PSNPs only in 60% of LC and HC samples, whereas none were detected in any Ctrl sample (Table 2).

Table 2: Proportion of samples of *Sparus aurata* blood, brain, liver, gut, muscle, and gills, were polystyrene nanoplastics were detected following a 28-day waterborne exposure under three experimental conditions (Ctrl = Control group, 0 µg/L; LC = Low concentration group, 100 µg/L; HC = High concentration group, 1000 µg/L).

	Blood	Brain	Liver	Gut	Muscle	Gills
Ctrl (0 µg/L)	0%	100%	0%	100%	0%	100%

LC (100 µg/L)	0%	100%	60%	100%	20%	100%
HC (100 µg/L)	0%	100%	60%	60%	0%	100%
All groups	0%	100%	40%	87%	7%	100%

Biometrics:

No significant differences were observed in either total length, weight, hepatosomatic index nor Fulton's condition factor between treatments (Table 3).

Table 3: Total length, weight, Fulton's condition factor and hepatosomatic index of *Sparus aurata* exposed to polystyrene nanoplastics over a 28-day period (Control = 0 µg/L; Low Concentration = 100 µg/L; High Concentration = 1000 µg/L). Expressed as group mean ± standard deviation.

	Control	Low Concentration	High Concentration
Total length (cm)	10.2 ± 1.57	10.6 ± 1.48	10.2 ± 1.55
Total weight (g)	13.5 ± 5.36	14.2 ± 5.81	13.3 ± 6.18
Fulton's condition factor	1.20 ± 0.17	1.14 ± 0.14	1.17 ± 0.09
Hepatosomatic index (HSI)	1.53 ± 0.29	1.58 ± 0.29	1.13 ± 0.43

Blood parameters:

The results obtained from the automated haematological analyser indicate no significant differences in any of the investigated parameters following the chronic waterborne exposure to PSNPs (Table 4). Similarly, the Erythrocyte Nuclear Anomalies (ENA) assay did not result in significant differences in the proportion of normal and abnormal erythrocyte nuclei between experimental groups (Table 4).

Table 4: Haematological parameters of *Sparus aurata* exposed to polystyrene nanoplastics over a 28-day period (Control = 0 µg/L; Low Concentration = 100 µg/L; High Concentration = 1000 µg/L). Expressed as group mean ± standard deviation. WBC: Leukocyte count; RBC: Erythrocyte count; HGB: Haemoglobin; HCT: Haematocrit; MCV: Mean Corpuscular Volume; MCH: Mean Corpuscular Haemoglobin; MCHC: Mean Corpuscular Haemoglobin Concentration; PLT: Thrombocyte count.

	Control	Low Concentration	High Concentration
WBC (10^3/µL)	36.4 ± 12	38.2 ± 10.1	29.3 ± 15.2
RBC (10^3/µL)	1.52 ± 0.24	1.52 ± 0.16	1.44 ± 0.20
HGB (g/dL)	3.09 ± 1.04	3.28 ± 0.44	3.25 ± 0.59
HCT (%)	25.7 ± 10.2	30.1 ± 6.49	25.5 ± 8.31
MCV (fL)	178 ± 28.6	190 ± 33.4	170 ± 20.5
MCH (pg)	21.5 ± 2.15	20.7 ± 1.81	22.7 ± 4.34
MCHC (g/dL)	12.4 ± 2.64	11.1 ± 1.15	13.6 ± 3.92
PLT (10^3/µL)	6.40 ± 4.74	4.44 ± 2.13	4.60 ± 2.41
ENA (‰)	19.5 ± 8.93	20.5 ± 4.40	20.6 ± 10.8

Histological analyses:

The direct observation of the prepared samples of gill (Figure 1A), gut (Figure 1B) and liver (Figure 1C) did not evidence any histopathological effect of the experimental conditions in *S. aurata* juveniles. In any case were the samples distinguishable between groups, and no cases of hyperplasia, dysplasia, inflammation or degenerative processes were found.

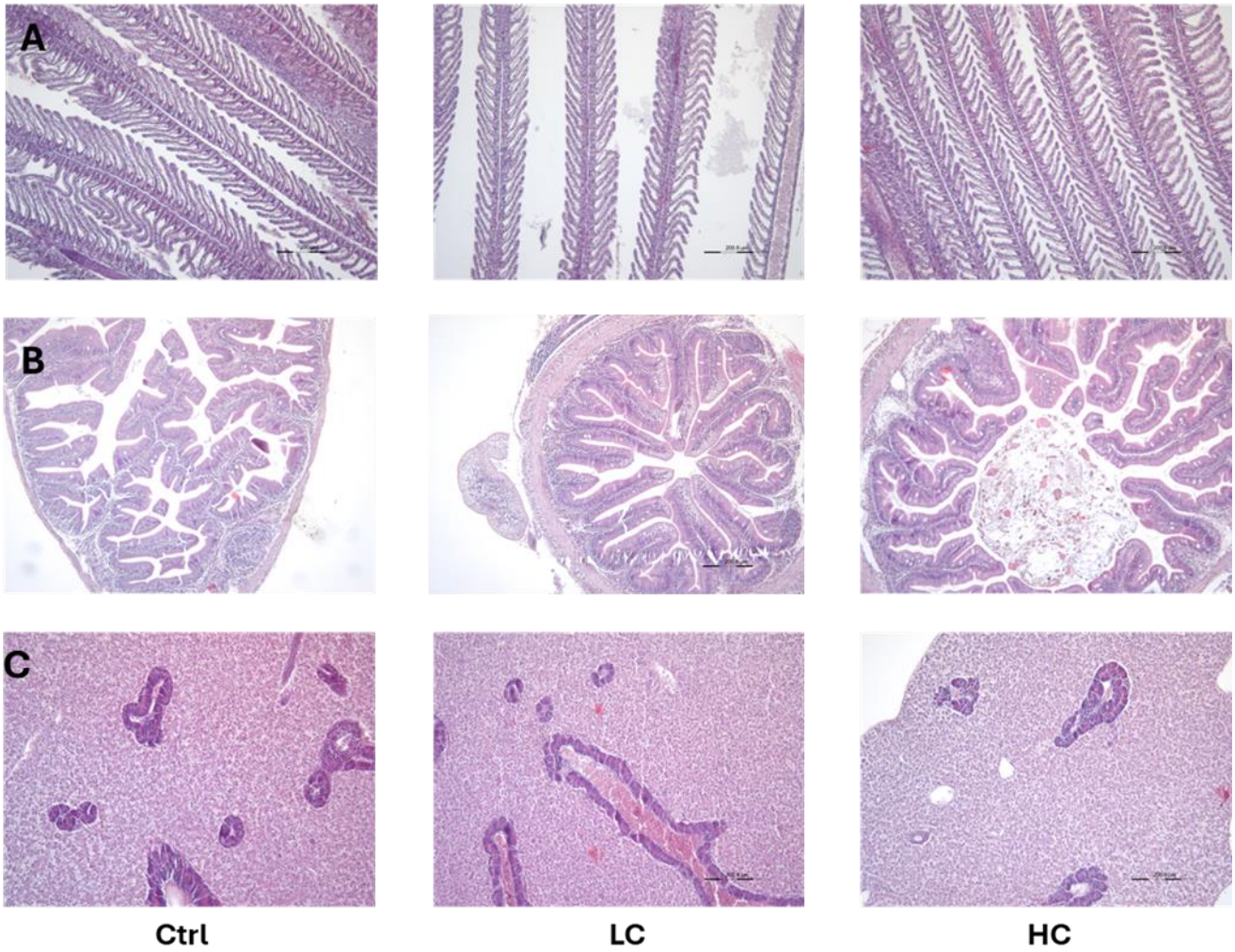

Figure 1: Histopathological observations of gills (A), gut (B), and liver (C) of juvenile gilthead seabream exposed to 0 µg/L (Ctrl), 100 µg/L (LC), and 1000 µg/L (HC) of waterborne polystyrene nanoplastics over a 28-day period.

Microbiome analysis:

Irrespective of experimental conditions, the most commonly identified bacterial phyla in the gut of *S. aurata* were *Proteobacteria* (i.e. family *Pseudomonadaceae*), followed by *Firmicutes* (i.e. families *Lactobacillaceae, Lachnospiraceae, Oscillospiraceae*, and *Clostridiaceae*), *Bacteroidetes* (i.e. family *Prevolellaceae*), and *Actinobacteria* (i.e. family *Bifidobacteriaceae*). The analysis of the 16S amplicon sequencing data revealed significant differences in gut microbiome between experimental groups. Statistically significant differences arose in exponential Shannon's entropy, with HC displaying a significantly lower exponential Shannon's Diversity Index compared to the Ctrl and LC groups, but no differences were found between Ctrl and LC (Figure 2). No differences were found in either species richness or inverse Simpson's diversity index between any group. *Pseudomonadaceae* resulted the most abundant family throughout the investigated samples, with a significantly greater representation in the HC group compared to the Ctrl. The differentially abundant taxa are shown in Figure 3 and Table 5. Regarding Table 5 only comparisons between control and high were significant, no differences were found in LC

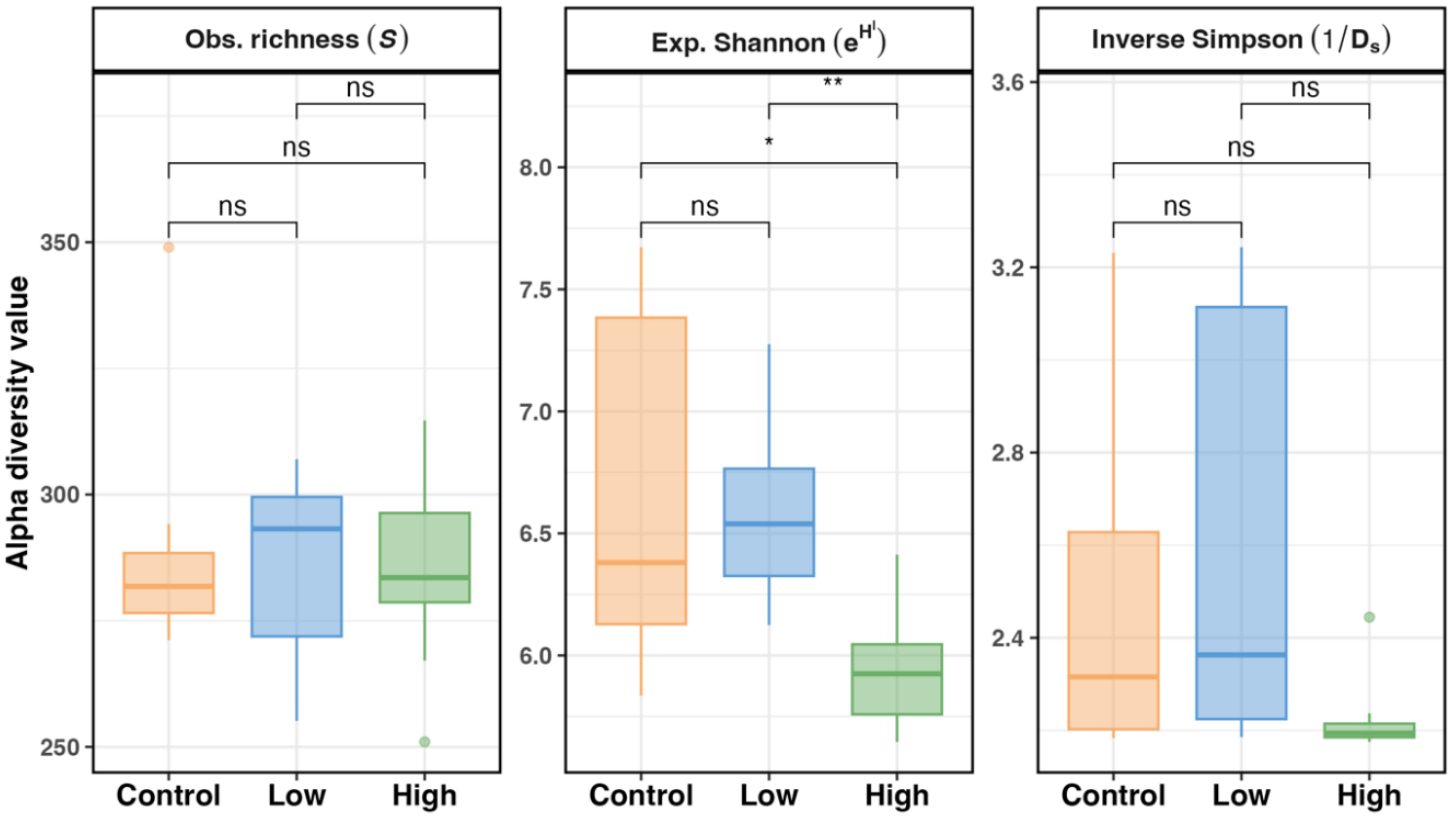

Figure 2: Gut microbiome alpha diversities of fish exposed to no (control), low (100 µg/L), and high (1000 µg/L) concentrations of polystyrene nanoplastics (ns = not significant, * = P-value < 0.05; **=P-value < 0.01).

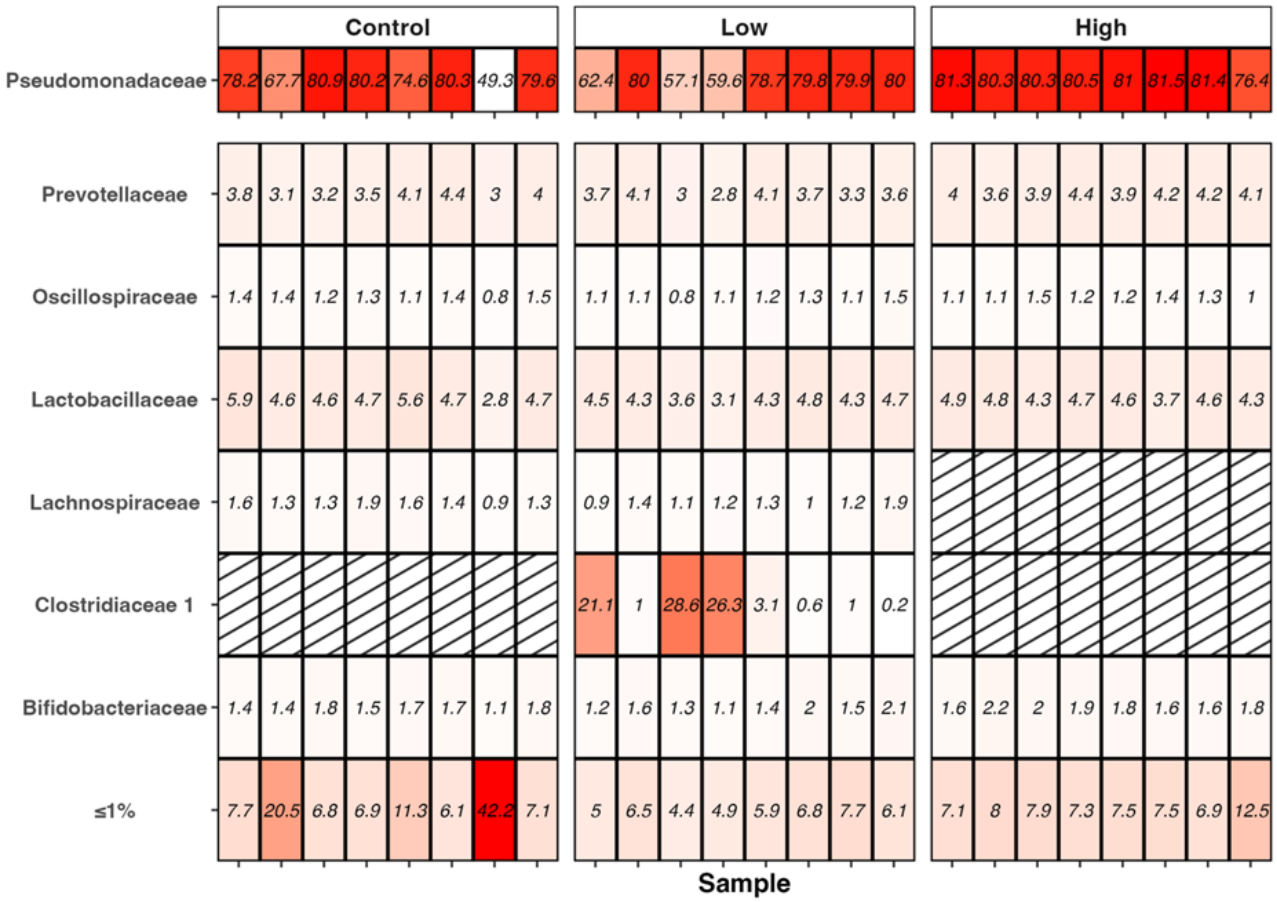

Figure 3: Heatmap of the family composition of bacteria community in the gut of fish exposed to polystyrene nanoplastics at 0 µg/L (control), low (100 µg/L), and high (1000 µg/L) concentrations. Stripped boxes indicated that the median relative abundance of the class in the experimental group is below 1%.

Table 5: Differentially abundant families in the gut microbiome between fish in the control and high group investigated using the linear discriminant analysis of effect size (LEfSe) method (Segata et al., 2011).

Taxa	Enriched group	Fold change	padj
Lachnospiraceae	Control	3.7	0.002
Parcubacteria_genera_incertae_sedis	Control	2.6	0.044
Methylococcaceae	Control	2.4	0.011
Microbacteriaceae	Control	2.4	0.015
Pseudomonadaceae	High	4.8	0.016
Methylobacteriaceae	High	2.3	0.008

NMR Metabolome analysis:

A total of 245 metabolites were detected, with 31 metabolites being identified and quantified (Spectra available in Suppl. 2). A PCA showed that 5 components explained over 86% of the differences observed between experimental groups (Suppl. 3). A discriminant analysis indicated that most samples were successfully separated by exposure condition, except for one LC sample which would have been classified as belonging to the Ctrl group (Figure 4). The statistical analyses of individual metabolites revealed significant differences between groups. HC group displayed significantly greater levels of glutamate, glycerol and formate when compared to the Ctrl group (Figure 5A, B and C). Additionally, proline levels resulted significantly higher in the HC group compared to both the Ctrl and LC groups (Figure 5D). The LC groups exhibited significantly lower values of valine compared to the two other experimental groups (Figure 5E).

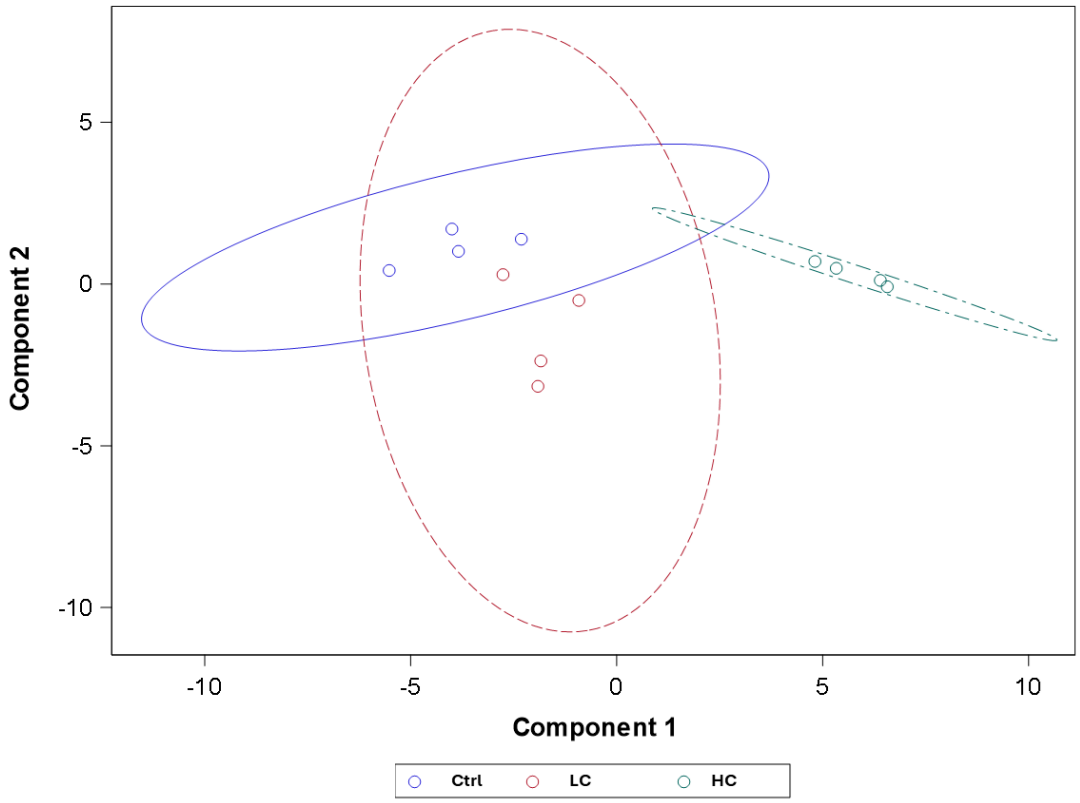

Figure 4: Grouping of the experimental samples by metabolomic profile obtained through nuclear magnetic resonance (NMR) of *Sparus aurata* plasma exposed to waterborne polystyrene nanoplastics at concentrations of 0 µg/L (Ctrl), 100 µg/L (LC), and 1000 µg/L (HC).

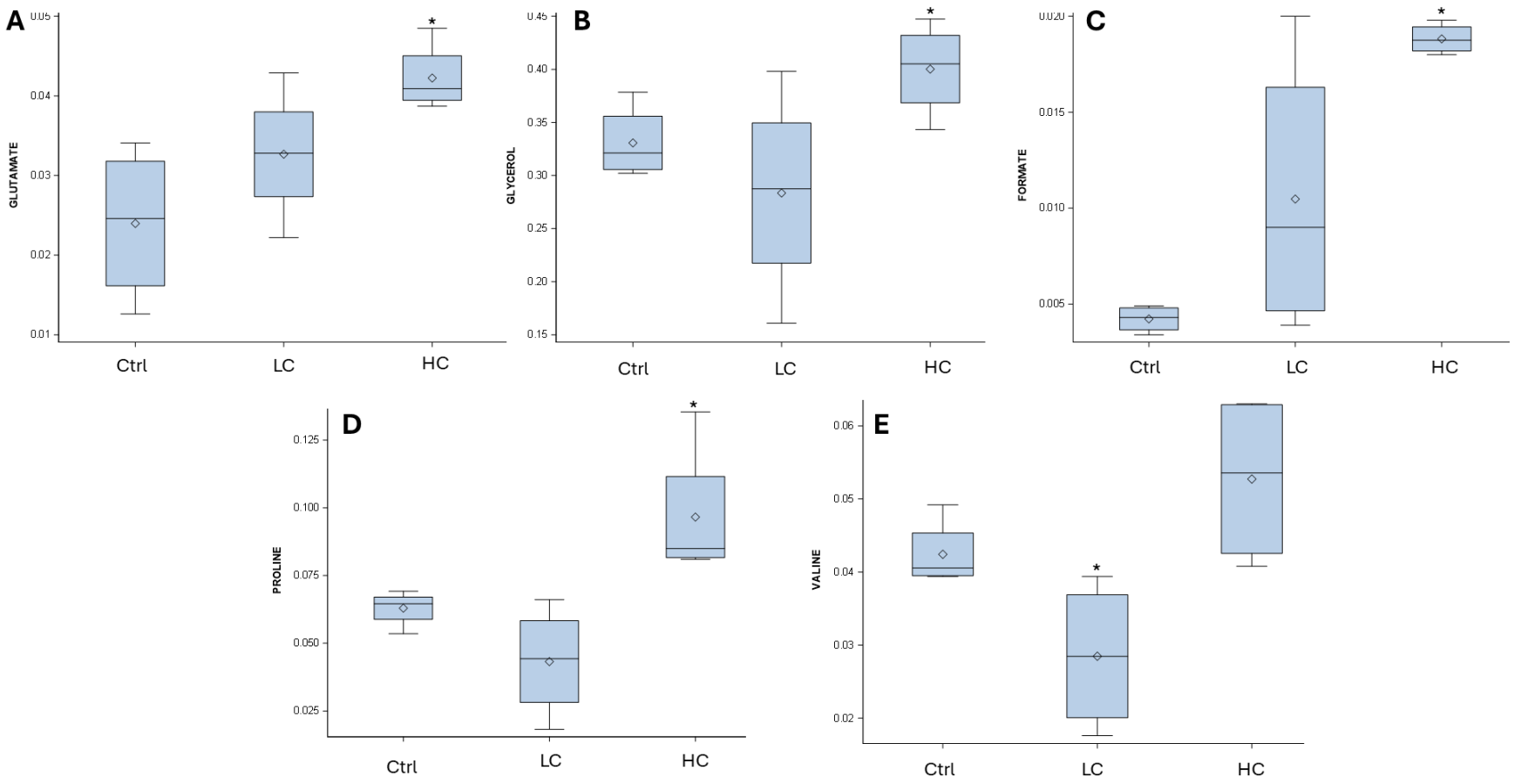

Figure 5: Variation of plasmatic levels of glutamate (A), glycerol (B), formate (C), proline (D), and valine (E) induced by a waterborne exposure to polystyrene nanoplastics at concentrations of 0 µg/L (control; Ctrl), low (100 µg/L; LC), and high (1000 µg/L; HC). Asterisks indicate significant differences with the control group (P-value <0.05).

Discussion

The present study investigated the potential consequences of a 28-day waterborne exposure to PSNPs in *S. aurata* juveniles under laboratory conditions, including internalisation and retention of particles, as well as impact on health. The characterisation of PSNPs in the experimental medium revealed the aggregation of the particles due to the physical properties of both the media and the PSNPs particles (Boughbina-Portolés & Campíns-Falcó, 2024). The ultrapure water maintains the electrostatic repulsive forces which limit aggregation, whereas the ions in ASW present positive charges which interact with the negatively charged PSNPs surface, leading to a marked decrease in stability and resulting in aggregation (Tallec et al., 2019). The possibility of the particles agglomerating rather than aggregating was discarded given the inability of PSNPs to recover their original size following sonication (Boughbina-Portolés & Campíns-Falcó, 2024).

The qualitative analysis of NPs in fish tissues indicated that the brain and gills are the most likely organs to internalise PSNPs, with 100% of these samples containing PSNPs, including those of the Ctrl group. Similarly, 100% of gut samples from the Ctrl and LC group contained PSNPs traces, compared to only 60% of those in the HC group. In the liver, no PSNPs were detected in the Ctrl group, but this contaminant was ese contaminants were identified in 60% of the exposed fish samples. The tissues that appeared to internalise the least PSNPs during the present experiment were muscle and blood, with 7% and 0% of all samples containing this pollutant, respectively (Table 1). This is in accordance with a number of past studies investigating the potential accumulation of PSNPs in fish and describing tissue-specific internalisation and retention rates. Sarasamma et al. (2020) reported that 70 nm fluorescent PSNPs were accumulated at a greater rate in zebrafish gonads than in any other organ, with gut, liver and brain samples containing similar PSNPs concentrations following a 30-day waterborne exposure. On the other hand, Hao et al. (2023) described significantly higher palladium-doped PSNPs levels in the gut of Nile tilapia (*Oreochromis niloticus*) compared to all other organs, irrespectively of size, following a 21-day exposure to 1 mg/L and a subsequent 7-day depuration period. In addition, the authors stated that muscle, gills and liver are more prone to accumulating smaller-sized NPs, with muscle samples displaying the highest quantified values of PSNPs. To the best of the authors' knowledge, no other study has investigated the accumulation of NPs in fish blood, and the absence of this compound in this tissue in the present study might be explained by the low retention time of contaminants in blood, which are rapidly translocated to the different organs. In addition, the inconsistency in results between previously published studies, and with the present results, regarding the affinity of different fish tissues to PSNPs is likely explained by the differences in particle size and detection method, as well as experimental media (i.e. freshwater vs. saltwater) and the species of interest. The presence of NPs in the muscle of fish, particularly those of commercial importance, as demonstrated by previous research, as well as by the present study, is of particular concern, as it represents an additional exposure route to this pollutant for humans through the ingestion of contaminated food (Blonç et al., 2023; Brandts et al., 2022; Hao et al., 2023).

Traditional health indicators

In the present study, all Fulton's condition factor values resulted around 1.15, indicating a generally appropriate health status for the experimental subjects, with no significant impact of the exposure on this parameter. Similarly, no effect of treatment was observed in length, weight and HSI. This is

consistent with previous research indicating a lack of significant alterations in these parameters following prolonged waterborne exposure to PSNPs in different species, such as *O. latipes* (Zhou, et al., 2023), *C. auratus* (Brandts et al., 2022), *Oreochromis niloticus* (Hao et al., 2023), and to a different polymer in *S. aurata* (Brandts et al., 2021). Nonetheless, these data should always be used complementary with other parameters, as drawing conclusions on the health status of a fish solely from biometrics incurs the risk of drawing wrongful or inaccurate conclusions (Morton & Routledge, 2006). Biometrics are common indicators of general health and fitness of individuals. In aquaculture, these data are used to infer on the performance of the activity. Significant alterations in length, weight or Fulton's condition factor might indicate disrupted feeding behaviour, poor or insufficient feed quality and quantity, or the overallocation of energetic resources into survival (e.g. due to disease). Fulton's condition factor values in *S. aurata* should be greater than 1, with lower values indicating poor fish health or nutritional quality (Faggio et al., 2014). Similarly, the hepatosomatic index is commonly used as a proxy for the energetic status of fish, as well as an indicator for water contamination (Hauser-Davis et al., 2012).

The haematological analyses hereby performed did not show any significant differences in the investigated parameters between any of the experimental groups. This is in accordance with previously published findings describing a lack of haematological alterations in *C. auratus* following a 30-day waterborne exposure to 100 µg/L of 44 nm PSNPs (Brandts et al., 2022). On the other hand, previous studies investigating the impact of PS micro and NPs in water on *O. niloticus* have reported significant disturbances to their haematological profile in a size- and dose dependent manner. Such results might indicate, amongst other things, the occurrence of inflammatory mechanisms and the triggering of immune responses, as well as problems associated with oxygen transport. For instance, Sayed et al. (2024) found significantly lower values of RBC, PLT, HCT, and HGB in individuals exposed to 5 mg/L of 50 nm PSNPs. Similarly, Vineetha et al. (2024) described significantly lower levels of RBC, HCT, HGB and WBC, following a sub-chronic exposure to 0.1 mg/L, 1 mg/L and 10 mg/L of 100 nm PSNPs, but no differences in PLT. Moreover, Hamed et al. (2019) observed a significant reduction in RBC and HGB but no effect on HCT, WBC or PLT following an experimental exposure to 1mg/L, 10 mg/L, and 100 mg/L of PSMPs and PSNPs (>100 nm).This indicates that even with the same species, different studies might obtain differing results, likely attributable to differences in analytical methods and particle size and shape. These three studies focusing on Nile tilapia all performed sub-chronic exposure lasting around two weeks. Therefore, differences between the present study and previously reported results might also arise from differences in exposure period and dosages.

The visual observation for ENAs revealed that the present experimental procedure did not induce significant genotoxic effects on *S. aurata* juveniles. In contrast, *C. auratus* exposed to similar concentrations of equally-sized PSNPs displayed significantly greater ENAs than control fish (Brandts et al., 2022). Such differences in results might be attributed to the intrinsic differences between both organisms, being a marine and a freshwater species, respectively.

In the present study, the exposure to waterborne PSNPs did not incur any histopathological changes in the gut, gills or liver of the experimental subjects. Similarly, a study investigating the impact of waterborne PSNPs (100 nm) at concentrations ranging from 0.001 to 10 mg/L reported no histopathological effects on the gills, liver, gut and kidney of *D. rerio*. Moreover, Zhang et al. (2021) found no histopathology arising from a 30-day dietary exposure to 100 nm PSNPs. Nonetheless, a number of available scientific articles describe significant alterations in the morphology of different

organs of a variety of species following both chronic and sub-chronic dietary and waterborne exposures, such as *O. niloticus* (Hao et al., 2023; Sayed et al., 2024; Vineetha et al., 2024), *C. auratus* (Abarghouei et al., 2021), and *S. schegelii* (Sun et al., 2023). Furthermore, it has been demonstrated that PSNPs do not induce histological lesions in the different tissues of the same species at the same rate, with some organs remaining morphologically normal after prolonged exposures to these contaminants (Abarghouei et al., 2021; Zhou et al., 2023).

Omics approach

Investigating the gut microbiome in fish can provide insightful information on their overall health status. Significant changes in relative abundance of different taxa, as observed here in treated groups compared to the control group, imply a disrupted community composition, and may indicate hindered physiological functions, reduced resistance to environmental pollutants and pathogens, and reduced overall fitness (Evariste et al., 2019; Xavier et al., 2024). Moreover, the dominance of *Pseudomonadaceae* family, which was even more pronounced in the HC group compared to the Ctrl, might be concerning, as members of this family are often associated with pathogenicity (Austin & Austin, 2012). The most abundant taxa found in the present study are in accordance with previous descriptions of the gut microbiome of *S. aurata* (Egerton et al., 2018; Estruch et al., 2015).

Overall, the present findings indicate an alteration of gut microbiome in *S. aurata* exposed to PSNPs over a prolonged period, specifically, an increased relative abundance of *Firmicutes* in the LC group, and a decrease in the HC groups. Although the information on the impact of waterborne nanoplastics on the gut microbiome of the gilthead seabream is scarce, numerous studies have investigated the effect of PSMP and PSNPs in other fish species, although the results are somewhat contradictory (Bao et al., 2024). For instance, Qiao et al. (2019) and Jin et al. (2018) both reported a significant increase in *Firmicutes* coupled with a significant decrease in the abundance of zebrafish gut *Proteobacteria* induced by waterborne exposure to PSMPs. The increase in *Firmicutes* was further corroborated after an exposure to PSNPs in *D. rerio* (Marana et al., 2022). On the other hand, Xie et al. (2021) described the opposite trend, with a significant reduction in the abundance of *Firmicutes* and an increase in *Proteobacteria* in the gut of the same fish species. Particle size and exposure duration seemed to play a major role in determining the effect of these contaminants in the gut microbiome and explain these inconsistent outcomes (Bao et al., 2024).

In this study, the major taxa were shared between experimental groups, and the observed differences are attributed to slightly shifted relative abundances of some taxa between control and treatment groups. However, the decrease in diversity as reflected by the significant drop in Shannon's Diversity value may indicate a certain degree of dysbiosis (Evariste et al., 2019). Disrupted gut microbiomes are often associated to morphological anomalies in the gastro-intestinal tract structure, such as inflammation (Jin et al., 2018), nonetheless, the gut histology results showed no evidence of structural alterations, implying that the decreased in the gut microbiome diversity did not induce an inflammatory response and might instead be intertwined with the alterations of the metabolomic profile of the experimental fish. Indeed, it has been suggested that in fish, as in humans, significant decreases in the diversity of intestinal microbiota might be reflected by changes in the metabolome (Qiao et al., 2019).

In the present study, the metabolomic profile of *S. aurata* juveniles resulted altered, to some extent. The significant increase in plasmatic glutamate, a non-essential amino acid, observed in the present

study may reflect disturbed metabolic processes in these fish. Glutamate is an abundant neurotransmitter in fish, and plays a major excitatory role (Trudeau et al., 1996, 2000). It appears as a versatile compound, involved in a variety of metabolic processes, playing a key function in gut health by modulating the structure of the gastrointestinal tract, and exhibiting antioxidant functions (Andersen et al., 2016). Moreover, glutamate is essential for the synthesis of gamma-aminobutyric acid (GABA), which acts as a inhibitory neurotransmitter (Park & Chu, 2022), and glutathione peroxidase, involved in antioxidant defences (Andersen et al., 2016). Therefore, the observed trend of glutamate hints towards inhibited GABA- or glutathione-synthesis-related pathways caused by the exposure to waterborne PSNPs. Ultimately, excessive accumulation of glutamate may lead to synapsis death (Aliakbarzadeh et al., 2023), causing serious neuronal damage and having deleterious consequences on the overall health and welfare of living organisms. Under some sorts of stress, such as starvation and physical injuries, glutamate levels have been described to drop, reflecting increased metabolic requirements (Andersen et al., 2016; Walker et al., 1996; Zhang et al., 2024). However, the response of this amino acid to MPs and NPs in fish displays, a high degree of dependence on a variety of parameters (*e.g.* polymer type, size and dose, and species). On one hand, zebrafish embryos exposed to PETNPs (70 nm) over a 24 h period, displayed significant decreases in glutamate (Bashirova et al., 2023), similar to what was observed when exposing zebrafish larvae to mask-derived MPs (1-20 µm) for 3 h (Hu et al., 2024), and adult marine medaka (*Oryzias melastigmas*) to PSMPs (10-200 µm) for 60 days (Ye et al., 2021). On the same note, Duan et al. (2020) described significant alterations in glutamate metabolism in zebrafish embryo exposed to 100 nm PSNPs over a 3-day lapse, stating that smaller particles had greater effects than their bigger counterparts. However, a 21-day waterborne exposure of adult Javanese medaka (*Oryzias javanicus*) to 5 µm PSMPs resulted in a significant increase in glutamate levels, in a clear dose-dependent manner (Usman et al., 2022), and *S. aurata* fed with PEMP-contaminated brine shrimp (*Artemia salina*; 10-20 µm MPs) over a 35-day period, displayed significantly higher glutamate levels (Jacob et al., 2021), as corroborated by the present results, further indicating that the toxicity of MPs and NPs, and the dynamics of glutamate response to these contaminants is dictated by a combination of polymer type, particle size and exposure type and duration.

Similarly to what was observed for glutamate, glycerol values were significantly greater in the HC group compared to the Ctrl. Glycerol is a metabolite resulting from the breakdown of triglycerides, and significant increases in this parameter might indicate strenuous physical activity or metabolic stress, reflecting a disturbed lipid metabolism. A similar trend has been observed in the Australian bass (*Macquaria novemaculeata*) exposed to PSNPs through contaminated *Artemia spp*., which exhibited significant alterations in the levels of glycerol in a size- dependent manner, with 50nm PSNPs causing the most substantial upregulation (Afrose et al., 2024). Other instances of altered glycerol metabolism have been reported in *Sebastes schegelii* and *O. melastigmas* following sub-chronic and chronic exposure to waterborne and dietary PSNP and PSMPs, respectively (Sun et al., 2023; Ye et al., 2021).

Proline, another non-essential amino acid involved in the response against oxidative stress (Krishnan et al., 2008), was also significantly increased in *S. aurata* by the exposure to PSNPs at a concentration of 1000 µg/L. Significant alterations in proline metabolism have been described numerous times in fish exposed to PSMPs and PSNPs (e.g. Chu et al., 2024; Usman et al., 2022). In rare minnow (*Gobiocypris rarus*), a 14-day exposure to PSMPs and PSNPs 1 µm at 1 mg/L and 100 nm at 1 mg/L

and 10 mg/L) led to a significant increase in the levels of this metabolite (Chu et al., 2024). Although such increases have also been observed in zebrafish embryos and adults (Qiao et al., 2019; Wan et al., 2019) exposed to PSMPs of sizes 5 and 50 µm, respectively, the effect of PS on proline metabolism has been suggested to be size-dependent, with smaller particles causing greater disruptions (Afrose et al., 2024; Ye et al., 2021).

On the contrary, valine levels in the plasma of juvenile *S. aurata* of the present study resulted significantly lower in individuals exposed to 100 µg/L, but not in those exposed to 1000 µg/L, once again indicating a dose-dependent effect. Information available in previously published literature supports this statement, given the variability in the response dynamics of this essential amino acid. Indeed, significant downregulations in measured valine concentrations have been reported in MPs-exposed marine medaka (Ye et al., 2021) and zebrafish embryos (Bashirova et al., 2023; Wan et al., 2019), as well as *S. schegelii* exposed to NPs (Sun et al., 2023). On the other hand, adult Javanese medaka and juvenile gilthead seabream exposed to PSMPs displayed significant increases in measured valine levels (Jacob et al., 2021; Usman et al., 2022).

To the best of the authors' knowledge, no study has described the dynamics of formate levels in the face of MPs and NPs in fish. In bacterial communities and mammals, this carboxylic acid has been described as an essential component of antioxidant defences (Thomas et al., 2016), and as playing a key role in intermediary and energy metabolism, being an important electron donor (Brosnan & Brosnan, 2016). Therefore, although the role of formate in fish is poorly understood, alterations in plasmatic levels of this metabolite might indicate disruptions in one-carbon and fatty acid metabolism, having serious implications for cellular function, energetic resources allocations and overall performance of individuals (Brosnan & Brosnan, 2016; Lamarre et al., 2013).

The discriminant analysis was able to successfully separate all samples from HC and Ctrl, whereas one sample from LC could have been considered as Ctrl, indicating that, under these experimental conditions, the high dose of PSNPs hereby employed had a greater impact on the metabolome of *S. aurata* juveniles than the low dose. Overall, the results obtained from the metabolomic assessment of *S. aurata* plasma revealed significant alterations in various metabolic pathways. In fish, similar trends have been observed, although the specific changes vary greatly between species and experimental setups. Rather than large changes in a few metabolites, the present study found that small variations in a wide range of apolar metabolites drives the metabolomic response of *S. aurata* to waterborne PSNPs. This has been previously described in fish exposed to a variety of both micro- and nano-sized PS particles through different routes, with the most frequently reported changes being involved in energy metabolism and oxidative stress management (Marana et al., 2022; Mattsson et al., 2015; Wan et al., 2019; Zhao et al., 2021).

General conclusions

In the present study, a multidisciplinary approach was employed in order to obtain a comprehensive understanding of the impact of PSNPs of emerging concern on fish. The results showed that waterborne PSNPs accumulated in multiple organs of juvenile *S. aurata* at different rates, particularly in the brain and gills which might have implications for the correct development of the fish, potentially affecting their neuronal health, as well as their respiratory and osmoregulatory capacities. Moreover, although lower than expected, the internalisation and retention of PSNPs in

the muscle of such a commercially important and gastronomically popular species represents an additional exposure route for humans to this contaminant. Nonetheless, histopathological lesions, although they could be expected due to the presence of PSNPs in gills, muscle and liver, were not detected. In contrast, alterations were found in the gut microbiome, and these might be linked to some extent, to changes observed in the metabolomic profile of the plasma. Together, these differences might translate into a disrupted energy metabolism and development, hindered antioxidant defences, that may result in an overall reduction in fitness and performance. Overall, under the present experimental conditions, waterborne PSNPs did not significantly affect the performance of juvenile gilthead seabream, although it is likely that a longer exposure period would lead to a possible decrease in growth and health performance, as suggested by the metabolic indicators. Future research should, thus, delve on longer waterborne exposures to different NPs polymers and size using comprehensive, multi-omics approach to unveil the long-term implications of NPs contamination for the fish food production industry and global health.

Ethical approval:

This work has received approval for research ethics from the Ethical Review Committees of the Autonomous University of Barcelona and a proof/certificate of approval is available upon request.

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6

Occurrence of micro-nanoplastics in a commercial recirculated aquaculture system and their translocation to cultured fish organs: A baseline study

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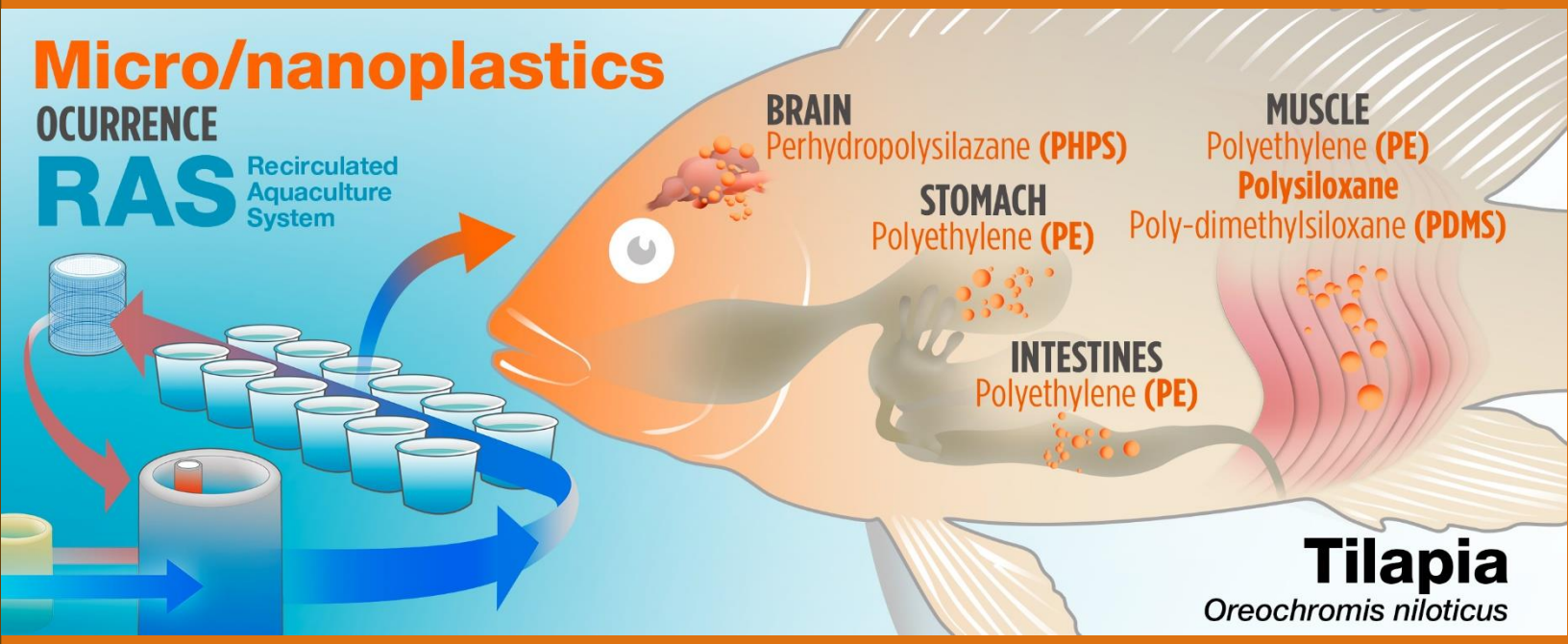

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Occurrence of micro- nanoplastics in a commercial recirculated aquaculture system and their translocation to cultured fish organs: A baseline study

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ABSTRACT

Micro- and nanoplastics (MNPs; < 5 mm and < 1 μm, respectively), are contaminants of emerging concern representing a major part of global plastic pollution, due to their ubiquity both in natural and urbanised environments. Although environmental concentrations of these pollutants have been measured in a variety of matrices, information on the occurrence of MNPs in recirculated aquaculture system (RAS) farms, is scarce. The present study aimed to investigate the presence of MNPs in a commercial European RAS farm, by identifying the occurring polymers in both the system water and in a variety of fish tissues and quantifying their concentration. To this end, adult *Oreochromis niloticus* (Nile tilapia) were sampled for brain, liver, gut, stomach, muscle and gonads, and water was collected from both the influent and the effluent of the system. Size exclusion chromatography coupled to high resolution mass spectrometry equipped with an atmospheric photoionization source was employed to identify five distinct polymers, namely polyethylene (PE), polyisoprene (PI), polysiloxane, perhydropolysilazane (PHPS), and poly-dimethylsiloxane (PDMS). Two polymers were present in the system water, with PI being found at considerably greater concentrations in the effluent than in the influent. By order, the tissues that retained the greater number of polymers were: muscle > gut = brain > stomach > liver = gonads. The analyses indicated that liver and gonads did not contain any MNPs particles, whereas muscular tissue contained up to 3 distinct compounds. The results may reflect different uptake pathways of MPNs depending on the polymer type and its respective properties. The presence of these emergent contaminants in the muscle represents an additional exposure pathway for humans, through the ingestion of contaminated RAS-farmed fish, adding to the long list of confirmed exposure routes. Investigating the input of MNPs in RAS facilities through the weathering of its plastic components and assessing non-plastic alternatives to these components (e.g. natural biofilters), as well as MNPs removal techniques from the system, is of utmost importance to minimise the presence of these contaminants in RAS, and their impact on global food security.

1. Introduction

Micro-nanoplastics (MNPs) are plastic particles smaller than 5 mm recognized as pollutants of emerging concern, due to their ubiquity and ability to accumulate along the trophic chain at every trophic level, including fish (Cedervall et al., 2012). Microplastics (MPs), the higher size range of MNPs are defined as plastic particles having a size between 1 μm and 5000 μm (Galgani et al., 2013). On the other hand, nanoplastics (NPs), the lower size range of MNPs are defined as particles smaller than 1 μm (Gigault et al., 2018), and it is thought that they might have the greatest ecological impact amongst plastic particles, due to their small size and colloidal characteristics (Mattsson et al., 2017). The presence of MNPs has been reported in a variety of environmental matrices including aerial (Rosso et al., 2023), terrestrial (Kumar et al., 2020), and aquatic systems, such as in the open sea (e.g. North Atlantic Gyre; Ter Halle et al. 2017), and in estuarine waters, with polystyrene (PS), polyethylene (PE), polyisoprene (PI) and polysiloxanes being some of the most commonly detected polymers, and some compounds

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reaching quantified concentrations as high as 7000 ng/L (Llorca et al., 2021). Most MNPs particles are generated from the fragmentation of larger plastic objects under natural environmental conditions, due to a sum of factors such as biological degradation, mechanical forces, and exposure to ultraviolet radiation (Andrady, 2011). This degradation of bulk plastic objects producing MNPs has been extensively demonstrated with plastic objects of daily use made from diverse polymers, such as PS (Lambert and Wagner, 2016), PE (Ekvall et al., 2022) or polyethylene terephthalate (PET) (Magrì et al., 2018). Due to their physicochemical properties, smaller MNPs can cross biological barriers and accumulate in tissues inside the organism (Ma et al., 2021; Shan et al., 2022). Moreover, these same particles can absorb other pollutants such as persistent organic pollutants (Bakir et al., 2014) and heavy metals (Brennecke et al., 2016), as well as microorganisms (Stenger et al., 2021), with the potential of transferring them into biota. In addition, the presence of MPs in aquaculture systems, such as recirculated aquaculture systems (RAS), has been linked to the occurrence of increased numbers of antibacterial resistant genes, with potentially significant impacts on the overall health status of the produced animals (Lu et al., 2019).

In teleost fish, MNPs can be absorbed through the gastrointestinal tract, gills, and skin (Bhagat et al., 2020; Brandts et al., 2021; Su et al., 2019) and their uptake pathway may condition the sites of retention and accumulation within the organism (van Pomeren et al., 2017). MNPs have been found to distribute throughout the fish body reaching the circulation as well as distant tissues, such as the brain or the eye (Brun et al., 2018, 2019; Kashiwada, 2006; Mattsson et al., 2017; Pitt et al., 2018; Skjolding et al., 2017; van Pomeren et al., 2017). In RAS, the presence of MPs has been reported in the system water, in the fish feed used, and in the cultured *Dicentrarchus labrax* (Matias et al., 2023). Indeed, the authors observed the occurrence of MPs in the fish's gastrointestinal tract, gills, liver and muscle. Interestingly, it was reported that the polymers used in the RAS components had very low occurrence in the analysed samples, indicating that the major sources of MPs in this system were the water and the feed. However, it is likely that the lack of detection of such polymers is linked to their nano-size, translating into an underestimation of their presence in the system. The presence of MPs in Asian RAS facilities has also been reported, although, surprinsingly, the authors indicated that the investigated RAS farms had significant lower MPs pollution levels compared to other traditional aquaculture settings (Huang et al., 2023; Lv et al., 2020). Detrimental effects of MNPs in fish have been documented, ranging from a broad general stress response to behavioural alterations, including neurotoxicity, haematological alterations, gastro-intestinal tract inflammation, and impaired growth (Balasch et al., 2021; Blonç et al., 2023; Brandts et al., 2021; Brandts et al., 2022, 2021; Brun et al., 2019; Chen et al., 2020; Iheanacho et al., 2023; Lee et al., 2023; Naidoo and Glassom, 2019; Pitt et al., 2018) . Altogether, MNPs have been shown to alter relevant functions in fish, such as the immune system, antioxidant/oxidant balance or lipid metabolism (Alomar et al., 2017; Bhuyan, 2022; Iheanacho et al., 2023; Solomando et al., 2020).

Nile tilapia (*Oreochromis niloticus*) is the third most important species for the aquaculture industry in terms of production, with around 7.3 million tonnes produced annually, with the pricings ranging from USD 1.6 to USD 5.96 per kg, depending on the country (FAO, 2021). Nile tilapias are notoriously known for their robustness and resistance to disease and stress, as well as for their rapid growth rate and adaptability in terms of water parameters (Barcellos et al., 1999; Chen et al., 2021; Grammer et al., 2012; Santos et al., 2013). It is an omnivorous species and one of the most sustainable for fish farming, being a major contributor to food security, especially in low- and middle-income countries, where affordable animal proteins are vital (Samaddar, 2022). Given these fundamental strengths, Nile tilapia farming has been rising rapidly in the last decades, with an increase of 400 % between 2000 and 2020 in terms of global production (FAO, 2022) and predictions of continued growth. Furthermore, this species is commonly farmed in RAS and is also particularly suitable for aquaponic systems (Dalsgaard et al., 2013; Rakocy, 1994). This is in accordance with the proposal of the Food and Agriculture Organisation of the United Nations' (FAO) for a transition towards more sustainable aquaculture practices (FAO, 2022), calling for RAS systems to be increasingly implemented (Li et al., 2023). However, in such systems, with up to 99 % of the water being reused, the potential accumulation of micro-contaminants within the system is a matter of concern that should be investigated (Martins et al., 2009).

MNPs of different polymers and sizes have been shown to occur in a wide variety of food commodities (De-la-Torre, 2020; Garrido Gamarro and Costanzo, 2022), amongst them, a range of both wild and cultured aquatic organisms of commercial importance, such as molluscs (Li et al., 2018; Ribeiro et al., 2020), crustaceans (Hossain et al., 2020; Karlsson et al., 2017; Timilsina et al., 2023), and fish (Garcia-Torné et al., 2022; Hosseinpour et al., 2021; Matias et al., 2023; Su et al., 2019). Furthermore, the presence of MNPs has also been shown in foodstuffs of terrestrial sources, including fruits, vegetables, and other plants (Azeem et al., 2021; L. Li et al., 2020; Oliveri Conti et al., 2020), poultry (Dong et al., 2023; Kedzierski et al., 2020), and bovine and porcine products (Dong et al., 2023; Urli et al., 2023). Additionally, MNPs have been detected and quantified in common beverages, such as water (Koelmans et al., 2019; Schymanski et al., 2018; Vega-Herrera et al., 2022), alcoholic drinks (Diaz-Basantes et al., 2020; Prata et al., 2020), and milk (Diaz-Basantes et al., 2020; Vitali et al., 2023).

Though several studies have already quantified MNPs in environmental samples and in fish from laboratory experiments, only a very limited number studies have tested samples coming from a RAS production system. Furthermore, these have mostly focused on larger-sized microplastics (Huang et al., 2023; Lu et al., 2019; Lv et al., 2020; Matias et al., 2023). For this reason, and due to the high number of plastic components in RAS, the present study is one of the first ones to investigate the occurrence of MNPs within a commercial European RAS fish farm. To this end, adult individuals of Nile tilapia of varying sizes were sampled for a variety of organs in order to detect, identify and quantify the different polymers that might be bioconcentrating within the farmed tilapia.

2. Materials and methods

2.1. RAS facilities description

O. niloticus were farmed in a decoupled aquaponic system, composed of a RAS, with 12 grow-out tanks made of fibreglass and PET with a volume of 2 m^3 each (24 m^3 in total; See Fig. 1). Each tank constantly receives filtered water at an average flow rate of 10 L per minute, which results in a total renewal of the water every 200 min. Each tank contains an aeration pipe connected to a central blower (KAESER Kompressoren BB52C, KAESER, Germany). Gravity pulls effluent water from each tank to a drum filter made of stainless steel and aluminium (Fish Farming Design, Denmark), a mechanical, self-cleaning microscreen filter designed for high-density fish farming RAS. Suspended solids are removed by the drum filter, after which water is returned to the system through the main sink, also manufactured from fibreglass and PET, with a total capacity of 7000 L. Water is pumped from the sink to a moving bed biological filter (height = 450 cm, diameter = 155 cm, volume = 8.5 m^3) made of stainless steel, with half of its volume filled with PE beads (around 4.25 m^3), serving as surface area for the nitrifying bacteria communities. The beads are constantly agitated by blowers situated at the bottom of the cylinder, to allow the bacteria living on the bio-elements to have constant access to oxygen and ammonia. This turbulence causes the PE beads and other filter elements to bump against each other. Once treated by the biofilter, water flows back to the main sink where it is then pumped to the grow-out tanks. All the pipework connecting the grow-out tanks together and to the different components of the system are made of polyvinyl chloride (PVC). Water temperature is kept above 27 °C year-round. Water pH is monitored daily and

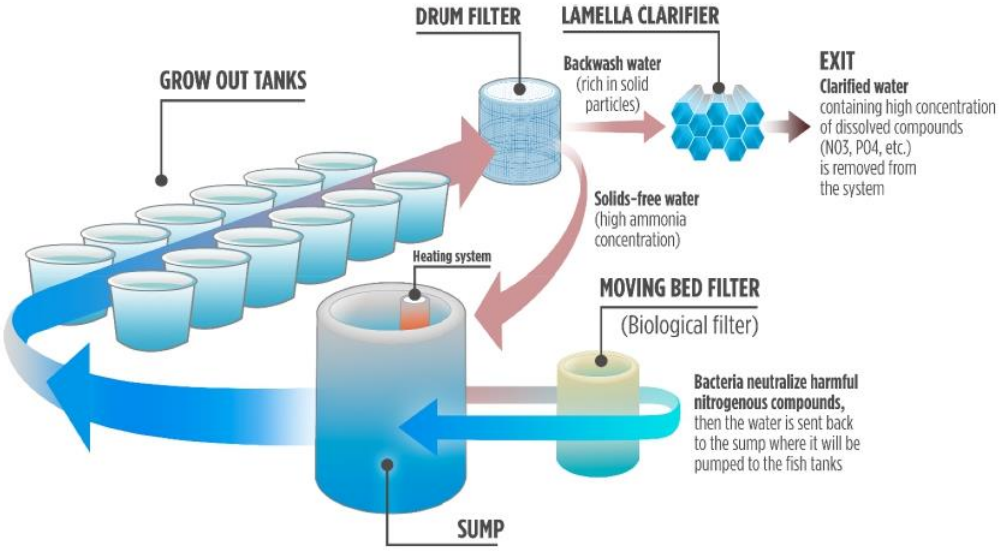

Fig. 1. Schematic design of the recirculated aquaculture system (RAS) farm where Nile tilapia (*Oreochromis niloticus*) organs and water were collected for micro- and nanoplastic (MNPs) analyses.

maintained between 7 and 7.8 by adding sodium bicarbonate ($NaHCO_3$) in order to balance the natural acidification of the farm water. Keeping the water slightly alkaline provides optimum growth condition for the essential nitrifying bacterial communities present in the biofilters. The fish are kept under a 12:12 (L:D, hours of light/darkness) photoperiod. On average, the farm discards 8.5 % (4000 L) of its water weekly.

2.2. Sampling

A total of 8 adult Nile tilapia were randomly selected from grow-out tanks of a commercial RAS farm in Spain. Fish were euthanised by immersion in 300 mg/L MS-222 buffered with sodium bicarbonate, in order to avoid unnecessary suffering of the animals, following recommendations for the ethical handling and slaughtering of fish ($NaHCO_3$; Rairat et al. 2021), and dissected for collection of liver, gut, stomach, gonads, brain, and muscle (Table 1). The wet weight of each collected organ was recorded, before inserting each sample in individual crystal vials with screw-on caps (Agilent Technologies). The samples were subsequently stored at −80 °C until processing. Water samples were collected from the farm's influent, as well as from the biofilter effluent, right before the water is being pumped back into the grow-out tanks, to determine whether these contaminants originate from the system itself, or if the incoming water is already polluted. Each sample was collected and stored in 2 L crystal bottles with screw-on caps (ISO 2000 mL, SIMAX, Czech Republic). Water samples were stored at 4 °C until processing.

2.3. Polymer analysis

2.3.1. Extraction of MNPs from water

MNPs from water samples were extracted according to Llorca et al. (2021). 2 L samples were filtered with 0.7 µm grade GF/F glass fibre filters (Whatman, United Kingdom), dried overnight at 40 °C and extracted with 10 mL of toluene by means of ultrasonic-assisted extraction (USAE) for 30 min. The supernatant was subsequently collected, introduced in a glass vial, and evaporated to near dryness under a nitrogen stream. The process was repeated twice, and the final evaporated volume was transferred to an LC vial for further evaporation until 1.0 ml. To rule out any possible cross contamination, a blank consisting on HPLC-grade water was extracted and analysed in parallel with the samples.

Table 1
Sample size (N) for each sample type collected for micro-nanoplastics (MNPs) Identification and quantification.

Sample type	Water (2 L)	Liver	Muscle	Gonads	Brain	Gut	Stomach
Sample size (N)	2	8	8	4	4	4	4

2.3.2. Extraction of MNPs from fish tissues

Liver, gut, stomach, gonads, brain, and muscle were lyophilised (CRYODOS-80, Telstar; 72 h, −75 °C, <0.15 m) and then homogenised (TissueLyser II, Quiagen). Samples were digested and MNPs extracted from fish tissues and purified as described in Brandts et al. (2022). Briefly, a 6 h digestion with KOH (10 %, 1:3 (w/v) at 60 °C was done and then samples were filtered. The fibreglass filters were then digested (HNO_3 20 %, 1 h) and the filters were then washed (MiliQ) and dried (60 °C). MNPs were extracted from the filters by ultrasonic-assisted extraction (30 min) with 10 mL of toluene and transferred to amber glass vials. To obtain the final extracts, the process was repeated twice, and extracts were concentrated through evaporation under a nitrogen stream. Samples were then centrifuged (5376 g, 10 min) and the supernatant was collected in LC vials and evaporated again under a gentle nitrogen stream to a final volume of 1.0 mL. To rule out any possible cross contamination, a blank consisting of "pure" fibre filter was extracted in parallel with the rest of the samples.

2.3.3. Polymer quantification by size exclusion chromatography

The analysis of the samples was based on Llorca et al. (2021). The compounds were separated by means of size exclusion chromatography on an Acquity LC (Waters, Milford, MA, USA) system equipped with an advanced polymer chromatography (APC) column (Acquity APC™ XT45 1.7 µm 150 mm). The chromatography was coupled to a Q-Exactive hybrid quadrupole-Orbitrap mass spectrometer (Thermo Fisher Scientific, San Jose, CA). The ionization of the polymers was performed under atmospheric pressure photoionisation source working in negative ionisation conditions ((−) APPI). The separation on APC column was achieved working with isocratic conditions with 100 % toluene at a flow rate of 0.5 mL/min for 5 min. The sample volume injected was 20 µL. Data was acquired in full scan mode (m/z 500–3000) at a resolution of

17,500 full widths at half maximum (FWHM). The identification of polymers was done by means of the Kendrick Mass Defect identification pattern (Llorca et al., 2021) while the quantification was only possible for those polymers with available calibration curves.

The confidence levels during tentative identification of chemical compounds were based on suspect and non-target screening strategies used when working with high resolution mass spectrometry. In this specific case, the procedure is based on Llorca et al., 2021):

- Level 1: the identification is unequivocal since it coincides in exact mass defect, Kendrick mass defect, retention times. The confirmation and quantification of the compound was finally done by comparing with pure analytical standard.
- Level 2: the identification is tentative at second level since it coincides in exact mass defect and Kendrick mass defect, but no quantification was done since the pure analytical standard was not available.

3. Results and discussion

The results show the presence of five different polymers in the collected samples, namely, PE, PI, polysiloxane, perhydropolysilazane (PHPS), and poly-dimethylsiloxane (PDMS; Figs. 2 and 3; Table 2). Both PE and PI were identified at confidence level 1, and quantified, whereas the other compounds were tentatively identified at level 2 of confidence. However, in order to obtain precise information on the size or shape of the collected particles, additional analytical techniques, such as Scanning Electron Microscopy (SEM) coupled with energy-dispersive X-ray spectroscopy (SEM-EDS), would be required (Klein, 2018).

In the collected water samples, both PI and polysiloxane were detected, although only PI could be quantified. Both polymers have been previously described in environmental samples, accounting for two of the most detected MNPs in Spanish river effluents (Llorca et al., 2021). In the present study, PI was detected both in the influent, indicating an input of this polymer from sources external to the farm, and in the RAS water. Relevantly, PI appeared in considerably higher concentrations in the RAS t water (257.73 ng/L) when compared to the influent water (156.3 ng/L), displaying an almost 2-fold increase. This is probably due to the presence of PI-based components within the RAS, such as anti-fouling coatings (Jellali et al., 2013), which could undergo thermo-degradation or mechanical weathering from water flow, and be transported throughout the system, accumulating in the culture water due to the low rate of water renewal. This increase in the concentration of PI when comparing water from the influent and the system suggests that the components of the RAS could be an additional source of MNPs for the surrounding aquatic ecosystems. More importantly, the input of MNPs into the culture water from plastic component within the system, combined to the intrinsically low water renewal rates of RAS translate into an accumulation of MNPs in the water, where they are prone to reaching high concentrations, potentially having deleterious effects on the health and welfare of the animals, and affecting production. However, no traces of this polymer were detected in any of the investigated tissue samples. The other polymer that was found in water, polysiloxane, was only detected in traces in the influent water, but not in the system . However, it is likely that the volume of water analysed (i.e. 2 L from the influent and 2 L from the biofilter effluent) give us only a snapshot of the system, meaning that the observed values could be an underestimation of reality.

Relevantly, polysiloxane was also detected in Nile tilapia muscle (Fig. 3). Therefore, the absence of polysiloxane in the biofilter effluent

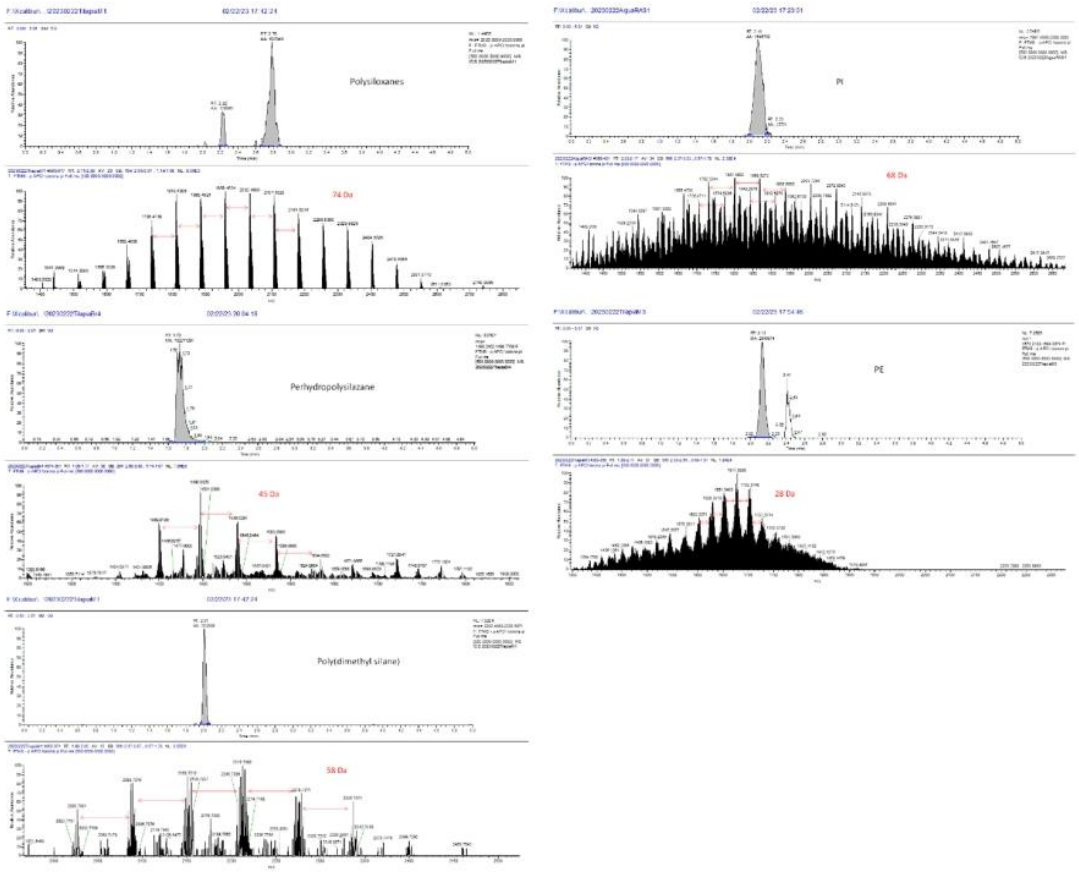

Fig. 2. Examples of chromatograms and spectra of the detected polymers in system water and organs of *Oreochromis niloticus* (Nile tilapia) of a European commercial recirculated aquaculture system (RAS) farm.

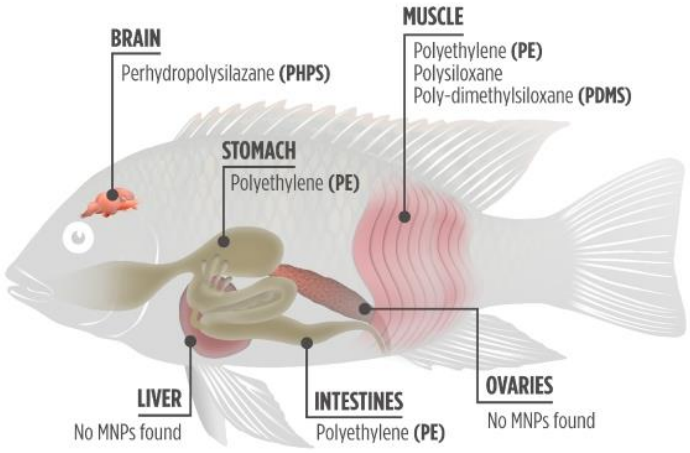

Fig. 3. Occurrence of distinct micro- and nanoplastics (MNPs) polymers in various organs of *Oreochromis niloticus* (Nile tilapia) of a commercial recirculated aquaculture system (RAS) farm in Europe.

Table 2
Presence of micro-nano-plastic (MNPs) polymers in different matrices (i.e. water and *Oreochromis niloticus* organs) in a commercial European recirculated aquaculture system (RAS) farm. The results are expressed both in number of samples, and in percentages, relative to the sample size of each matrix. Similarly, the total number of samples, regrouping all considered matrices where each polymer was detected, is displayed.

Polymer **Sample type and size (N)**	Polyisoprene (PI)	Polyethylene (PE)	Polysiloxane	poly-dimethylsiloxane (PDMS)	Perhydropolysilazane (PHPS)
Water ($N = 2$)	**2 (100 %)**	0	**1 (50 %)**	0	0
Muscle ($N = 8$)	0	**8 (100 %)**	**7 (87.5 %)**	**3 (37.5 %)**	0
Gut ($N = 4$)	0	**4 (100 %)**	0	0	0
Stomach ($N = 4$)	0	**1 (25 %)**	0	0	0
Brain ($N = 4$)	0	0	0	0	**4 (100 %)**
Liver ($N = 8$)	0	0	0	0	0
Gonads ($N = 4$)	0	0	0	0	0
Total Samples ($N = 34$)	**2 (5.8 %)**	**13 (38.2 %)**	**8 (23.5 %)**	**3 (8.8 %)**	**4 (11.8 %)**

water could be partially explained by the internalisation and retention of this polymer in fish muscle, removing it, to some extent, from the surrounding water. In the present study, polysiloxane was not detected in any other organ. However, the internalisation and accumulation of this polymer in gastrointestinal tract of several fish species, both marine and freshwater, has been reported in Spanish coastal and riverine waters (Garcia-Torné et al., 2022). The analysed muscular tissues also contained traces of PDMS, a specific type of polysiloxane, relatively immiscible with water (Powell et al., 1999) used for distinct industrial purposes, such as protective coatings and lubricants (Fendinger, 2000; Stevens et al., 2001). However, quantification of this polymer to determine real concentrations was not feasible. This specific polymer was not detected in any other sample type analysed. It is likely that these samples fail to accurately reflect their occurrence within the system, as polysiloxanes and specially PDMS have been reported to accumulate in sludge (Fendinger et al., 1997). Therefore, it would be of interest to investigate the presence of MNPs in sediment or solid waste samples in the commercial fish farm in future studies. (Feng et al., 2012) reported the effect of PDMS on the development of both the Atlantic purple sea urchin (*Arbacia punctulate*), on the freshwater medaka fish (*Oryzias latipes*). Their study suggests that surface associated molecules and mixtures leaching from PDMS have significant impacts on the development of these two model organisms. Reported effects included decreased hatching success, delayed development, lower larval survival rate and buoyancy issues. Additionally, other studies indicate that increasing concentrations of this pollutant lead to significant alterations in water quality, consequently affecting the overall fish's nutritional quality, by reducing its crude protein content and significantly increasing its lipid content (Anyachor and Sikoki, 2022).

Muscle samples also contained quantifiable amounts of PE (Fig. 3), ranging from 0.23 ng/g to 79.57 ng/g. This polymer was the most detected compound across tissues, being present in 38.2 % of the analysed samples (Table 2) and in 3 of the 6 sampled organs, namely muscle, gut (12.28 – 90.76 ng/g), and stomach (83.55 ng/g). As mentioned in the description of the RAS facilities (Section 2.1) the biofilter beads are made of PE and could be a possible source of the PE found in fish samples. Indeed, this contaminant was quantified in 100 % of the muscle and gut samples, as well as in 25 % of the stomach samples analysed (Table 2). The presence of PE-MNPs in tilapia gut and stomach is in accordance with previous research, that demonstrated the occurrence of this compound in the gastrointestinal tract of a variety of Spanish fish species (Garcia-Torné et al., 2022). Several studies have investigated the effects of PE-MNPs in aquatic organisms. For instance, exposure to PE-MNPs has been described to significantly alter acetylcholinesterase (AChE) activity, as well as cause oxidative and genotoxic damage in marine and terrestrial invertebrates (Avio et al., 2015; Chen et al., 2020; Prüst et al., 2020). Similarly, exposure to PE-MNPs has also led to significant changes in behaviour, enzymatic activity (e.g. AChE) and oxidative stress parameters in both marine and freshwater fishes (Fonte et al., 2016; Luís et al., 2015; Mak et al., 2019; Prüst et al., 2020; Wen et al., 2018). In the Korean bullhead (*Pseudobagrus fulvidraco*), acute exposure to PE-MPs led to the accumulation of this compound in the fish's organs, inducing alterations on both haematological and plasma biochemical parameters, and triggered antioxidant responses (Lee et al., 2023). Bobori et al. (2022) demonstrated that particle size influences the hazardous effects of PE-MPs, with smaller particles (10–45 μm) being more potent to induce noxious effects (alterations in the lipid metabolism, DNA damage and ubiquitination. Furthermore,

this compound stimulated signal transduction pathways leading to autophagy and apoptosis in both *Danio rerio* (the zebrafish), and *Perca fluviatilis* (the European perch). Moreover, Varó et al. (2021) reported that bigger PE particles were more prone to engender physical alterations of the gastrointestinal tract.

The effects reported in the mentioned literature showed considerable dependency on species, particle size, sampled organs, and co-exposure with additional contaminants (Prüst et al., 2020). PE was expected to be detected in the water, as some components of the RAS (e.g. biofilter beads) are made of this material and are constantly subjected to the mechanical action of water. However, it is likely that the water samples were not representative of the entire system, as the presence of this compound was confirmed within the system through detection in fish tissues and future improvements of the methods should be considered.

The only polymer detected in cerebral tissue was PHPS (Fig. 3), indicating that these particlescross the blood-brain-barrier. Brain was the only organ where this compound was detected (Table 2). Similar occurrences have been described for polystyrene (PS) NPs in both mammals (Shan et al., 2022), and fish (Mattsson et al., 2017), where retention of PS-NPs in brain was reported. PHPS is a versatile hyper-hydrophobic polymer and is therefore used as a precursor for a variety of coating (e.g. anticorrosive, antirust) and sealing materials, amongst others (Song et al., 2018; Zhang et al., 2015). To the best of the authors' knowledge, no scientific publication investigating the effects of PHPS pollution on natural ecosystems is available. Although it can be hypothesised that internalisation and retention of this contaminant in cerebral tissue may have similar effects as those seen with other MNPs, further research is required to fully understand the environmental effects of PHPS. Surprisingly, the present results do not indicate the occurrence of MNPs of any polymer in neither liver nor gonads of tilapia. Other MNPs, such as PS have been reported to bioconcentrate in the liver of both freshwater and marine organisms, although the tissue-specific accumulation of MNPs appears to vary between species (Brandts et al., 2022; Pedersen et al., 2020). Similarly, previous research has demonstrated the accumulation of PS-NPs in the gonads of *D. rerio* (Sarasamma et al., 2020).

Differences in bioconcentration between polymers and organs have previously been reported, describing that the mechanisms and rates of MNPs internalisation are species-, life stage-, tissue- and polymer-dependant (Habumugisha et al., 2023; Zhou et al., 2022). The results hereby presented reveal that no organ retained all detected compounds, and no polymer was detected in all sample types. Therefore, the present results further corroborate the idea that retention of MNPs is somewhat tissue-specific, in addition to being influenced by the polymer's specificities, and the studied species, as varying bioconcentration rates have been observed in the same organ of different species exposed to the same compound (Brandts et al., 2022; Garcia-Torné et al., 2022; Pedersen et al., 2020).Finally, some of the expected polymers were not detected in any of the analysed samples, including PS, PVC and polypropylene (PP). PVC and PP are used in main components of the RAS and PS is reported to be one of the most commonly detected plastic pollutants in Spanish rivers, coastal and tap water, and fish tissues (Garcia-Torné et al., 2022; Llorca et al., 2021; Vega-Herrera et al., 2022). The absence of these polymers in the detected MNPs could be explained by the use of protective coatings, to prevent fouling, corrosion and oxidation of the farm components (e.g. polysiloxane-, PHPS-, or PMDS-based products). However, a more comprehensive sampling campaign, with greater water volumes and an increased sample size would allow for a better understanding of the MNPs contamination in the system's water and organisms. Additionally, it could prove interesting to investigate the presence of MNPs in the aquafeed used by the farm, in order to further determine the potential origin of the MNPs detected within the RAS and the different fish organs.

4. Conclusions

A total of 5 distinct polymers were detected in the analysed samples, accounting for 6 tilapia organs (i.e. liver, gut, stomach, gonads, brain, and muscle; Fig. 3) and system water. MNPs were detected in fish muscle, brain, gut, stomach, but not in the gonads or liver. MNPs were also detected in water from the influent of the system and effluent of the biofilter. Importantly, within the samples analysed in the present study, muscular tissue was the most prone to retain MNPs, with 100 % of the muscle samples containing at least 2 distinct polymers. Moreover, muscle accumulated 3 out of the 5 different polymers found in the present data. This may have implications for human health, representing an additional pathway for exposure (i.e. the internalisation and retention of these contaminants through the consumption of contaminated fish; Garcia-Torné et al., 2022). Nonetheless, it is important to note that fish are far from being the only food source that has been demonstrated to be contaminated by MNPs. Moreover, RAS farms, being isolated from the external environment, offer an opportunity for an increased level of control on the presence of contaminants, both in the system and in the cultured organism, allowing for a better monitoring of the degree of MNPs contamination compared to wild catch or offshore systems. With MNPs being ubiquitous in both environmental matrices and human food sources, gaining a comprehensive understanding on the effects of these compounds on human health is of utmost importance. In addition, investigating possible solutions to remove MNPs from natural ecosystems and mitigate their environmental impacts, as well as creating and implementing legislations to further regulate the production, use and discard of plastics, is of greatest urgency. Furthermore, it is crucial to determine the impact of MNPs pollution in aquaculture systems, not only on fish health and welfare, for ethical reasons, but on the seafood production sector, and, ultimately, global food security.

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CRediT authorship contribution statement

M. Blonç: Conceptualization, Investigation, Writing – original draft, Writing – review & editing. **F. Husson:** Conceptualization, Investigation, Writing – original draft, Writing – review & editing. **M. Llorca:** Investigation, Writing – review & editing. **M. Farré:** Funding acquisition, Investigation, Writing – review & editing. **L. Tort:** Conceptualization, Funding acquisition, Writing – review & editing, Supervision. **I. Brandts:** Writing – original draft, Writing – review & editing. **M. Teles:** Conceptualization, Funding acquisition, Investigation, Writing – original draft, Writing – review & editing, Supervision.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

Acknowledgments

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Stress response to centrifugal pumping in Salmo salar in a recirculated aquaculture system: A case study.

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Fish Physiology and Biochemistry: Under Revision

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Stress response to centrifugal pumping in *Salmo salar* in a recirculated aquaculture system: A case study.

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Abstract

Salmo salar, as the most economically important salmonids, is commonly reared in recirculated aquaculture systems (RAS) during its land-based production phases. In RAS, pumps are commonly employed to transfer fish, although information on their impact on fish is scarce. This study is the first to follow a transfer performed through centrifugal pumping in a commercial RAS, investigating its effect on the stress of fish, and their ability to recover from the stress over a week. This was assessed through the analysis of physiological, biochemical and molecular indicators of stress responses and inflammation, in plasma and relevant organs. The primary and secondary stress responses were triggered, as indicated by the alterations in biochemical parameters. Cortisol levels were considerably lower than expected for non-stressed fish, indicating a possible exhaustion, or decreased sensitivity, of the hypothalamus-pituitary-interrenal (HPI) axis. In the gills and head kidney, all genes displayed significant alterations, whereas no differences were detected in brain. The results indicate that, in this specific industry-based case, centrifugal pumping was stressful to *S. salar* smolt, but this stress was finally not maladaptive, and the fish were able to maintain homeostasis. This study, having been performed in industrial settings, provides results of greater representativeness of real-life scenarios compared to laboratory experiments or simulations. Further research following the stress status of *S. salar* in industrial settings throughout the production cycle is required to investigate to which extent rearing conditions in commercial RAS do indeed alter the HPI axis responsiveness.

Keywords: Hypothalamus-pituitary-interrenal (HPI) axis, physiological response, cortisol, Recirculated Aquaculture System (RAS), Atlantic salmon

1. Introduction

Atlantic salmon (*Salmo salar* L.) is the most produced salmonid species by the aquaculture industry, representing over 70% of total salmonid production and 30% of the total fish production from the marine aquaculture industry, equalling over 2.7 million metric tonnes per year (FAO, 2022; Pandey et al., 2023). The farming of this species is dominated by five countries, with Norway, Chile, Canada, the United Kingdom and the Faroe Islands accounting for over 90% of the total global production (FAO, 2022; Iversen et al., 2020). *S. salar* is therefore one of the most economically important fish, and an increasing number of producing companies are implementing recirculating aquaculture systems (RAS) for the land-based phases of salmonid production (Lazado & Good, 2021).

RAS, characterised by major water reutilisation rates (>90%) and a variety of filtration mechanisms (*e.g.* mechanical and biological), sterilisation and oxygenation systems, allow for the rearing of aquatic organisms in indoors, closed settings, virtually isolated from the surrounding environment (Ahmed & Turchini, 2021; Badiola et al., 2012). Therefore, these systems allow for increased control over water quality and overall monitoring, enhancing fish welfare. In addition, by minimising the interactions between the farm's components and the external environment, they reduce both external pressure to the cultured species (*e.g.* absence of predators, protection from climatic events) and the environmental impact of the aquaculture facility (*e.g.* lack of escapees, increased outlet water quality, decreased possibility of disease transmission), all while allowing for increased stocking densities (Ahmed & Turchini, 2021; Ayer & Tyedmers, 2009; Chang et al., 2019). For these reasons, *Salmo salar* producers in the Nordic countries are prioritising smolt production in RAS (Crouse et al., 2021). In such systems, like in all aquaculture practices, fish are subjected to handling and transportation. In addition, in RAS, fish might be transferred from a tank to a truck or a boat as part of transport to sea or to slaughtering facilities, or from one tank to another in order to reduce density, and the use of pumps to this end is common practice (Noble et al., 2018). Although highly common and often inevitable, these transfers are inherently stressful events, and are most often preceded by a crowding episode to facilitate the transfer process (Hoem & Tveten, 2023; Norwegian Scientific Committee for Food Safety, 2008).

In fish, the primary stress response is mediated by the activation of the hypothalamus-pituitary-interrenal (HPI) axis, characterised by a rise in cortisol levels, and the brain-sympathetic-chromaffin (BSC) axis characterised by the release of catecholamines (Schreck & Tort, 2016). When the hypothalamus initiates the activation of sympathetic fibres, chromaffin cells in the head kidney release catecholamines which, in turn, lead to an increase in the availability of energetic resources to aid in the "fight or flight" response. In addition, the activation of the HPI axis induces the release of corticotropic releasing factor (CRF) from the preoptic area in the brain. In the pituitary gland, CRF starts a process resulting in the synthesis of adrenocorticotropic hormone (ACTH) which prompts the production and release of cortisol into the bloodstream (Madaro et al., 2016; Schreck & Tort, 2016). This triggers the secondary response, which encompasses a variety of physiological and metabolic adaptations related to allostasis (*e.g.* increase in circulating glucose and lactate; Raposo de Magalhães et al., 2020). Significant alterations in these pathways might have cascading effects, impacting the immune capacity of the fish, its reproduction, growth, and overall performance (Schreck & Tort, 2016). Furthermore, it has been reported that stress induces immunodepression and fragilizes living organisms, and that subsequent mild stressors following a major stressing event are more likely to result in mortalities (Sampaio & Freire, 2016; Sandodden et al., 2001). It is clear that

exposure to stress in cultured fishes might have strong repercussions on the sustainability and profitability of the aquaculture sector by increasing susceptibility to disease and causing mortality events. Therefore, it is of greatest interest to precisely understand how common commercial practices affect the overall stress and welfare status of fishes in order to develop and implement mitigation techniques, both from an economic and an ethical point of view.

Over the past few decades, a number of studies have investigated the impact of live transportation both in salmonids (Barton & Peter, 1982; Hoem & Tveten, 2023; Iversen et al., 1998; Sandodden et al., 2001; Schreck et al., 1989; Specker & Schreck, 1980) and non-salmonid species (Davis et al., 1994; Dobšíková et al., 2009). Live transportation by itself has been demonstrated to induce the activation of the stress response in fishes. However, it has been reported that the loading process (*e.g.* through crowding and pumping) might have a stronger impact on the organisms than the actual transport, and that different loading procedures stress the fish to different extents (EFSA, 2009; Dobšíková et al., 2009; Hoem & Tveten, 2023; Merkin et al., 2010). Indeed, according to previously published research, the practice of vacuum pumping to transfer fish is more stressful than other loading techniques, such as netting, and might significantly extend the recovery period (Davis et al., 1994; Wagner & Driscoll, 1994) in addition to significantly decreasing product quality by reducing the *rigor mortis* onset time (Roth et al., 2012). However, many of these studies have focused on the whole process, without differentiating between stages (*e.g.* loading, transport, and unloading; Dobšíková et al., 2009; Urbinati et al., 2004). Previous research that has specifically focused on the effects of pumping in fish separately from transportation has investigated mainly biochemical parameters in the plasma (Davis et al., 1994; Espmark et al., 2016; Wagner & Driscoll, 1994), behaviour (Nomura et al., 2009), muscle pH (Merkin et al., 2010), survival (Iversen et al., 2005), or onset time of *rigor mortis* (Gatica et al., 2008; Roth et al., 2012).

Although previous research has investigated the impact of pumping in fish in laboratory conditions, to the best of the authors knowledge, no study has focused on the impact of fish centrifugal pumping on biochemical parameters and molecular endpoints in *S. salar* smolt directly in a commercial RAS setting. While conducting this type of investigation in commercial settings, following routine operations without any sort of intervention from the researchers, does not allow for tank replicates, it provides insightful information on what occurs in a real-life scenario. Therefore, the present study is the first to examine the effect that a standardised transfer operation (*i.e.* with density reduction as primary objective) might have on the general stress and welfare status of *S. salar* in an industrial RAS, and the capacity of the fish to recover from the event. To this end common physiological indicators of the primary and secondary stress response (*i.e.* circulating cortisol and glucose levels, in the plasma), liver activity and damage (*i.e.* aspartate aminotransferase, AST; alanine transaminase, ALT), and immune and inflammatory regulation (adenosine deaminase, ADA) were researched. In addition, the relative abundance of genes relevant to the general stress response (*i.e.* glucocorticoid receptor 1, *gr1*; glucocorticoid receptor 2, *gr2*; mineralocorticoid receptor, *mr*; corticotropin releasing factor, *crf*), homeostasis (*i.e.* heat shock protein 70, *hsp70*), and pro-inflammatory cytokines (*i.e.* tumour necrosis factor alpha, *tnfα*; interleukin 1 beta; *il1β*) were investigated in brain, head kidney, and gills. Furthermore, the *star* (steroidogenic acute regulatory) protein was assessed in the head kidney, and mucin-coding genes (*i.e. muc2* and *muc18*) were analysed in the gills. Gills were also selected for the present study as, being a highly sensitive mucosal surface in direct contact with the surrounding environments, they are known to be influenced by both acute and chronic

stressors, and be involved in maintaining homeostasis (Hoem & Tveten, 2023; Takei & Hwang, 2016). On the other hand, the head kidney and the brain were investigated given their involvement in the regulation of both primary and secondary stress responses, as well as in neuro-immuno-endocrine signalling pathways (Madaro et al., 2023; Tort, 2011). Thus, the selected endpoints reflect, to some extent, the response of both the peripheral and central systems.

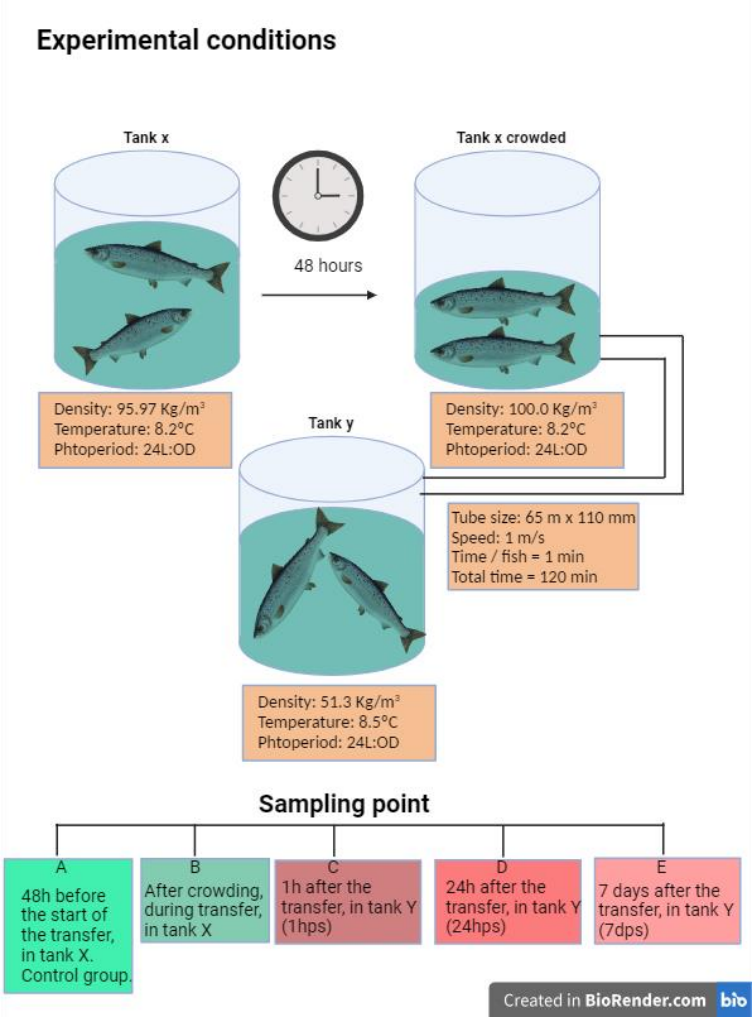
The hypotheses were two-fold. Firstly, it was expected that the transfer from one tank to another through pumping would trigger the stress response in *S. salar*, which would be reflected by an almost immediate rise in plasmatic cortisol levels, followed by an increase in glucose, and variations in both biochemical parameters and in the expression of relevant genes. Secondly, it was hypothesised that all investigated parameters would return to control levels shortly after the start of the recovery period (up to 7 days post stress).

2. Materials and methods:

2.1. Sampling

A total of 40 *S. salar* smolt (64.7 ± 18 g total weight, 18.7 ± 1.5 cm total length) were sampled from an industrial RAS farm at five different time points (A-E; Figure 1). The sampling took place around a standardised pumping episode destined to transfer fish from one tank (X) to another (Y) in the same facility to reduce density and prevent overcrowding stress. The sampling points were distributed over time as follows:

A- 48h before the start of the transfer, in tank X. Control or pre-treatment group.
B- During transfer after crowding, in tank X
C- 1h after the transfer, in tank Y (1hps)
D- 24h after the transfer, in tank Y (24hps)
E- 7 days after the transfer, in tank Y (7dps)



No specific indications were given to the farm operators as to how to perform the transfer, ensuring that the event was not influenced by the sampling, and that the results would reflect as much as possible a real-life, commercial scenario (Iversen et al., 2005). The fish were subjected to a 24h light photoperiod throughout the land-based

Figure 1. Description of the sampling procedure. (hps=hours post stress, dps=days post stress). Source: *Biorender*.

production phase and were food-deprived 24h prior to the beginning of the transport. Directly before the transfer, the water level in tank X was slightly lowered, resulting in a slight crowding of the fish, although the entire transfer process lasted less than 2 hours, meaning that the crowding time was considerably short. The transfer was performed through centrifugal pumping, during which fish were transported via a plastic hose (65m x 110mm), at a speed of *ca*. 1 m/sec (approximately 1 min from tank to tank). The water temperature in tank X did not differ greatly from that in tank Y, with 8.2 and 8.5ºC, respectively. On the other hand, the initial density (in tank X) was estimated at 95.97 Kg/m^3, whereas the final density (in the receiving tank, Y) was estimated at 51,3 kg/m^3.
Eight fish were randomly collected from the industrial tanks and immediately anaesthetised by immersion in 300 mg/L of MS-222 (Finquel, MSD Animal Health) buffered with 600 mg/L of sodium bicarbonate. Once the fish had reached stage 3 of anaesthesia (*i.e.* surgical), they were carefully removed from the anaesthesia tank, checked for injuries, malformations, and signs of disease, weighed, and measured. Once the fish was deemed healthy, blood was extracted using heparinised syringes equipped with 24G needles. The obtained blood was placed in individual Eppendorf tubes and stored at 4ºC under dark conditions until further processing. Subsequently, the fish were euthanised by re-immersion in the anaesthesia tank until stage 4 was attained (*i.e.* medullary collapse), and death was confirmed with a sharp cut through the spine. Once the individual was dead, the head-kidney, brain, and gills were extracted and immediately stored individually in RNA Later® (Ambion), and subsequently stored at -80ºC until further processing and analysis.

2.2 Plasma extraction and biochemical endpoints

The collected blood samples were centrifuged at 2000 *g* over a 10-minute period in a cooling centrifuge maintaining the temperature at 4ºC in order to obtain plasma for subsequent biochemical analyses. Cortisol levels were determined with an automated analyser (Immulite 1000, Siemens Health Diagnostics) using a commercial immunoassay (LKC01, Siemens Health Diagnostics). Glucose levels were also analysed with an automated analyser (Olympus Diagnostics) following the manufacturer's recommendations. ADA activity was determined with commercially available kits (Adenosine Deaminase assay kit, Diazyme Laboratories, Poway, CA, USA) as per the provider's instructions. AST and ALT were measured in the plasma of fish using commercial kits (Olympus Systems Reagents; Olympus life and Material Science Europe GmbH, Hamburg, Germany). All automated assays were previously validated for fish (Blonç et al., 2023).

2.3. RNA extraction, complementary DNA synthesis and real-time quantitative Polymerase Chain Reaction

A total of 20 mg of each organ samples was placed in RLT lysis buffer (Qiagen GmBH, Germany) homogenised with the TissueLyser II (Qiagen) at 30Hz for 4 minutes on each side to ensure an equal degree of mixing throughout the samples. RNA was then extracted using the RNeasy Mini Kit following the manufacturer's directions, with minor modifications in the centrifugation steps, increasing the centrifuge's speed to 9400 *g*. Complementary DNA (cDNA) was synthetised by reverse transcription using the commercially available kit LunaScipt™ RT SuperMix Kit (NEB E3010L, New England BioLabs Inc, USA) following the manufacturer's recommendations. The efficiency of all selected primers (Table1) was tested through RT-qPCR of serial dilutions of pooled cDNA (Pfaffl, 2001) to ensure that all efficiencies were in the range of 90-110%. RT-qPCRs were performed using 384-well plates, each containing a final volume of 5µL, comprising 2.5µL of Universal SYBR® Green Supermix (Bio-Rad, USA), 250nM of the corresponding primer pair (forward and reverse) and 1µL of cDNA at the

previously determined optimal dilution. The qPCR protocol, ran through the BIO-RADCFX384 Real-Time PCR Detection System (Bio-Rad), was the following: 95ºC for 3 min, and a cycle of 10 s at 95ºC and 30 s at 60ºC repeated 40 times.

2.4. Data analysis

The pre-selected reference genes (Table 1) were tested for their stability throughout the samples using the GENorm algorithm already incorporated into the Bio-Rad CFX Maestro Software (Bio-Rad), with the results indicating organ specific combinations of the most suitable reference genes for the present study (Brain: *elf1* and *rbs5*; Gills: *18S* and *βactin*; Head kidney: *18S* and *βactin*). The gene expression of the target genes was then calculated relative to that of these reference genes following the Bio-Rad CFX Maestro User Guide's indications. All collected data were tested for normality and homoscedasticity, and data that met the assumptions were analysed with parametric one-way ANOVA and Tukey's post-hoc tests. On the other hand, data that did not meet the assumptions were treated with the non-parametric equivalents, namely, Kruskal-Wallis tests, and False Discovery Rate tests. All data were analysed using GraphPad Prism 8. Moreover, the sampling effect was tested to evaluate the impact of the absence of tank replicates (data not shown), and the results indicated no significant effect of sampling.

3. Results

3.1. Plasma biochemistry

The analyses conducted indicate significant differences in the plasmatic biochemical profiles of *S. salar* (Figure 2). The determined values of circulating cortisol ranged between 0.05 and 15 ng/ml. A significant increase in cortisol levels was observed at sampling points B and C, with a subsequent significant decrease afterwards. Glucose levels in *S. salar* plasma displayed a significant increase from point B to C followed by a significant decrease after sampling point E The levels of plasmatic AST followed a significant increase towards point C, followed by a decrease until reaching control levels in the subsequent sampling points. ALT levels in plasma displayed a slight but non-significant increase during pumping, but levels significantly decreased at point D and E relative to the control. The determined ADA levels in plasma displayed a significant increase at point B, with a slight, non-significant decrease at point C. These levels returned to control values at point D, with a subsequent significant increase at point E.

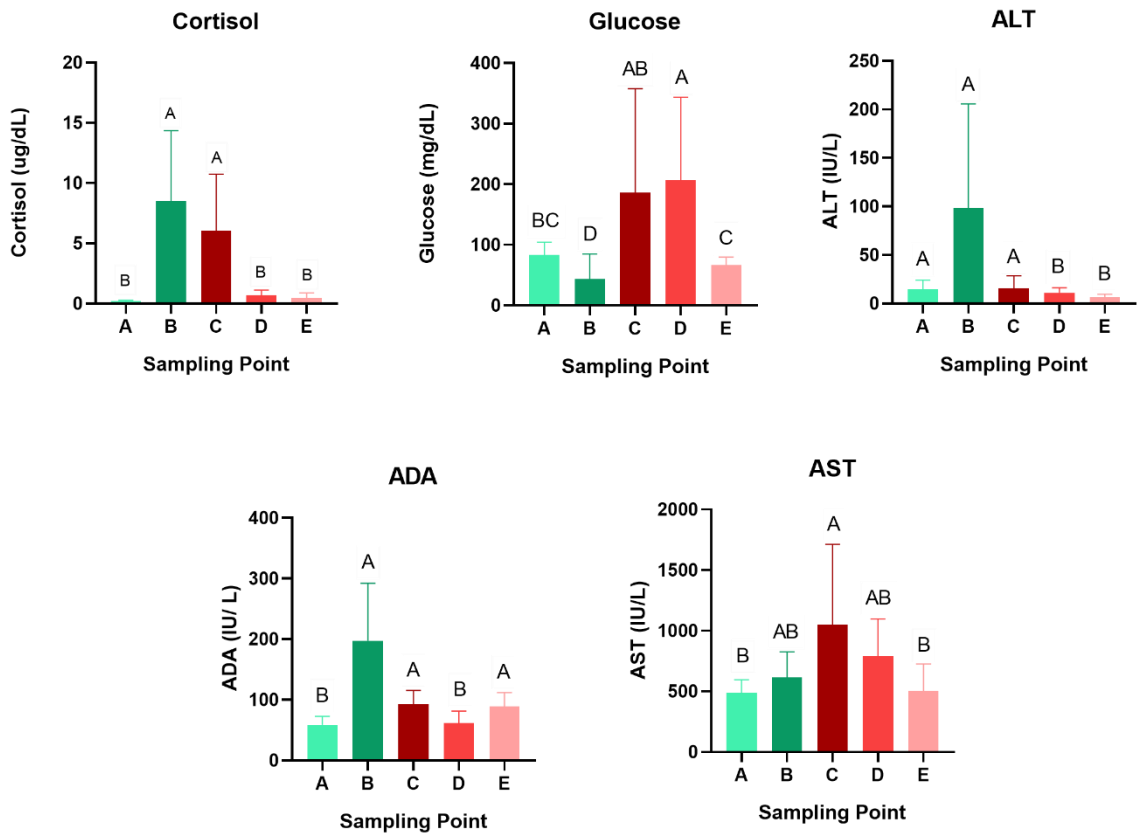


Figure 2. Biochemical parameters investigated in *Salmo salar* plasma during a centrifugal pumping episode in a commercial recirculated aquaculture system (RAS). Cortisol; glucose; AST: aspartate aminotransferase; ALT: alanine transaminase; ADA: adenosine deaminase. A: 48h before exposure; B: during transfer; C: 1hps; D: 24hps; E: 7dps. Different letters above columns indicate significant differences between sampling points ($P<0.05$).

3.2. Gene expression

In the head kidney (Figure 3), *gr1* was significantly downregulated in all sampling points compared to the control (point A), with mRNA levels of this gene stabilising at point C, and subsequently decreasing significantly at point E. In addition, the expression of *crf*, *gr2* and *hps70*, displayed decreasing trends, with significant downregulations only at point E. However, the results indicate a significant decrease in mRNA abundance of *tnfα* directly after the stress started (point A), remaining stable up to 24hps, and further decreasing at point E. On the other hand, *mr* was not affected in the head kidney in the present study. Similarly, the relative abundance of *il1β* in this organ was not significantly affected during the pumping event, although a significant reduction was observed in point E compared to all other sampling points. Likewise, *star* did not result significantly altered during the pumping process, but a significant downregulation was observed in point E compared to control values.

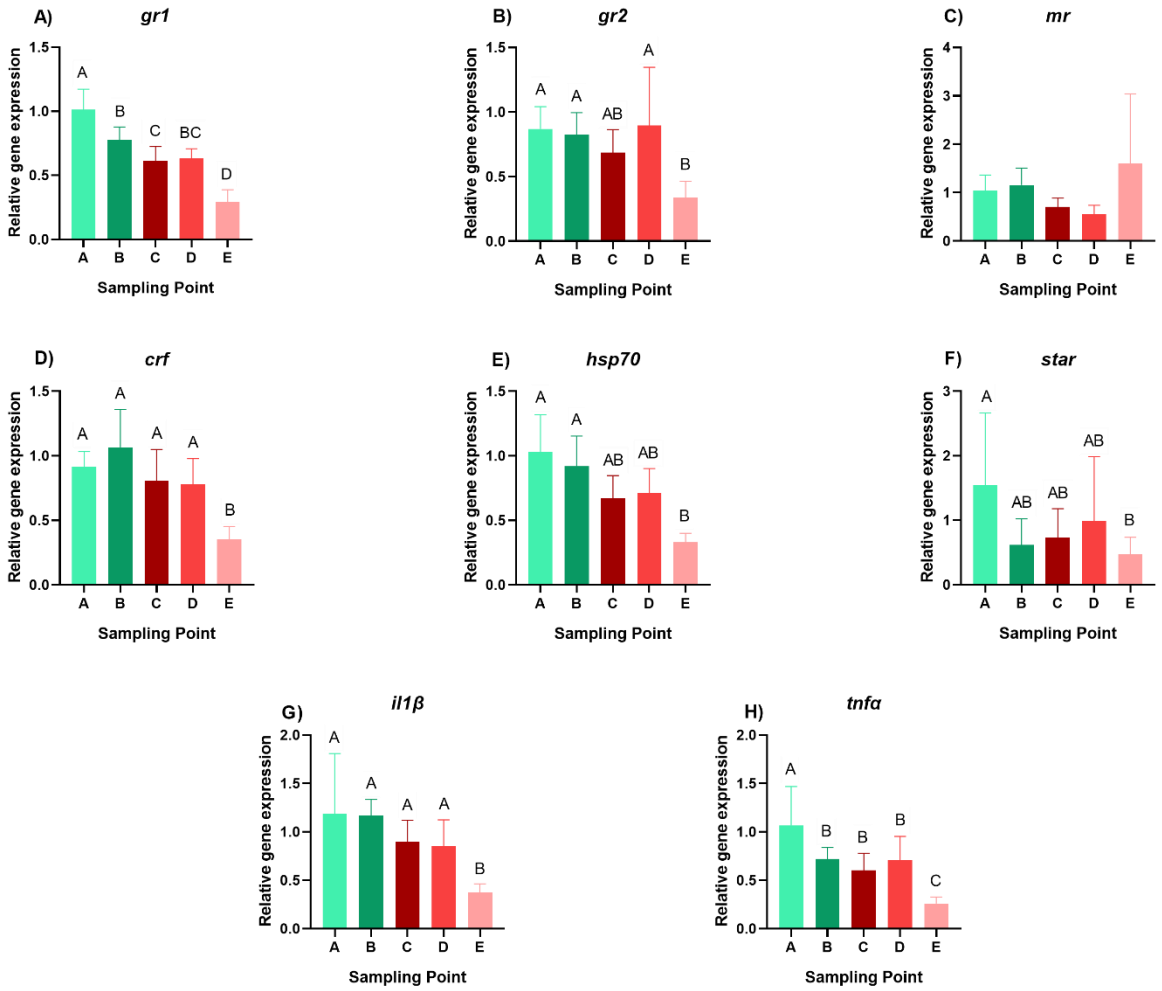


Figure 3. Relative gene expression analysed in head kidney of *Salmo salar* during a centrifugal pumping episode in a commercial recirculated aquaculture system (RAS). A: 48h before exposure; B: during transfer; C: 1hps; D: 24hps; E: 7dps. Different letters above columns indicate significant differences between sampling points ($P<0.05$).

In the gills of *S. salar* (Figure 4) all studied genes exhibited a significant increase in abundance at point B compared to the control, with the exception of *mr* which displayed a significant upregulation only at point C. In no case was there any significant differences in mRNA abundance of the studied genes between points B, C and D, and in all cases the expression of genes observed at point E was comparable to that measured at point A, with no statistical differences arising. Although the same genes were investigated in the brain (data not shown) no significant differences were observed in their expression.

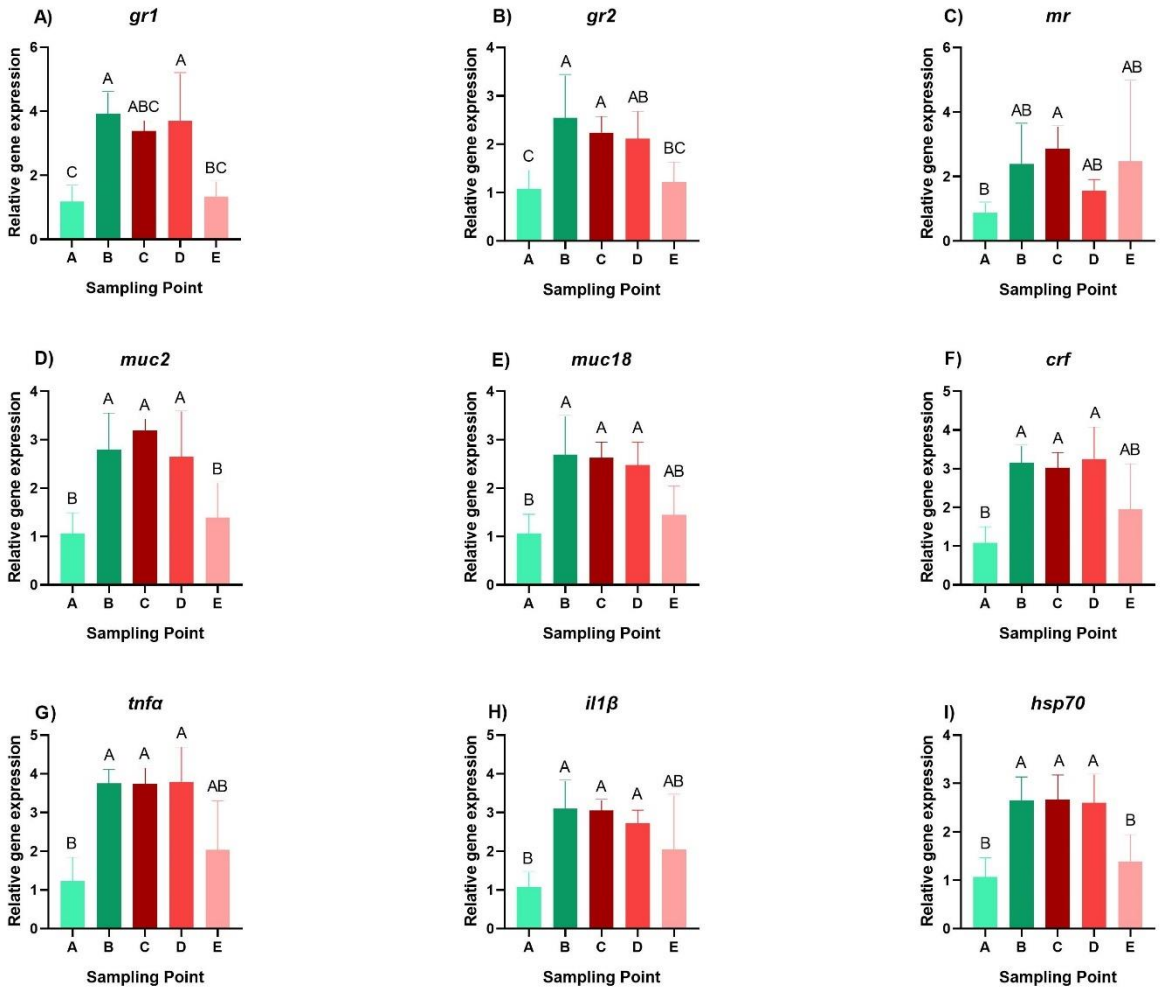


Figure 4. Relative gene expression analysed in gills of *Salmo salar* during a centrifugal pumping episode in a commercial recirculated aquaculture system (RAS). A: 48h before exposure; B: during transfer; C: 1hps; D: 24hps; E: 7dps. Different letters above columns indicate significant differences between sampling points (P<0.05).

4. Discussion

Few studies have been carried out in industrial salmon facilities, following the exact same procedures used for commercial fishes. Therefore, the present study is not a laboratory or pilot experiment closely simulating the farm situation, but a direct measurement during an actual salmon production process. This provides more accurate data on the effects of stress linked to industrial husbandry practices during commercial fish production.

4.1. Physiological indicators in plasma

The observed increase in plasma cortisol levels during the stress and 1hps indicates the triggering of the primary stress response (Schreck & Tort, 2016) as expected after an acute stress, corroborating previous findings in salmonids (for instance, see García-Meilán et al., 2022). Although previous

research conducted on the direct impact of pumping on *S. salar* has reported contradictory results, with some describing no significant changes in plasma cortisol levels (Davis et al., 1994; Espmark et al., 2016; Merkin et al., 2010; Nomura et al., 2009), there is evidence that this practice might significantly increase circulating cortisol levels (Iversen et al., 2005). In addition, available literature describes significant increases in plasma cortisol as a response to crowding (Einarsdóttir & Nilssen, 1996; Merkin et al., 2010; Veiseth-Kent et al., 2010). Therefore, the variation described in the current study might be attributed to the pre-transfer crowding of the fish in the commercial tanks. However, the aforementioned studies have focused on the impact of vacuum-pumping and turbine pumping, while the transfer event hereby investigated was performed through centrifugal pumping. Thus, differences between the present results and past studies might also be attributed to these methodology-related variations, the experimental vs. industrial environment, as well as the pre-stress conditions. Furthermore, differences between reported tendencies in plasma cortisol fluctuations in past research might arise from dissimilarities in pumping design (*e.g.* hose dimensions, velocity). Other possible explanations for the disagreements between studies regarding cortisol behaviour are differences in analytical methods and sampling techniques (including time of day of sampling). As expected, these measurements returned to control levels by sampling point D (*i.e.* less than 24 hps). This is in accordance with previously published results indicating a return to basal cortisol levels in the plasma of *S. salar* 6 h after pumping (Iversen et al., 2005). Nonetheless, it is noteworthy that, although significantly different, the values of plasma cortisol amongst all groups are situated between 0.05 and 15 ng/ml, even in the groups B and C, that display the highest values. In contrast, it has been reported that basal levels of circulating cortisol for *S. salar* smolt oscillate around 4 ng/mL and that, following a stressful episode, these might rise to considerably higher levels than those observed in the present study (Ding et al., 2023; Fast et al., 2008). Although differences in analytical methods might explain, to some extent, the considerable differences in circulating cortisol levels detected, the technique hereby employed has been previously validated for salmonids (Franco-Martinez et al., 2019), and it is, thus, highly unlikely that such differences arise solely from the quantification method. Instead, the low values shown by the present results may indicate that the HPI axis has not been greatly activated or that the HPI axis has been somehow desensitized due to feedback mechanisms, given that, in many fish species, levels below 10 ng/ml may be considered basal levels (Baßmann et al., 2023; Odhiambo et al., 2020; Ruane & Komen, 2003). Therefore, a reasonable explanation is the possibility that these fish show a lower HPI-axis responsiveness due to previous prolonged and regular exposure to stressors common in RAS, such as overcrowding, noise, or constant light (Calabrese et al., 2017; Hang et al., 2021), thus possibly either exhausting the axis, or reducing its ability to show a more powerful cortisol release response. Similar patterns of inhibited stress axes as a consequence of chronic exposure to stress have been described in birds, with both decreased basal cortisol levels and lower increases in this parameter following novel acute stressors (Rich & Romero, 2005). Similarly, in humans exposed to chronic stress and/or experiencing PTSD, lower cortisol levels and a hindered responsiveness of the hypothalamic-pituitary-adrenal (HPA; analogous to the HPI axis in fish) axis have been reported (Lehrner et al., 2016). Nonetheless, further research and additional data is needed to confirm this hypothesis in fish.

The observed initial decrease in plasmatic glucose levels agrees with published literature describing a significant decrease in circulating glucose levels shortly after a crowding event, followed by a significant increase in this parameter, both relative to control values (Veiseth et al., 2006), with the latter indicating the successful triggering of the secondary stress response. In addition, a return to

basal levels of plasmatic glucose after a stress-induced increase has been reported to take over 24 h (Sandodden et al., 2001), further agreeing with the present results. This is also in accordance with the low but significant increase in cortisol, which would induce the catabolic increase of plasmatic glucose levels (Schreck & Tort, 2016).

Past studies investigating liver damage resulting from pumping in fish have reported species-specific variations in certain indicators (*e.g.* ALT, AST). Xu et al. (2017) described significant differences in the fluctuations of biochemical parameters in the serum of various cyprinid fishes, including these liver health biomarkers, with some species displaying significant increases in circulating levels of ALT and AST following pumping, and others not showing any differences. The present study showed a significant increase in AST 1h after the procedure with a subsequent return to control levels after 24h. On the other hand, ALT displayed a considerable increase after transfer, although this remained below the threshold for significance. Nonetheless, at 24 hps, the measured levels of plasmatic ALT significantly decreased compared to the control. Reportedly, prolonged truck transport induces a significant reduction in plasmatic ALT, coupled with a significant increase in AST, following the same patterns as observed in the present study (Dobšíková et al., 2009). ADA has been demonstrated to play an important role in the modulation of inflammatory and homeostatic processes in fish following exposure to stressful events (Piato et al., 2011; Zimmermann et al., 2016). It has been reported that shortly after an acute stress by direct exposure to air, plasmatic levels of ADA display a significant increase, returning to control levels 24 hps (Franco-Martinez et al., 2022), mimicking the results from the present study over the same period. Since crowding and transfer stress finished after point B, the progressive return of indicators back to control values, as well as the recovery of liver homeostasis was expected.

4.2. Molecular indicators in organs

Gills are a commonly targeted tissue when investigating the stress response in fish given their degree of exposure to external stressors and their fragility (Harper & Wolf, 2009). Given this direct contact with the surrounding environment, it is not surprising that this organ displays the highest degree of variation in terms of expression of relevant stress genes. Three distinct sets of genes (*i.e.* inflammation-related, mucins and endocrine receptors) were investigated in the present study. The upregulation of *tnfα*, *il1β* and *hsp70* may indicate the triggering of responses to cellular stress, promoting inflammatory and cell repair processes (Østevik et al., 2022). It has been suggested that these three genes might display significant upregulations as a response to mechanical stressors, such as handling (Idriss & Naismith, 2000; Kaneko et al., 2019; Østevik et al., 2022). The results suggest that the potentially pro-inflammatory mechanisms induced by the pumping episode were halted over the course of a week following the stress. Therefore a trade-off between resources directed to face stress versus immunity could be applied to our case (Segerstrom, 2007).

In *S. salar*, as in mammals, the expression of mucins-related genes, such as *muc2*, has been described to be stimulated by *tnfα* (Ahn et al., 2005; Hoem & Tveten, 2023), and similar patterns can be observed in the present study. Mucins can be classified into large secreted gel-forming mucins (*e.g. muc2*), and membrane-bound mucins (*e.g. muc18*), displaying both shared and distinctive properties, and playing essential roles in the protection of an organism against pathogens, environmental fluctuations and physical harm (Pérez-Sánchez et al., 2013; Vatsos et al., 2010). Although the mechanisms influencing the expression of mucin-encoding genes in fish are still poorly

understood, previous research has shown that branchial *muc2* is significantly upregulated following a handling episode (Sveen et al., 2017), as observed during the fish pumping hereby investigated. On the other hand, *muc18* is reportedly downregulated in the gills of fish exposed to amoebic gill disease (Marcos-López et al., 2018), but significantly upregulated after transfer to sea by pumping (Hoem & Tveten, 2023). Similarly to what was observed with the genes involved in homeostasis and inflammation, the mucin-coding genes investigated here returned to control values 7 dps. Overall, it appears as if mucin-coding genes are relevant and suitable for the determination of stress levels in *S. salar* following centrifugal pumping.

It has been reported that chasing stress induces a significant upregulation of endocrine receptors *gr1*, *gr2*, *mr*, and *crf* in the pituitary gland of *S. salar*, but does not lead to any significant changes in the expression of these genes in the brain (Madaro et al., 2016), indicating a clear organ-dependent response to such physical stressor. Furthermore, a previous study conducted on the response to air-exposure and handling in *Sparus aurata* suggests that these stressors lead to the modulation of *gr1*, *mr*, and *il1β* in the gills, but no in the skin (Vallejos-Vidal et al., 2022), further corroborating the hypothesis that organs differ in their response to similar stressors, particularly when the tissues are involved in systemic or local regulation. Moreover, the same study described a lack of significant alterations in *gr2*, *tnfα*, or *hsp70* in gills, all of which have been upregulated in the present study, hinting towards species-specific response patterns to somehow similar stressors. The present results show closely related patterns of modulation between the four stress-related genes investigated in the gills (*i.e. gr1*, *gr2*, *mr*, and *crf*), agreeing with previous data published on the expression of these in a salmonid species (Teles et al., 2013).

In the head kidney, the investigated molecular endpoints display completely different patterns of modulation following the stress when compared to the gills. This is not surprising, given that previous literature has described differing response dynamics to the same stressor between organs. In the head kidney, Martorell Ribera et al., (2020) reported significant alterations in *il1β* only 24 h following a direct *in-vitro* insertion of cortisol. In the present study, the significant downregulation of this gene was only visible at 7 dps, although it is possible that it occurred between 1 and 7 days and wasn't detected due to the absence of sampling points during this period. It might also be that, as the present study is *in-vivo*, the interaction between cortisol and the head kidney cells is delayed compared to an *in-vitro* due to the increased complexity of the matrix and the consequent systemic interrelations. Furthermore, the authors investigated the *in-vivo* impact of a non-virulent injection on the mRNA abundance of *il1β* in the head-kidney, and described a significant downregulation 72 h post injection (Martorell Ribera et al., 2020). This is potentially more representative of the present results, given the handling and *in-vivo* nature of the experiment. As mentioned above, the present study did not include a sampling point at 72h post stress, making the significant decrease in the abundance of this transcript only visible at 7 days, even though it is likely to have occurred earlier on. Pelusio et al. (2022) has stated that confinement and crowding induces a significant downregulation of *gr1* in the head kidney, as the present results display. This trend has also been observed with different types of stressors, including bacterial infections (Pelusio et al., 2022). This might be explained by *gr*'s expression not being solely influenced by cortisol levels, but also depending on immune-endocrine interactions through the stimulation of immune processes. It has also been reported that this gene is often downregulated during exposure to chronic stress, acting as a regulatory mechanism of the stress response (Peixoto et al., 2024). Furthermore, previous studies

show that cortisol-dependent modulation differs amongst corticoid receptors (*i.e. gr1, gr2, mr*) in the head kidney of salmonids (Teles et al., 2013), as observed in the present study. In rainbow trout, it was reported that acute handling and hypoxic stress induced a significant downregulation of *hsp70* in the head kidney, without indications of recovering basal values even 24hps (García-Meilán et al., 2022). Furthermore, it appears that, in percids, handling stress regulates the expression of certain genes such as *hsp70* even 144hps, in a clear temperature-dependent manner (Eissa et al., 2017). Therefore, it cannot be discarded that the mRNA abundance of the genes hereby investigated is still affected by the pumping episode 7dps. At this point, it should be reiterated that the cortisol response observed in the present study is weak, with low amounts of circulating cortisol. Moreover, not only stress-associated genes are moderately expressed, but responses of the stress-regulatory organs like the head kidney show low responsiveness. Therefore, this reinforces the hypothesis of the reduced reactivity of the overall HPI axis under the conditions of the investigated RAS.

The steroidogenic acute regulatory protein (StAR) is responsible for the transport of cholesterol into inner cellular membranes, playing a crucial role in steroidogenesis, and therefore in the onset of the stress response in vertebrates (Clark et al., 1994). Expression of *star* has been reported as displaying significant decreases in *S. aurata* head kidney cells following *in-vitro* exposure to ACTH (Castillo et al., 2008). However, an *in-vivo* experiment performed by the same authors involving netting stress in *S. aurata* resulted in no significant differences in the expression of *star* in the head kidney. It is worth noting that *star* is activated only under high levels of circulating cortisol (Geslin & Auperin, 2004), although other mechanisms are probably at play when regulating the expression of this gene (Castillo et al., 2008). It is possible that the fluctuations in mRNA abundance of *star* observed in the present study are partly modulated by ACTH levels. However, this parameter was not analysed for in the collected samples. All in all, this data further indicates the previously suggested decreased responsiveness of the HPI axis.

5. Conclusions

The data obtained from the present case study indicate a significant activation of the primary and subsequent secondary stress responses, characterised by the rise in circulating cortisol levels and a slightly delayed increase in plasmatic glucose, respectively (Schreck & Tort, 2016). This is further corroborated by the upregulation of *gr1, gr2, mr*, and *crf* in the gills, which are recognised indicators of the generalised stress response in fishes (Faught et al., 2016). It also seems as if centrifugal pumping had an impact on the immune status of the fish, inducing inflammation (*i.e.* as evidenced by the upregulation of *tnfα* and *il1β*), and slightly triggered antioxidant defences and cellular repair processes.

The modulation of the investigated genes clearly follows different patterns in gills, head kidney, and brain, which might provide further evidence for the occurrence of negative feedback loops or compensatory mechanisms from the central to the peripheral system in order to prevent abrupt dysregulations with potentially strong repercussions on the overall allostatic capacity of the fish. However, additional research is required to further investigate this hypothesis. As previously mentioned, although information available on the impact of centrifugal pumping on the stress status of fish is lacking, the present results suggest that the primary response has been moderately activated, particularly at the peripheral level, triggering inflammatory mechanisms and antioxidant processes, as well as inducing the significant upregulation of mucin-coding genes. According to the present data, the fish manage to cope with the transfer and return to control values in most of the

investigated parameters around 7 days post stress, indicating that, although the pumping event is stressful to the fish, it is likely not maladaptive. However, the allocation of energetic resources to the activation of the primary and secondary stress responses, as well as allostatic processes, might translate into an increased susceptibility of the fish to additional insults (Sampaio & Freire, 2016; Sandodden et al., 2001). Therefore, ensuring stability in the rearing environment during those days might be critical for the prevention of elevated mortality events. Nonetheless, some of the studied endpoints (*e.g.* mRNA abundance of relevant genes in the head kidney and plasmatic ALT) remained significantly altered even 7dps, suggesting that homeostasis was still not attained at this point, and that additional time is required for the fish to fully recover from the transfer.

Discrepancies between the present results and previously published data might arise mainly from variations in fish production conditions, methodology, including stressor intensity (*e.g.* hormesis; Schreck, 2010), as well as the studied species and specific organs. Furthermore, it is important to remark again that the present study was conducted in a fully commercial setting and is therefore highly representative of what occurs in a *S. salar* RAS, whereas most of the available literature deals with laboratory experiments, mimicking, to the best of their abilities, industrial conditions. Indeed, it is possible that the prolonged exposure to common stressors in RAS (*e.g.* crowding, soundscape, constant light) might induce a decrease in responsiveness of the HPI axis to subsequent insults, without significantly harming the fish, as, although the response is considerably lower than expected, homeostasis was partly recovered by the end of the experimental period. It is also worth considering that the relatively low increase in circulating cortisol levels hereby observed might translate into a milder immunosuppression, compared to fish which would have displayed a stronger increase. Further research is necessary to confirm this hypothesis, and it would be of great interest to compare fish originating from the same batch being reared in non-recirculating aquaculture settings, and in RAS. Moreover, it could prove interesting to follow the same batch of fish throughout the production cycle in a commercial RAS, investigating the response of individuals at each significantly stressful event (*e.g.* during and after each transfer episode) to determine whether it decreases over time as the fish are repeatedly exposed to additional stressors.

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GENE NAME	SYMBOL	SEQUENCE		ACCESSION NUMBER (GENBANK)	SOURCE (ARTICLE)
		Forward	Reverse		
ACTIN BETA	*βact*	CCATCCAGGCAGTGTTGT	CGGAGTCCATGACGATACC	AF012125	https://doi.org/10.1038/s41598-018-32019-8
18S RIBOSOMAL RNA	*18s*	TACAGTGAAACTGCGAATGG	GCATGGGTTTTGGGTCTG	AJ427629	https://doi.org/10.1016/j.aquaculture.2011.05.038
ELONGATION FACTOR-1 ALPHA	*ef1α*	TGCCCCTCCAGGATGTCTAC	CACGGCCCACAGGTACTG	XM_014141923.1	https://doi.org/10.1007/s10499-023-01084-w
GLUCOCORTICOID RECEPTOR 1	*gr1*	ACGACGATGGAGCCGAAC	ATGGCTTTGAGCAGGGATAG	AF209873.1	10.1242/jeb.120535
GLUCOCORTICOID RECEPTOR 2	*gr2*	TGGTGGGCTGCTGGATTTCTGC	CTCCCTGTCTCCCTCTGTCA	XM_014213196.1	https://doi.org/10.1016/j.aquaculture.2021.737882
MINERALOCORTICOID RECEPTOR	*mr*	TCGTCCACAGCCAAAGTGTG	TTCTTCCGGCACACAGGTAG	AF209873	10.1242/jeb.120535
CORTICOTROPIN RELEASING FACTOR	*crf*	AACCAGCTCGACGACTCGATGG	GCTATGGGCTTGTTGCTGTAACTG	BT057824	10.1242/jeb.120535
HEAT SHOCK PROTEIN 70	*hsp70*	CCTGCCTACTTCAACGATTCACAGAGACA	CCAGCGATCACTCCAGCGTCCTTA	NM_001141684	https://doi.org/10.1038/s41598-018-32019-8
INTERLEUKIN 1 BETA	*il1β*	GTATCCCATCACCCCATCAC	GCAAGAAGTTGAGCAGGTCC	NM001123582	https://doi.org/10.1016/j.fsi.2010.11.017
MUCIN 18	*muc18*	AAGAGCAGCGAGGTGGTG	TCCGTTGACTTGGCAGATGA	XM_014213637	https://doi.org/10.1038/s41598-018-32019-8
MUCIN 2	*muc2*	CGACTGCCACAAAGCCATTAGG	GCGTGTTGCTGCGTGTCTT	XM_014183074	https://doi.org/10.1038/s41598-018-32019-8
STEROIDOGENIC ACUTE REGULATORY PROTEIN	*star*	AGGATGGATGGACCACTGAG	TTCTCCCATCTGCTCCATGT	DQ415678.1	10.1242/jeb.120535
TUMOR NECROSIS FACTOR ALPHA	*tnf-a*	GACAACTGGCGACATGGAGA	ATGCTGACACCAGGCAAAGA	NM_001123589.1	https://doi.org/10.1016/j.fsi.2023.108887

Supplementary table 1: Name, symbol, sequence (forward and reverse) and accession number (Genbank) of the Atlantic salmon (*S. salar*) primers used for gene expression analysis through RT-qPCR.

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Discussion and Conclusions

8

8. Discussion

8.1 General discussion

Fish in RAS are exposed to a plethora of stressors, whether environmental, physical, chemical, or social. Depending on the duration and the intensity of the stressing events, these might have maladaptive consequences, translating into impaired performance and affecting the productivity of the aquaculture industry, with both economic and societal implications. Identifying causative agents of stress that compromise fish welfare is key to managing the issue, enhance the quality of life of the cultured organisms, and avoid unnecessary losses.

The first part of the present thesis attempted to elucidate the impact of CEC in fish of particular scientific or economic relevance (Blanco et al., 2018). PSNPs were selected, as it is the most commonly employed polymer in studies investigating the toxicity of NPs, with over 90% of available literature focusing on this compound (Eliso et al., 2024; Pelegrini et al., 2023). On the other hand, GEM was chosen given its higher prevalence compared to other lipid regulating agents (Zhang et al., 2020), and its worldwide ubiquity in aquatic environments. As CECs, including NPs and GEM have been detected in city waters, with particularly high concentrations measured in Catalan river systems (Domínguez-García et al., 2024), they are expected to be present in aquaculture systems, including RAS.

An increasing number of scientific literature is available on the impact of these CECs on aquatic organisms. Experimental exposures to GEM have been conducted in a variety of fish species, and the results, although sometimes divergent, often conclude that this contaminant acts as an endocrine disruptor, leading to a disrupted lipid metabolism, and impaired gametogenesis as one of its consequences, with potential effects on both reproductive success and the performance of offspring (Al-Habsi et al., 2016; Fraz et al., 2019; Galus et al., 2014; Skolness et al., 2012). Given the fact that GEM is a single compound, it is reasonable to speculate that the punctual discrepancies between results obtained in different studies might be attributed to methodological variations, such as exposure route, dose and duration, as well as the characteristics of the studied organisms *e.g.* marine vs freshwater, species, life stage, sex; Blonç, et al., 2023).

A similar but more complex scenario occurs with NPs. Firstly, NPs denotes not a single compound, but a large variety of plastic polymers, with distinctive and changing properties (Ivleva, 2021). Indeed, the myriads of factors to consider and the versatility of these compounds with regards to shape, size, charge, density, and affinity to co-contaminants, amongst others (Shi et al., 2024), makes it extremely difficult to draw meaningful, generalised, conclusions regarding their ecotoxicity, and to fully grasp their impact on fish.

Moreover, NPs ecotoxicity has become a "*hot topic*" in research, with a considerable increase in the number of studies conducted on this field in the last decade, and resulting in an overwhelming quantity of data (Atugoda et al., 2023; Pelegrini et al., 2023; Shi et al., 2024). This, in combination with the increasing habit of "*Publish or perish*" in academic culture, which may lead to unethical research practices, which, together with the significant rise of the so-called "*Predatory journals*", makes it extremely time-consuming and complicated to filter reliable or thoroughly obtained results from the available literature (Moosa, 2024; Rawat & Meena, 2014). It is particularly relevant in this context to employ thorough experimental designs, standardised analytical methods, and clear descriptions of the methodology in scientific publications, to optimise the comparison between studies. Moreover, it is of utmost importance to investigate a vast variety of endpoints, and to recognise the inherent limitations of laboratory research as to avoid drawing inaccurate conclusions.

Given the comprehensive discussions of individual results in each chapter, this section of the thesis focuses on comparing the responses of the studied species to the different selected stressors trying to detect patterns between responses

Study 1 (see Blonç et al., 2023) and Study 2 (see Blonç et al., 2023) of this thesis focused on exposing *C. auratus* to different waterborne CECs at environmentally relevant concentrations. Both studies complement published research following similar experimental designs, adding to already existing knowledge and allowing for some comparison between the ecotoxicological impact of PSNPs and GEM in the same model organism. These prolonged exposures indicated some similarities and discrepancies in the toxicity of these CECs.

Firstly, the quantification of GEM through liquid chromatography coupled to high-resolution mass spectrometry (LC-HRMS) as performed in the present thesis indicated the accumulation of this compound at similar rates in both muscle and liver samples of fish exposed to spiked concentrations (*i.e.* higher than environmental values; HC). Furthermore, GEM was detected in samples both from the control group and the group exposed to environmentally relevant concentrations (*i.e.* Ctrl and LC), although below quantification limits. Moreover, this compound was identified and quantified in blood plasma of *C. auratus* using liquid chromatography tandem mass spectrometry (LC-MS/MS; Mimeault et al., 2005), with results indicating bioconcentration of GEM in all experimental groups, including control groups and groups exposed to environmentally relevant and spiked concentrations. Similarly, the quantitative analysis of PSNPs through LC-HRMS in goldfish tissue following a prolonged exposure to environmentally relevant concentrations showed that these particles were present in liver and muscle samples of both exposed, and control individuals, with the latter being below quantification limits (Brandts et al., 2022).

Altogether, these results corroborate the presence of these emerging contaminants in local waters, as they were detected in the experimental media of control groups, and hint towards

the global occurrence of both compounds, including in aquaculture facilities and RAS, as observed with other NP polymers and CECs (Blonç, et al., 2023; Martins et al., 2009).

However, contrarily to what was observed with GEM, PSNPs appeared to show organ-specific internalisation and retention rates, with liver having significantly higher concentrations of PSNPs compared to muscle (Brandts et al., 2022). Moreover, PSNPs were not detected in the blood of *C. auratus*. These differences are further emphasised by the lack of effect of PSNPs on the haematological profile of goldfish (Brandts et al., 2022) compared to GEM, significantly altering haemoglobin levels in goldfish exposed to spiked concentrations (Study 2 of this thesis: Blonç et al., 2023).

GEM appeared to have more severe effects on the biochemical profile of goldfish than PSNPs. Indeed, GEM significantly decreased testosterone (Mimeault et al., 2005), cortisol and glucose (Study 2: Blonç et al., 2023) levels in the plasma of individuals exposed to both environmentally relevant, and spiked concentrations, hinting towards a disrupted lipid metabolism. However, important molecular indicators of lipid metabolism in the liver (*e.g. stAr, ppara, pparγ*) remained unaltered (Study 2: Blonç et al., 2023; Mimeault et al., 2005), suggesting that the observed decreases in plasmatic metabolites might instead reflect the relocation of energetic resources in face of a prolonged insult (Schreck & Tort, 2016). On the other hand, these common indicators of physiological stress were not significantly altered by PSNPs (Brandts et al., 2022). Nonetheless, both CECs triggered, to some extent, antioxidant responses in this fish. This is evidenced by the significant increase in total antioxidant capacity (TAC) measured after exposure to either PSNPs or spiked concentrations of GEM. In the case of GEM, significant increases were also observed in the mRNA abundance of hepatic molecular markers (*e.g. gpx, gst*), as well as enzymatic activity (*i.e.* catalase, glutathione peroxidase) indicative of antioxidant defences although mostly at spiked exposure concentrations (Study 2: Blonç et al., 2023; Mimeault et al., 2006).

Overall, it appears that at, environmentally relevant concentrations, GEM had little effect on the health status of *C. auratus*, as the majority of changes were seen after exposure to spiked concentrations. However, it is important to note that all individuals considered for this study were adults, and that the toxicity of GEM at environmentally relevant concentrations might be considerably greater in larvae or sub-adults (Henriques et al., 2016).

Regarding PSNPs, only an environmentally relevant concentration was selected, and it appeared to elicit some sort of stress response in goldfish. Thus, it could be suggested that, in the current scenario, and in adults of a freshwater species such as *C. auratus,* PSNPs are more worrisome contaminants compared to lipid regulators, particularly GEM. Although no effects of either of these compounds on fish biometrics were evidenced by the obtained results, it could be expected that longer exposures, or exposures from a younger age would negatively impact these parameters. Moreover, the concentrations of the studied CECs in RAS are likely greater than those measured in natural environments due to the minimal water exchange and the inability of the filtration mechanisms to remove them from the system,

leading to their accumulation in RAS water, and having potentially more adverse effects than those hereby observed.

The toxicity of PSNPs also presented similarities and differences between the freshwater goldfish and marine gilthead seabream studied in the present thesis, and these might be explained by a variety of factors. Firstly, and perhaps most importantly, the intrinsic behaviour of PSNPs in saltwater due to their physio-chemical properties, led to the aggregation of the particles. Thus, the expected exposure to PSNPs of around 40nm in size turned out to be an exposure to particles of around 550nm (Study 3). Secondly, the more robust experimental design and more comprehensive set of analytical methods used in the experiment with the gilthead seabream provides significantly more information than what was obtained from goldfish. These factors make it delicate to compare the results obtained from both studies. Overall, it can be observed that, under environmentally relevant concentrations of PSNPs, gilthead seabream displayed less changes than goldfish. In both cases, the uptake and retention of PSNPs displayed strong tissue-specific tendencies. In neither case did PSNPs induce significant changes in the haematological profiles of the studied fishes, nor did they lead to differences in morphological parameters of importance to the aquaculture sector, such as weight, length and hepatosomatic index (Brandts et al., 2022). However, Brandts and colleagues (2022) reported a significantly increased number of erythrocyte nuclear anomalies (ENAs), a marker of genotoxicity, in goldfish, an effect that was not observed in the gilthead seabream. Moreover, *S. aurata* displayed altered metabolomic profiles after exposure to PSNPs at both environmentally relevant and spiked concentrations, indicating a potentially unbalanced or transiently altered allocation of energetic resources. The less severe impact of NPs in the gilthead seabream might be partly explained by the size of the particles in the experimental media, as it has been widely reported that smaller particles have more deleterious effects on aquatic biota (Liu et al., 2024). Differences in response to environmental pollutants can also be expected between freshwater and marine organisms. The variability of observed differences between investigated endpoints further highlights the idea that single-biomarker approaches are insufficient to understand the impact of CECs in aquatic organisms, and might lead to erroneous conclusions, significantly affecting the credibility of the obtained results, and of scientific approaches in general.

The second part of the thesis consisted of two case studies undertaken in commercial RAS facilities in Europe, studies that are not often performed in these conditions but rather in laboratory or pilot-scale set-ups

Study 4 confirms the hypothesis that NPs of distinctive natures occur in commercial RAS (in Spain) and are internalised by the reared fish. The results indicate that many of the identified compounds matching the composition of different RAS compartments (*e.g.* pipes, biofilter media, coating). Although unexpected, the absence of PS amongst the detected polymers could be explained by the small sample size obtained for this study, mainly due to economic

restraints. However, given the omnipresence of PS in natural and urban waters, it is likely that this compound is also present within RAS. Moreover, the analysed water displayed different NPs compositions between inlet and outlet water samples, with outlet water containing a greater diversity of NPs. Thus, NPs contamination in RAS potentially originates from both endogenous and exogenous sources. Viable, eco-friendly, plastic-free alternatives for some of these components are already subject to studies (Mnyoro et al., 2022), indicating that the issue of NP pollution in RAS might be addressed at the source without compromising the efficiency of the system. Given the diversity in observed effects (or absence of effects) of experimental exposure to NPs, it is highly difficult to predict the real impact of NPs pollution on aquatic ecosystems. Thus, endeavours should prioritise the design of alternative materials and mitigation measures to minimise the future input of NPs into the environment and reduce the already existing pollution.

Study 5 provides strong evidence that intrinsic factors in RAS are significantly influencing the HPI axis responsiveness in *Salmo salar*, as shown by the unexpectedly low physiological response of this species to the investigated stressor. Given the fact that the measured values of circulating cortisol during the centrifugal pumping episode equalled what is expected for unstressed *S. salar*, this study showcases the importance of having internal controls (*i.e.* baseline values for a given population) for all assessed endpoints in order to appropriately evaluate the impact of stressing events. Although, as the name "case study" indicates, this might be an isolate scenario, it should serve as motivation to further investigate the dynamics of the HPI axis efficiency throughout the production cycle to try to identify the potential factors causing this. It would be unwise to compare the impact of centrifugal pumping to that of CECs due to the completely divergent nature of the stressors. Nonetheless, this study highlights the importance of conducting studies under industrial conditions. Although such research inherently has several limitations (*e.g.* a myriad of factors, some of them that cannot be controlled), it provides interesting insights into what happens under real-life scenarios. It is expected that, under chronic stress conditions induced by RAS settings, such as soundscape, 24h light photoperiod, or high stocking densities, the impact of any additional stressor, as could be CECs, would be significantly different to what is observed under laboratory conditions.

8.2 Some considerations for present and future research:

Both laboratory and field experiments were conducted for this thesis, each presenting a series of advantages and limitations. On one hand, the controlled conditions offered by laboratory-based research allow for increased repeatability of the experiments, and to isolate specific factors to focus the investigation, providing insightful data, although with limited representativeness of real-life situations. On the other hand, field-based research provides information on more realistic scenarios, although the possible interference from uncontrolled fluctuations of both biotic and abiotic factors should be taken into account. Moreover, in studies investigating such a small portion of the whole population, sampling

bias resulting from differences in individual personality traits (*e.g.* boldness; Biro & Dingemanse, 2009) and fitness (*i.e.* ability to escape) is likely to affect the obtained results, even if sampling is conducted randomly.

Regarding research on CEC toxicity, future endeavours should investigate the combined impact of CECs with relevant environmental factors (*e.g.* fluctuations in temperature, dissolved oxygen concentration, co-occurrence of CECs or pathogenic agents), employing a multi-biomarker approach, and, when possible, prioritising little-to-non-invasive assays. In parallel, it is essential to explore alternative materials to reduce the presence of plastic components in RAS, as well as mitigation measures to alleviate NPs contamination.

Further research should also be undertaken to deepen the understanding on the impact of RAS settings on fish stress and welfare. For instance, investigating the impact of biofilter efficiency and microbial communities would be of great relevance to RAS managers. Similarly, comparing the responsiveness of the HPI axis of fish cultured under slightly different conditions (*e.g.* photoperiod, density) in RAS, and in traditional aquaculture systems (*e.g.* flowthrough systems), throughout the production cycle, and its impact on the fish's ability to cope with subsequent stressors, could allow to modify management practices accordingly. One physiological aspect that also deserves further investigation is the effect of permanent stressors on the ability of fish to overcome further challenges. The results found in Study 5, where cortisol levels showed a significant low value, opens interesting new questions on the function of HPI axis under these situations and on the sensitivity, fragility, and resilience capacity of cultured fish. Moreover, the physiological and biochemical mechanisms behind these altered responses deserves further research.

The use of little-to-non-invasive matrices to evaluate fish stress and welfare needs to be further investigated. Although biochemical analyses or metabolomic profiling of the plasma seem like promising alternatives, assessing the efficiency of eRNA, water cortisol and behaviour in indicating the welfare status of fish should be prioritised.

8.3 Conclusions:

- Fish reared in recirculated aquaculture systems are potentially subjected to a wide variety of insults, both endogenous and exogenous to the system, triggering primary and secondary stress responses. It appears that RAS characteristics interfere with fishes' ability to respond to physical stressors, significantly altering the sensitivity of the HPI axis.
- Some contaminants of emerging concern, such as NPs, originating from both external and internal sources occur in RAS and accumulate in fish tissues. Evidence obtained from laboratory experiments suggests no significant impact on performance (*e.g.* growth) of fish after 28-day challenges to environmental concentrations, although it can be expected that longer exposures have more

pronounced effects with potential implications for the aquaculture sector, in a species-specific manner.

- The toxicity of NPs on aquatic organisms depends upon a vast range of factors, making it extremely difficult to predict their impact on the aquaculture industry. Efforts should focus on measures to prevent and mitigate NPs pollution.
- Multi-biomarker, and potentially multi-omics, approaches are essential to accurately elucidating the response of fish to different stressors. Little-invasive matrices such as blood plasma provide insightful results, depending on the sensitivity of the equipment. However, depending on the assay, sample size might be a limitation. Thus, particular attention should be given to non-invasive assays such as water-based indicators, including water cortisol and eRNA, and behaviour.
- Achieving a comprehensive understanding of the impact of a particular stressor on fish is possible through a combination of -omics and non-invasive methods, although some of these are still under development. The considerable quantity of data obtained through these assays might provide contradictory information and requires thorough mining and analysis. The possibility of employing artificial intelligence to this end should be explored.

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