UNIVERSIDADE DE LISBOA
FACULDADE DE CIÊNCIAS
DEPARTAMENTO DE BIOLOGIA ANIMAL

Investigating the impacts of microplastics and venlafaxine contamination on fish physiology and behavior

Neuza Sofia Antunes Dias Nobre Fonseca

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Ana Rita Lopes

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Abstract

The continuous increase in plastic production and their degradation into microplastics (MPs), coupled with the growing prescription and consumption of antidepressants, has raised concerns about their isolated and cumulative impacts on marine organisms. This study aimed to evaluate the effects of MPs and venlafaxine (VFX), a selective serotonin and noradrenaline reuptake inhibitor (SNRI) antidepressant, on the body condition, metabolism and behavior of juvenile *Sparus aurata*. Fish were weighed, measured and their Fulton index was calculated. Oxygen consumption was measured, following the 2-week exposure period, to assess metabolic impacts, while focal observations of swimming activity and agonistic behaviors were made to determine behavioral changes during the exposure and elimination periods. A significant decrease in the Fulton index was observed between the control and the MP treatment groups during the elimination period, and between periods within the MP treatment. A reduction in weight was also noted in the control group between periods. Juveniles exposed to MPs or VFX spent more time stationary compared to the control, with VFX exposure – both isolated and in combination with MPs – reducing aggressiveness. Oxygen consumption decreased in all treatments, although not statistically significant. The results suggest a possible interaction between MPs and VFX that could potentially alter their individual effects, but only when considering aggressive behaviors. With this in mind, the precise consequences and mechanisms of this possible interaction remain unclear, highlighting the need for further research to better understand and mitigate the impacts of these contaminants on marine life.

Keywords: *Sparus aurata*, microplastics, venlafaxine, metabolism, behavior

Resumo

O aumento contínuo da produção de plásticos e a sua subsequente degradação em microplásticos (MPs), juntamente com o uso crescente de fármacos como antidepressivos, tem levado a uma preocupação crescente em relação aos seus efeitos nos ecossistemas marinhos. Os MP são partículas de plástico com menos de 5 mm de diâmetro, que entram no ambiente marinho através de várias fontes, como as estações de tratamento de águas residuais, deposição atmosférica, e má gestão de resíduos. Por sua vez, os antidepressivos, como a venlafaxina (VFX), um antidepressivo inibidor seletivo da recaptação da serotonina e da noradrenalina (SNRI), entram nos sistemas aquáticos devido à excreção pelos pacientes que os consomem ou à eliminação inadequada de medicamentos. Estes compostos, muitas vezes persistentes, não são completamente removidos nas estações de tratamento de águas residuais, contribuindo para a sua acumulação nos ecossistemas aquáticos.

A interação entre MPs e compostos farmacêuticos, como a VFX, no ambiente marinho, tem levantado questões sobre os seus efeitos cumulativos nos organismos marinhos. Embora existam estudos sobre os efeitos isolados destes contaminantes, pouca atenção tem sido dada aos seus efeitos combinados em organismos marinhos. Este estudo teve como objetivo principal investigar os efeitos isolados e combinados dos MPs e da VFX na condição corporal, metabolismo e no comportamento de juvenis da espécie *Sparus aurata*, uma espécie com grande importância comercial, tanto na pesca como em aquacultura.

De modo a alcançar os objetivos mencionados, foi realizada esta experiência com a duração de 36 dias, tendo sido estabelecidos 4 tratamentos experimentais: o grupo de controlo, um grupo exposto a MPs, um grupo exposto a VFX e um grupo exposto a uma combinação de MPs e VFX. Os juvenis desta espécie foram recolhidos da Estação Piloto de Piscicultura do Instituto Português do Mar e da Atmosfera (IPMA), de Olhão, tendo sido mantidos em quarentena por 11 dias no biotério LabVivos do IPMA, Algés. Após o período de quarentena, os peixes foram pesados e medidos, e aleatoriamente distribuídos pelos aquários experimentais. Após 3 dias de aclimatação aos mesmos, os peixes (n = 128) foram expostos aos tratamentos durante 14 dias, após os quais se seguiu um período de eliminação de 7 dias, em que deixou de existir a contaminação da água, permitindo a observação dos possíveis efeitos pós-exposição.

Para determinar os efeitos na condição corporal os indivíduos foram pesados e medidos, tanto no final da fase de exposição como no final da fase de eliminação, tendo sido posteriormente calculado o índice de Fulton. Para o comportamento, foram feitas observações focais, que consistiram no registo da atividade natatória (tempo, em segundos, passado a nadar ou imóvel) e comportamentos agonísticos (frequência de perseguições e mordidas entre conspecíficos) durante 2 minutos por peixe, tendo sido observados 2 indivíduos aleatórios por aquário, durante 9 dias alternados (6 durante o período de contaminação e 3 durante o período de eliminação). O comportamento é um indicador sensível da saúde dos peixes, desta forma, mudanças nos padrões comportamentais podem ser um dos primeiros sinais de stress causado por fatores ambientais, tais como contaminantes. Por fim, e de forma a avaliar os efeitos da exposição aos contaminantes no metabolismo, mediu-se o consumo de oxigénio de rotina (RMR). O RMR é uma medida do consumo de oxigénio necessário para suportar as funções metabólicas de um organismo, considerando a atividade espontânea dos organismos, fornecendo uma visão mais realista do custo energético total necessário para manter funções fisiológicas básicas, sem influência da alimentação ou do exercício. O consumo de oxigénio foi medido individualmente, ao colocar um indivíduo dentro de uma câmara de respirometria, de forma a calcular a sua RMR após o período de exposição. A taxa de consumo de oxigénio serve como indicador da saúde metabólica dos peixes, sendo

que variações significativas no consumo podem indicar que os contaminantes estão a desviar energia de outros processos fisiológicos essenciais, como o crescimento ou a resposta ao stress.

Os resultados deste estudo mostram que, em termos de peso, foi observado um decréscimo nos indivíduos do grupo de controlo entre períodos de exposição e eliminação, não tendo sido encontrada nenhuma justificação para este decréscimo, dado que a seleção dos indivíduos foi aleatória e não houve variações de peso nos outros grupos experimentais. Foi também registada uma diminuição do índice de Fulton entre o grupo controlo e o tratamento dos MPs no período de eliminação, indicando que os MPs podem continuar a influenciar negativamente a condição corporal dos peixes mesmo após a sua remoção da água. O índice de Fulton também diminuiu entre períodos de exposição e eliminação no tratamento dos MPs, tendo esta observação sido associada a um possível efeito cumulativo dos MPs, uma vez que estes foram encontrados nos tecidos dos indivíduos após o período de eliminação, podendo os 7 dias não ser suficientes para existir descontaminação e a reposição de homeostasia nos peixes. Em termos de comportamento, os juvenis expostos à VFX e MPs isoladamente, passaram mais tempo estacionários do que os do grupo controlo. No que diz respeito à agressividade, observou-se uma redução significativa no número de mordidas entre os peixes expostos à VFX, tanto de forma isolada, como em combinação com os MPs, quando comparados com o grupo exposto apenas a MPs. A exposição a MPs já revelou diminuir o comportamento exploratório e de fuga dos peixes, o que pode possivelmente explicar este comportamento estacionário. Relativamente à resposta com a VFX, este comportamento está alinhado com os efeitos sedativos conhecidos do antidepressivo, já documentados noutras espécies de peixes, que podem ser explicados pelo aumento dos níveis de serotonina (5-HT) no cérebro. A serotonina é um neurotransmissor que desempenha um papel fundamental na regulação de várias funções corporais, incluindo o controlo da agressividade e da resposta ao stress. A exposição à VFX, ao aumentar os níveis de 5-HT, pode ter contribuído para a redução da atividade e dos comportamentos agressivos observados nos juvenis expostos a este fármaco.

Durante o período de eliminação, os níveis de atividade e interações agonísticas não diferiram entre tratamentos, sugerindo que o comportamento dos peixes voltou aos níveis do controlo assim que os contaminantes foram removidos. Relativamente ao metabolismo, todos os tratamentos apresentaram uma diminuição do consumo de oxigénio (RMR) em comparação com o controlo, embora estas diferenças não tenham sido estatisticamente significativas. A diminuição observada no consumo de oxigénio após exposição a MPs pode estar associada a pequenas perturbações nas trocas iónicas ao nível das brânquias, enquanto a redução observada após exposição à VFX pode ser explicada pelos seus efeitos ansiolíticos, que reduzem a atividade metabólica e, consequentemente, o consumo de oxigénio. A co-exposição a MPs e VFX parece amplificar estes efeitos, possivelmente devido à capacidade dos MPs de facilitar a bioacumulação de VFX, aumentando os seus efeitos nos peixes.

Os resultados do presente estudo sugerem uma possível interação entre MPs e VFX, contudo apenas quando considerando os comportamentos de agressividade, e que esta possível interação pode potencialmente afetar os efeitos resultantes da exposição individual de cada contaminante. Tendo isto em mente, e em conjunto com as limitações deste estudo, não ficou explicito quais as consequências e mecanismos desta possível interação. Algumas destas limitações passaram pelo curto período de exposição, e pela redução do número de indivíduos por aquário entre períodos. Desta forma, é necessário desenvolver futuros estudos, que tenham em conta estas limitações, de forma a determinar quais as consequências da interação destes dois contaminantes, e criar estratégias para mitigar os impactos na vida marinha.

Palavras-chave: *Sparus aurata*, microplásticos, venlafaxina, metabolismo, comportamento

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List of abbreviations, acronyms and symbols

MPs - Microplastics

VFX - Venlafaxine

PAHs - Polycyclic aromatic hydrocarbons

VOC - Volatile Organic Compound

Cu - Copper

PPCPs - Pharmaceutical and personal care products

WWTP - Wastewater treatment plants

SSRI - Selective serotonin reuptake inhibitor

SNRI - Serotonin and norepinephrine reuptake inhibitor

TCA - Tricyclic antidepressants

ODV - *O*-Desmethylenlafaxine

NDV - *N*-Desmethylenlafaxine

DDV - *N,O*-Desmethylenlafaxine

TDV - *N,N,O*-Tridesmethylenlafaxine

CYP2D6 - Cytochrome P450 2D6

CYP2C9 - Cytochrome P450 2C9

CYP2C19 - Cytochrome P450 2C19

CYP3A4 - Cytochrome P450 3A9

UDPGT - Uridine diphosphate glucuronosyltransferase

FAO - Food and Agriculture Organization

IPMA-EPPO - Estação Piloto de Piscicultura de Olhão

IPMA – Instituto Português do Mar e da Atmosfera

UHMWPE - Ultra-high molecular weight polyethylene

PC - Polycarbonate

DI – Deionized

C17H27NO2.HCl - Venlafaxine hydrochloride

K - Fulton condition

RMR - Routine metabolic rate

ClNaO - Sodium hypochlorite

Na2S2O3·5H2O - Sodium thiosulfate pentahydrate

EE2 - 17-alpha ethinyl estradiol

5-HT - Serotonine, hydroxytryptamine

UV - Ultraviolet

1. Introduction

1.1. Contamination in Marine Environments

Human activities are constantly shaping marine environments. Anthropogenic activities, such as mining, chemical combustion, agriculture run-off, medical waste and sewage discharge, are rising the levels of pollutants in aquatic systems, especially when considering coastal areas (Ansari and Matondkar, 2014; Häder et al., 2020). In fact, an overwhelming 80% of marine pollution originates from land-based activities. Pollutants, such as pesticides, fertilizers, chemical contaminants, heavy metals and marine litter, have demonstrated detrimental effects on marine organisms and ecosystems (Verma et al., 2020).

Agriculture, aquaculture and domestic waste run-off increase nutrient pollution (i.e. nitrogen and phosphorus pollution) in coastal areas, resulting in an increased growth of microorganisms, such as microalgae, causing algae blooms. Algae blooms, whether toxic or non-toxic producing algae, will lead to the reduction of oxygen levels in marine environments, leading to hypoxic or "dead zones" disrupting marine life, making the environment unsuitable for water breeding animals. This will ultimately affect fisheries and human health (Meyer-Reil and Köster, 2000).

Additionally, sea-based activities, such as shipping, aquaculture, off-shore activities, seabed mining and dredging, result in the release of chemical contaminants into the marine environment (Tornero and Hanke, 2016). Chemical contaminants, such as polycyclic aromatic hydrocarbons (PAHs) a volatile organic compound (VOC), often related to accidental oil spills, are known to cause harmful effects on marine organisms (Sammarco et al., 2013). For example, VOC like crude oil (469 μg/L total petroleum hydrocarbon) have been associated with high mortality rates in shrimp larvae (Keitel-Gröner et al., 2021). Moreover, copper (Cu), a compound used in antifouling paints in vessels, have been linked to a decrease in settlement success of coral larvae and growth inhibition on diatoms (Cid et al., 1995; Reichelt-Brushett and Harrison, 2000). Furthermore, Cu is also responsible for the disruption of matting behaviors in crabs, that fail to detect female pheromones and form pairs (Krång and Ekerholm, 2006). Still within the chemical contamination category, pharmaceutical and personal care products (PPCPs) refer to a wide range of products that include both medicine available without a prescription, such as pain relievers and antibiotics, as well as ingredients found in everyday personal care items like soaps, shampoos, perfumes, and skin care products. These substances are used for therapeutic, cosmetic, and hygiene purposes, spanning from human health to veterinary care (Ohoro et al., 2019). The presence of PPCPs, along with their metabolites, in the environment is raising concerns. Their widespread use is leading to a continuous release either via excretion of their components or metabolites through discharges in wastewater treatment plants (WWTP) directly into the oceans (Fenet et al., 2014), or even through their use and loss directly into water, for example in the case of sunscreens or insecticides (Langford and Thomas, 2008). These chemical contaminants exhibit remarkable persistence, resisting dissolution or breakdown within marine ecosystems, and are among the substances that endure and accumulate within the tissue cells of marine organisms. This process, known as bioaccumulation, not only disrupts underwater life but also poses risks of mutations in aquatic species (Verma et al., 2020).

Lastly, marine litter, often defined as "*any persistent, manufactured or processed solid material discarded, disposed of, or abandoned in the marine and coastal environment*", impacts the environment, human and animal health (Werner et al., 2016). An alarming 80% of ocean debris is composed of plastic waste. The presence of discarded plastic in the ocean poses a significant threat to marine and terrestrial wildlife, often leading to fatal consequences such as suffocation and entanglement (APA, 2021; Verma et al., 2020). The concentration of plastic in the ocean has been increasing over the years (Mattsson et al., 2014), mainly due to the drastic increase in plastic production since 1950, and the poor management of plastic waste, which leads to 10% of this waste ending up in the ocean (Thompson, 2006). Currently,

it is estimated that the ocean carries over 150 million tons of plastic waste. If the current practices persist, by 2025, the ratio of plastic to fish in the ocean is expected to reach one ton of plastic for every three tons of fish. Even more concerning, by 2050, the weight of plastic debris in the ocean is projected to surpass the total weight of fish (Ellen MacArthur Foundation et al., 2016).

1.2. Biological impacts of antidepressants on marine organisms

Antidepressants are a drug used to treat psychiatric diseases, such as anxiety and depression, acting on different neurotransmitter systems, like serotonin, norepinephrine and dopamine. The global increase in the prescription of this type of pharmaceuticals has led to the increase of their concentration in aquatic environments (Salahinejad et al., 2022; Silva et al., 2015). Antidepressants enter the aquatic environment mainly through wastewater effluent discharge, after being excreted by patients, either in their original form or their metabolites, being directly disposed into the wastewater system by manufacturing facilities, hospitals or improper household disposal (e.g. toilet or sink flushing), or even by leach of landfills into surrounding water bodies (Bound and Voulvoulis, 2005; Corcoran et al., 2010; Salahinejad et al., 2022; Sehonova et al., 2018).

There are three major classes of antidepressants, the selective serotonin reuptake inhibitor family (SSRI), the serotonin and norepinephrine reuptake inhibitor family (SNRI) and tricyclic antidepressants (TCA) (Gould et al., 2021). The antidepressants of the SSRI family, such as the fluoxetine, sertraline, paroxetine, citalopram, escitalopram and fluvoxamine, inhibit the reuptake of serotonin by biding to their neurotransmitter in the presynaptic neuron (Blier and El Mansari, 2013; McDonald, 2017). The antidepressants of the SNRI family – e.g.: venlafaxine, duloxetine, milnacipran and desvenlafaxine - inhibit the reuptake of serotonin and norepinephrine by biding to their neurotransmitters, also in the presynaptic neuron (Naseeruddin et al., 2020; Stahl et al., 2005). The antidepressants belonging to the TCA family, such as amitriptyline, nortriptyline, protriptyline, imipramine, also inhibit the reuptake of serotonin and norepinephrine neurotransmitters.

The presence of these antidepressants in the aquatic environment has exhibited effects in non-target living organisms (Sehonova et al., 2018). One of the most prescribed SSRI antidepressants – fluoxetine - that, consequently, is also one the most detected antidepressants in the water, has been proven to induce neurotoxicity, and affect the normal behavior of fish, such as swimming, social interactions and fear or stress responses (Salahinejad et al., 2022). For example, Saaristo et al. (2017) proved that wild guppies after 28 days of exposure to 16 ng/L of fluoxetine, exhibited longer periods of freezing, were less active and spent more time under plant cover when compared to the control group, after an encounter with a simulated bird predator. The most prescribed TCA antidepressant is amitriptyline and has been detected in water bodies worldwide (Breyer-Pfaff, 2004; Schmieg et al., 2020). It has been observed tachycardia and increased mortality in the offspring of zebrafish exposed to amitriptyline (0.8 μg/L) for 21 days. The larvae also exhibited decreased locomotor activity and duration of thigmotaxis (tendency to stay close to the walls of the aquarium), in which the last behavior indicates a lower anxiety state in the exposed parental offspring (Liu et al., 2024). Brown trout exposed to 100 and 300 μg/L of amitriptyline also showed altered behavior, specifically, the individuals exhibited ataxic movements like side and upside-down swimming and were less active than the ones of the control group (Schmieg et al., 2022). Venlafaxine (VFX), an antidepressant belonging to the SNRI family, is one of the most prescribed antidepressants on a global scale, used to treat anxiety and depression (Magalhães et al., 2014). It is excreted from the human body through urine, either in its unchanged form (approximately 4.7%) or in its metabolized form, after undergoing metabolic transformation within the human body (Figure 1.1).

These include O-desmethylvenlafaxine (56%), N,O-didesmethylvenlafaxine (16%), N-desmethylvenlafaxine (1%), and N,O-didesmethyl-N-desmethylvenlafaxine (Magalhães et al., 2014).

Even though VFX is primarily excreted in its metabolized state, it has been detected, in its original form, in untreated wastewaters in concentrations only 3 times lower that its principal metabolite, O-desmethylvenlafaxine (Schlüsener et al., 2015). Schlüsener et al. (2015) concluded that VFX and its metabolites, showed low removal rates during biological wastewater treatment, which results in their continuous discharge into rivers and streams through WWTPs. The authors also indicate that, after the discharges, the concentration ratios of VFX and its metabolites remain constant from the spring to the mouth of the river, which vividly suggests that these contaminants do not suffer more biodegradation in the river course.

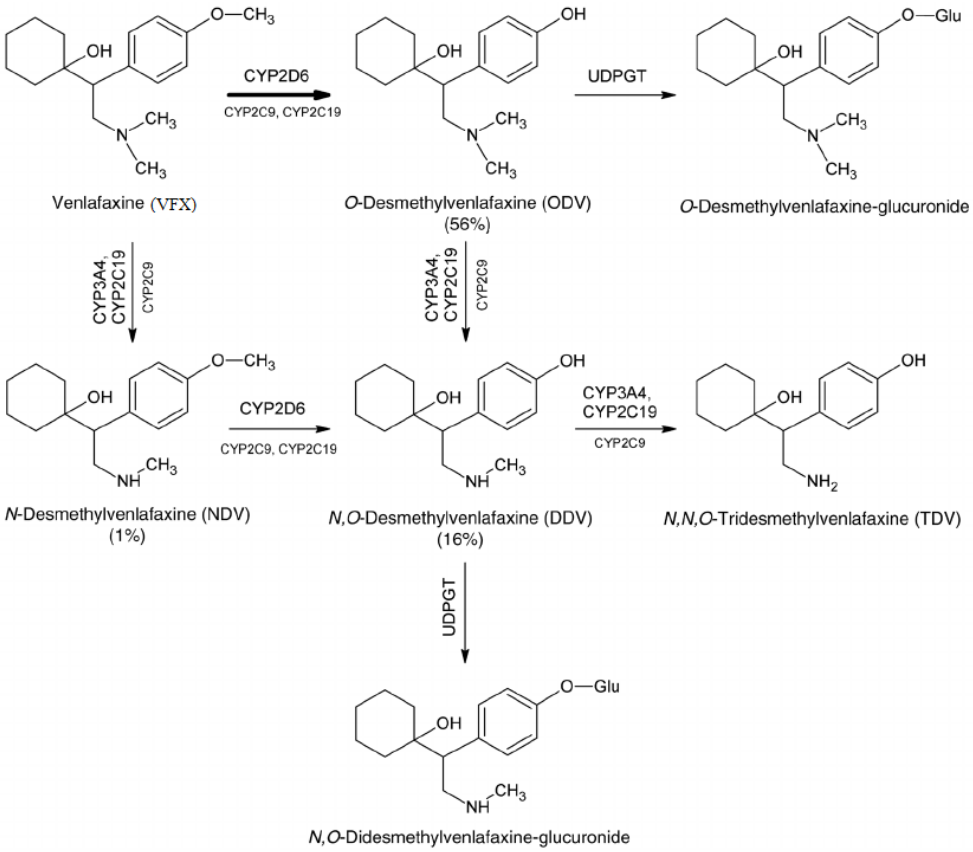


Figure 1.1. VFX metabolization process in the human body. In each arrow is present the enzyme(s) responsible for the correspondent metabolite catalyzed. The bold arrow indicates the principal metabolic pathway, and principal metabolite formed. (Source: Magalhães et al., 2014)

The potential bioaccumulation of VFX by fish was observed in a study where it was registered the presence of VFX in fish's liver, gonads and muscles, but not in their brain (Arnnok et al., 2017). In addition, Santos et al. (2020) showed that juvenile fish meagre bioaccumulate VFX, once this antidepressive isn't extensively metabolized by the species. The concentration of VFX in the meagre tissues was higher in the liver, followed by the brain, then in the plasma, and finally it was lower in the muscle.

When facing stressful situations, fish release epinephrine and norepinephrine into their circulatory system, which increases the production of glucose (by the breakdown of glycogen) and the affinity of

oxygen to blood cells, assuring their delivery to the tissues (Fabbri et al., 1998; Perry and Wood, 1989; Reid et al., 1998). Best et al. (2014) suggested that venlafaxine affects the normal metabolic response in glucose production, that is essential to provide the energy necessary to regain homeostasis after a stressful event in fish. A study performed in hybrid striped bass showed that VFX caused a decrease in brain serotonin concentration, which resulted in the increase of time to catch prey (Bisesi Jr et al., 2014). Additionally, since serotonin acts at the endocrine and regulatory levels, its alteration can generate various toxic effects, that can be observed at the metabolic and behavioral levels (Maulvault et al., 2018, 2019; Santos et al., 2010). Metabolically, exposure to VFX affected the antioxidant defense mechanisms in juvenile meagre fish, powering cellular damage pathways (i.e. increasing lipid and protein damage). Behaviorally, juvenile meagre fish show increased exploratory behavior, spending longer periods of time in the top area of the tank and displaying a decreased tendency to stay within the shoal formation (Maulvault et al., 2018).

1.3. Impacts of microplastics on marine organisms

Plastics can be released into the marine environment directly or indirectly, for example through WWTP, waste handling or aerial deposition (Nowack and Bucheli, 2007). Once in the marine environment, this plastic waste undergoes degradation, producing smaller fragments, known as microplastics (MP) (Derraik, 2002). MP particles range in size from 0.5 to 5.0 mm (Chubarenko et al., 2016). Due to their small size, buoyancy and eye-catching coloration, MP can be mistaken for prey (e.g. phytoplankton), resulting in their ingestion by fish. This ingestion of MP can also occur indirectly when they are inside or attached to consumed prey, like for example when MP are ingested or adsorbed by organisms at the primary trophic level, such as phytoplankton and zooplankton (Jovanović, 2017; Lusher, 2015).

Fish can enter in contact with MP through feeding (active uptake), ventilation, and swimming processes (passive uptake) (Figure 1.2). This means that the MP can get caught in the buccal cavity, gills, intestine and skin mucosal barrier, interfering with physiological processes (Al-Salem et al., 2020; Liang et al., 2023). The gills are the primary target to waterborne contaminants (Duarte et al., 2009), and it was observed an increase in oxygen consumption after an uptake of MP via the gills (Watts et al., 2016).

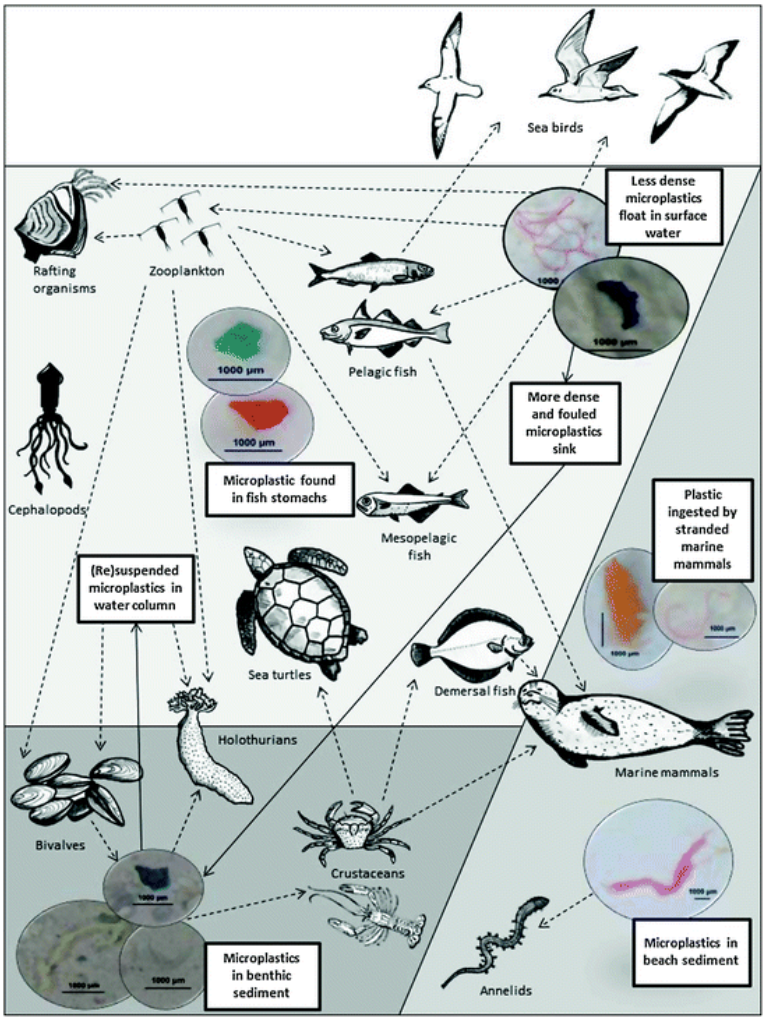


Figure 1.2. Interactions between MPs and marine organisms and their potential transmission through the marine food chain. Source: Lusher (2015).

MP are not biodegradable and, after ingestion, they are not digested and accumulate. It has been shown that the ingestion of MP by adults redtail fish results in a reduction of digestive enzymes, which negatively affects the digestion process, induces oxidative stress and causes hepatic disfunction. This combination of hepatic disfunction and abnormal digestive performance will compromise the metabolism and energy generation crucial to life and growth (Xiao et al., 2023). This affects the energy necessary to other functions and behaviors that are fundamental to their survival, swimming activity, escape from predators, predatory attacks, among others. When these are compromised, individuals, populations and even the trophic chain are at risk (Warren et al., 2017).

Liang et al. (2023) observed that after exposing juvenile goldfish to MP, it was registered a decrease in food intake and inhibited feeding behaviors, like predatory failure, freezing behavior and bottom-dwelling, that was associated to changes in the mucus secretion and diverted energy. These results also suggest that MPs interact more and in higher concentrations with the gills and buccal cavity, once the changes in the mucosal barrier of these preceded the changes in the guts tissues.

MP exposure has been found to increase oxygen consumption and ammonia excretion in juvenile black rockfish. It has also showed impacts in the behavior of the same species, with a decrease in swimming speed and explored space, presence of shoaling behavior, which can negatively affect foraging and exploration behaviors (Yin et al., 2019).

1.4. Microplastic and Venlafaxine: a double threat

MPs can absorb and collect other pollutants present in the water, such as pharmaceuticals, due to their small size and significant surface area in relation to their volume (Santos et al. 2021). As a result, MPs can facilitate the interaction between pharmaceuticals and aquatic organisms, acting as vectors. This can potentially lead to the accumulation and magnification of pharmaceuticals throughout the food chain, when the MPs are ingested, and can alter the toxic effects of pharmaceuticals, either by enhancing (synergism) or reducing (antagonism) them (Figure 1.3).

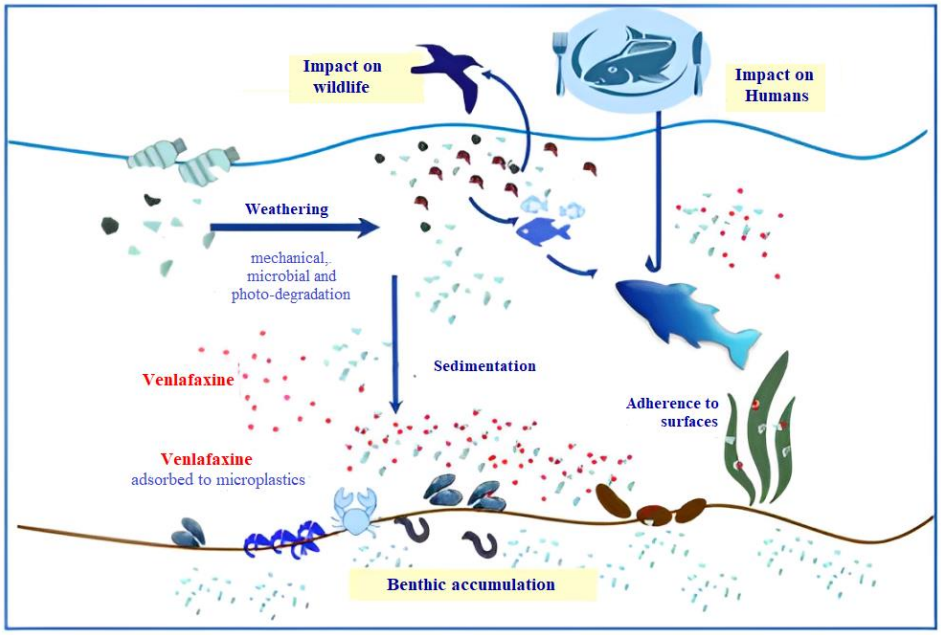


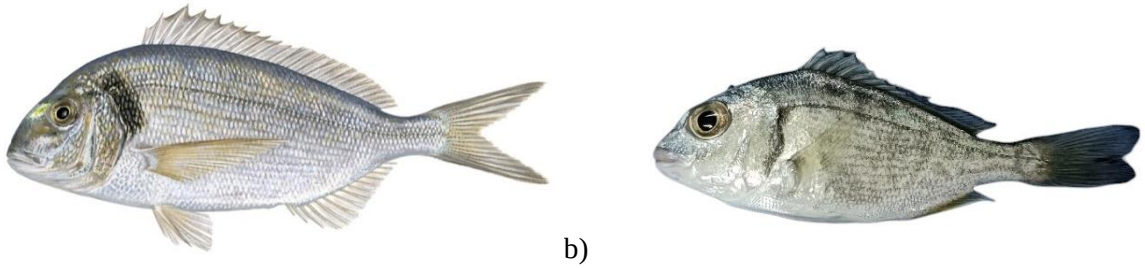
Figure 1.3. Diagram of possible interaction of VFX and MPs and their impacts on living organisms. (Adapted from: Munoz-Pineiro, 2018)

The increase concentration of microplastics in the aquatic environment can potentially extend the duration of pollutant presence and result in long-term adverse effects on both aquatic organisms and the environment (Qu et al., 2018). To date, few studies focused on the combination of MPs and VFX, one in particular, on a freshwater, benthic fish, the loach *Misgurnus anguillicaudatus*, showed that when in the presence of MPs in the water, there was a lower concentration of VFX. They also observed that, after ingestion, VFX was found in higher concentrations in fish liver tissues, supporting the evidence that MPs can not only adsorb, but also increase the bioaccumulation of VFX (Qu et al. 2019).

1.5. Study species

Sparus aurata (Figure 1.4), commonly known as gilthead seabream, is a marine fish, that lives in the Atlantic coast of Europe and the Mediterranean Sea (Figure 1.5), being one the most important marine species in fisheries and aquaculture (Arabacı et al., 2010). The most common environment for *S. aurata* is marine and brackishwater like coastal lagoons and estuarine areas, especially in the initial stages of its life cycle. Breading season occurs in the Winter, between November and December, in the sea. Juveniles migrate in the Spring to protected coastal waters, where they find abundant food and warmer temperatures, making such sensitive life stages more prone to the higher levels of contaminants near

coastal zones. In the autumn, due to the low temperatures and their sensitivity to them, *S. aurata* return to the open sea, where the adult individuals breed. This species is frequently found in rocky and seaweed bottoms, but also in sandy grounds. While young fish are found at low depths, adults reach deeper zones (up to 150m) (Moretti et al., 1999). *S. aurata* is an opportunistic feeder that adapts its diet to the prey found in the environment, being mainly carnivorous. In the southern Portugal, the diet of *S. aurata* mainly consists on bivalves and gastropods. Juveniles prey on small zooplanktonic crustaceans (larvae of shrimps, Isopoda and Gammaridae), annelids and small pelagic fish and fish larvae (Pita et al., 2002; Taieb et al., 2013). Adults, due to their bigger sizes, prey on teleosts, molluscs and crustaceans (Taieb et al., 2013).



a) b)

Figure 1.4. a) Adult specimen of gilthead seabream (*Sparus aurata*) (https://fish-commercial-names.ec.europa.eu/fish-names/species/sparus-aurata.pt); b) Juvenile specimen of gilthead seabream (*Sparus aurata*)

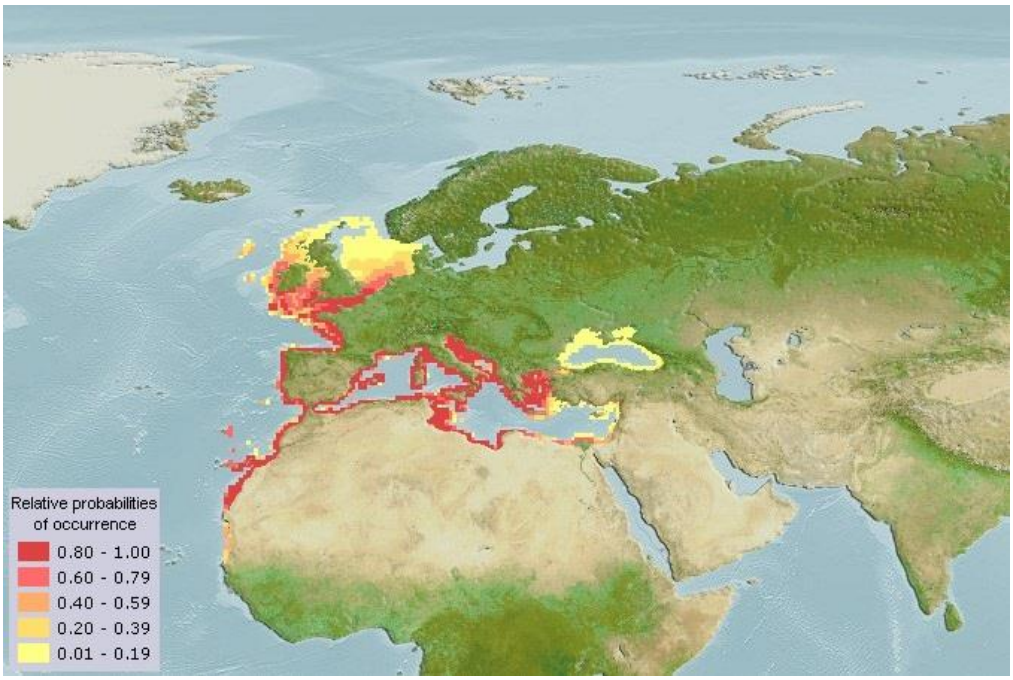


Figure 1.5. Map of the geographical distribution of *Sparus aurata* (Adapted from: https://www.aquamaps.org/receive.php?type_of_map=regular&map=cached)

For the first two years of its life cycle, *S. aurata* is a functional male, and when it exceeds 30 cm, it can turn into a female (Arabacı et al., 2010) (Figure 1.6). According to Mhalhel et al. (2023), FAO estimated that the production of gilthead seabream was at 258,754 T in 2020, which made this species classified 33rd among the most bred fish.

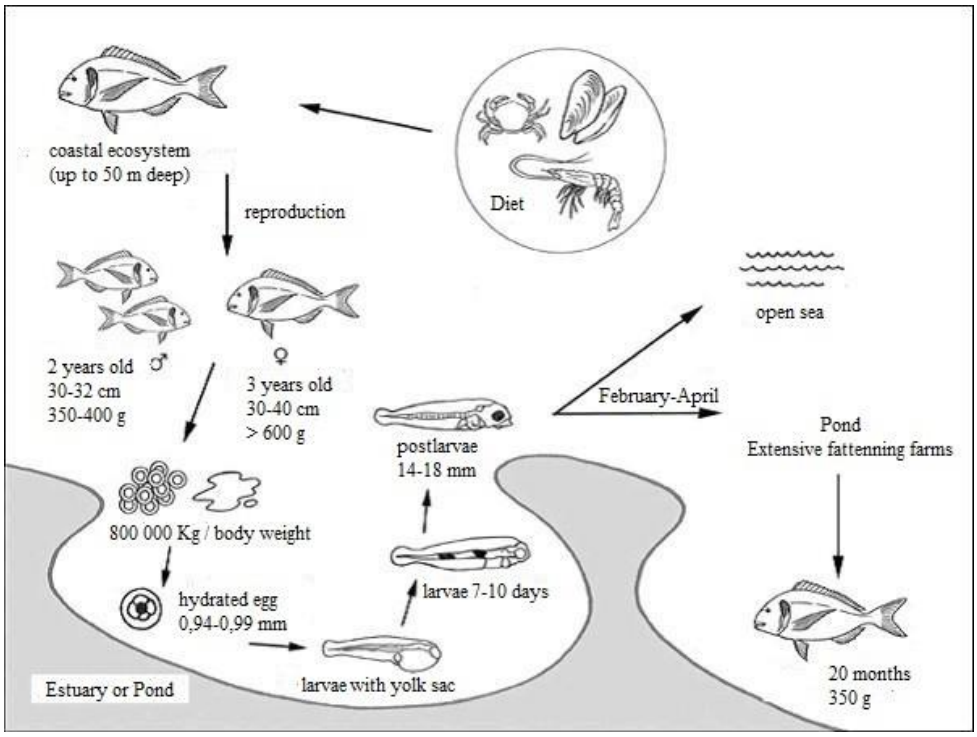


Figure 1.6. Life cycle of *Sparus aurata* (Adapted from FAO, 2009)

1.6. Objectives

There is a gap in the literature regarding the potential effects resulting from the combination of MPs and VFX. This scarcity highlights the importance of the current study for advancing our understanding on the complex interactions between MPs and VFX and to assess their possible impacts in the marine environment. Understanding these interactions is crucial for comprehensively assess the environmental risks posed by these pollutants.

Another important aspect of this study is its focus on juvenile organisms. As IWAMA (1998) referred, it's important to consider that repeated or prolonged exposure to aquatic contaminants can result in the long-term redistribution of energy resources away from essential biological processes crucial for overall fitness like growth, immune function, and reproductive capabilities. This can potentially impact the juvenile fish transition into adults, leading to a subsequent impairment in the reproductive capability and a decrease in the number of individuals and consequently a decline at the population level.

With this in consideration, the goal of the current study was to assess the effects of MP and VFX, isolated and combined, on: i) the physiology of juvenile *S. aurata*, focusing on their survival rates, growth and oxygen consumption, and; ii) on behavior, specifically swimming activity and agonistic interactions.

2. Methods

2.1. Acclimation and experimental set-up

Sparus aurata specimens were reared at the aquaculture pilot station, Estação Piloto de Piscicultura de Olhão (IPMA-EPPO), until reaching the juvenile stage, and transported to the live marine organisms bioterium facilities (LABVIVOS), at the Instituto Português do Mar e da Atmosfera (IPMA). At arrival, fish were maintained in 500L quarantine tanks for 11 days under similar conditions to rearing site (19ºC,

35 salinity). Following that period, fish were randomly transferred to 20L aquariums, where they remained for 3 days to acclimatize to the new environment, after which the experimental period began.

Following 3-days acclimation, fish were exposed to 4 different treatments: a) control, with uncontaminated water; b) MP, with water only contaminated with MP in order to observe its isolated effect; c) VFX, with water only contaminated with VFX in order to observe the isolated effect of this contaminant; d) MP_VFX, in order to observe the interactive effect of these two contaminants. Each treatment consisted of 4 aquariums filled with 20L seawater, with 8 fish in each. A total of 128 juvenile fish were exposed for a period of 15 days, followed by a 7-day elimination period, during which the water was no longer contaminated for all the treatments (Figure 2.1). From the 128 fish, a subset of n=24 were weight at the beginning of the experiment to access fish weigh and adjust the food provided (8.66 ± 1.97 g)

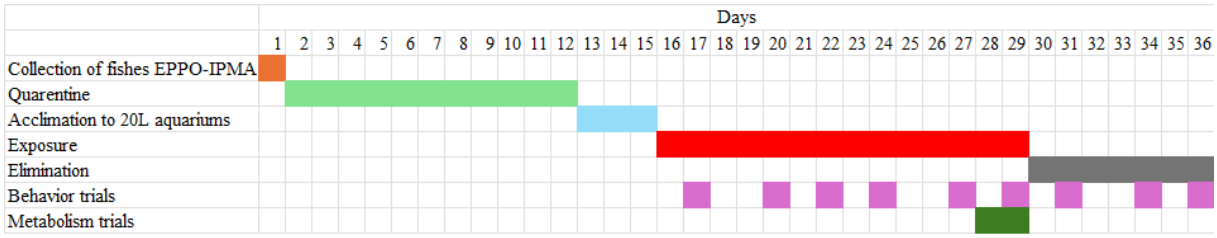


Figure 2.1. Experimental timeline, showing the days at which behavior and metabolism trials were performed.

The study was carried out in glass aquariums to avoid contamination from other plastics and adsorption of the compound to the aquarium walls. To avoid the adsorption of MP and VFX by the diffuser stones, and to ensure oxygenation of the water, air lines were connected to glass pipettes. Two glass pipettes were placed in each aquarium to ensure proper oxygenation of the water (Figure 2.2).

To ensure that the MPs and VFX concentration was kept at the desired concentration, around 70% of the water in each aquarium was renewed every day, while also removing feces and excess uneaten food. After adding new water, it was again contaminated according to the corresponding treatment. The temperature in the aquariums was maintained by placing the aquariums in a water bath fitted with thermostats and coolers that kept the temperature at the desired level (~18ºC, see supplementary table S1). Each treatment was composed of 2 water baths, each with 2 aquariums (Figure 2.3).

The individuals were fed dry food pellets at a rate of 10% of the fish's weight, divided in two meals per day, one in the morning and another in the afternoon. These amounts were adjusted throughout the experimental period, according to fish weight.

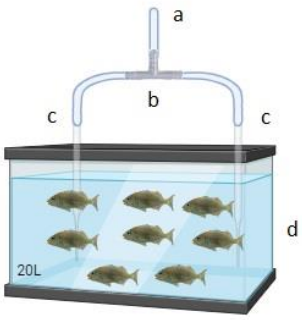


Figure 2.2. Experimental set-up of the aquariums: a – air line; b – T-shaped connecter; c – glass pipettes; d – 20L aquarium.

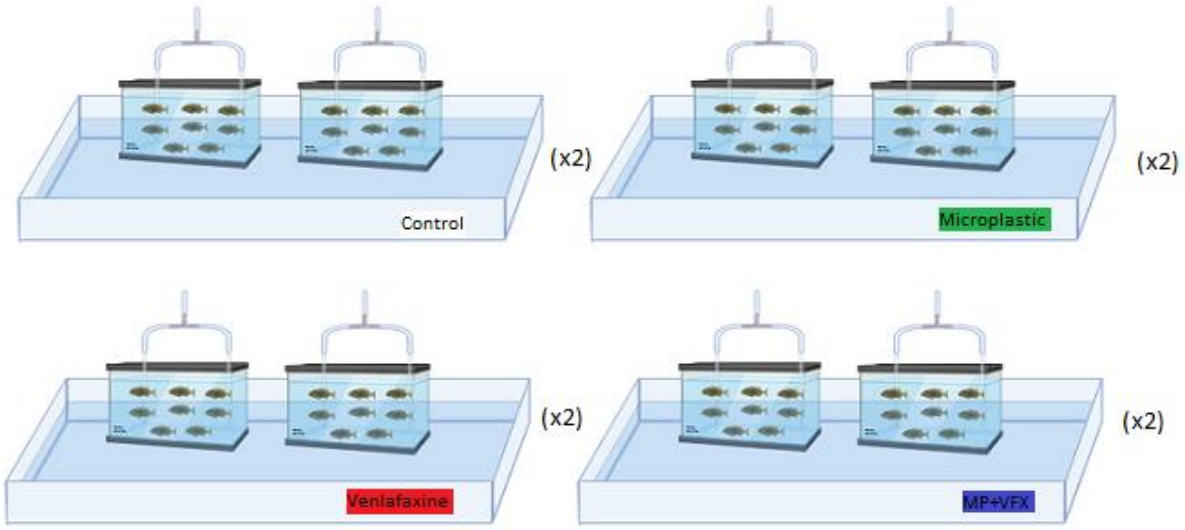


Figure 2.3. Experimental set-up for each treatment. Each treatment was composed of 2 water baths, each with two 20L aquariums, in a total of 4 aquariums per treatment. Each aquarium contained 8 fish, totalizing 32 fish/treatment.

2.1.1. Microplastics preparation and exposure

The MPs selected for this study consisted of ultra-high molecular weight polyethylene (UHMWPE) fragments (Goodfellow; ET306010; density: 0.94 gcm-3) with a 125-250 µm dimensions. The selected fragments dimensions resulted from sieving 20g of the original supplied powder, firstly with a 250 µm sieve (2x) and then with a 125 µm sieve (2x). Subsequently, fragments were dyed, to allow a fluorescence-based detection during samples observation. To that end, the dying protocol developed by Karakolis et al., 2019 was employed, using the Jacquard iDye Poly PINK (JID I 456). Briefly, 0.005g (≈3500 fragments) of the 125-250 µm fragments were placed in a glass Petri dish, where we added 1mL of the iDye solution (100 mg iDye/mL DI water), repeating this process for a total of 50 Petri dishes (≈175 000 fragments in total). Afterwards, the Petri dishes were placed in a tray and left to incubate for 2h at 55ºC in the dark. Following that period, the content of each Petri dish was individually vacuum filtered (WP6122050, Milipore) onto 20 µm PC filters, resuspended in DI water and filtered again (3x) to remove any excess of the iDye. Each PC filter was then placed in a new petri dish and left to incubate again at 55ºC for 1h in the dark. At the end, we proceeded to the transfer of the fragments from each PC filter to the same petri dish (stock) with a paint brush, storing in the dark.

To infer the number of fragments within each 20L aquarium (i.e. denoting the number of fragments passing through the feeding radius of a juvenile fish throughout the 12-hour diurnal interval, as *S. aurata* holds to diurnal behavior patterns (Velázquez, 2004)), a systematic approach was employed, incorporating several assumptions and approximations considering findings from field observations. Briefly, the water volume intersected by an individual fish, to subsequently estimate the count of particles transiting within the 12-hour daylight period was calculated as V=Base area x Height (h). According to Müller et al., 2020, the parameter 'Base area' consists on the feeding radius of the fish, aligning with the dimensions of the 20L aquarium (0.24m x 0,39m), while 'h' represents the length of water passing by an individual fish during the 12-hour period, considering the equation vx60x60x12 = 5616 m, where 'v' is the average current velocity found within the Sado estuary (0.13 ms^{-1}; Biguino et al., 2021), one of *S. aurata* habitats in Portuguese waters. Consequently, the volume 'V' of water passing by an individual fish, considering the equation above, was estimated to be 525,66 m^{-3}. Given that there are 1.5 microplastic particles per m^{-3} (Bessa et al., 2018), the number of particles coursing through the feeding radius of a juvenile fish over the 12h daylight was estimated to be approximately 789. Based on the established assumptions, approximately 800 fragments were sprinkled into the water surface. Noteworthy, the number of each particle was based on the resultant weight of the particles rather than the counts of individual particles, consisting of ≈ 1mg per aquarium.

2.1.2. Venlafaxine preparation and exposure

Venlafaxine exposure occurred via water, by daily spiking the correspondent 20L aquariums during the 14 days of exposure, with a stock solution of 60 mg L^{-1} of venlafaxine hydrochloride (C17H27NO2.HCl;, >98%, CAS 99300-78-4, TargetMol) dissolved in deionized water (1L total volume), to achieve a nominal VFX concentration of 20 µg L^{-1} in each aquarium (Maulvault et al., 2018). The determination of the nominal concentration of VFX was established based on the lowest VFX concentration previously reported to induce significant behavioral changes in fish (50 µg L^{-1}; Bisesi Jr et al., 2014).

2.2. Body condition

Fish weight, standard and total length were measured at day 14 (the last of contamination) and at day 21 (after 7 days elimination). A total of 72 fish were measured, 36 at the end of contamination and elimination phases (9 treatment/day). It was determined the Fulton's index (K) for each treatment according to the following equation (2.1):

$$\textbf{(2.1)} \quad K = 100 * \left(\frac{weight}{standard\ length^3}\right)$$

2.3. Behavior

Based on a defined ethogram, focal behavioral observations were made three times a week, throughout the entire experiment. The focal observations were carried out by just one person to avoid bias. The immobile observer analyzed a random fish for 2 minutes, recording its behavior using the BORIS software (Friard and Gamba, 2016). Focal observations were always carried out at the same time of day, in the morning between 9h and 10h30, before maintenance and feedings, in order to avoid agitating the fish and post-feeding biases. Two individuals were observed per aquarium, making a total of eight

individuals analyzed per treatment each day. The 4 behaviors observed were swimming, stationary, chasing and biting (Table 2.1).

Table 2.1 – Ethogram of *Sparus aurata* describing activity and aggression behaviors (adapted from Pimentel et al., 2016)

Behavorial categories	Description
Swimming (time, seconds)	Movement against the current in which the individual uses the pectoral and caudal fins to move
Stationary (time, seconds)	Individual remains motionless in the water column, with only movements of the pectoral fins to maintain his position
Chase (frequency)	A fish attacks or pursues a feeing fish
Bite (frequency)	Individual bites conspecific, after chasing

2.4. Routine metabolic rate (RMR)

In order to measure routine oxygen consumption, 7 fish per treatment were placed individually in respirometry chambers of around 300 mL each, where their oxygen consumption was recorded for a period of 3 hours, with the first hour of reading being discarded once it was the period of acclimatization of the fish to the chambers. A total of 8 chambers were used in each run, 7 of them with one individual in each, and one chamber only with water to account for the background respiration. The chamber that measured the background respiration was changed in each run. The fish were not fed for the previous 24 hours in order to avoid an increase in metabolic rates due to digestion (Killen et al., 2013).

The procedure followed these steps:

1- The fish were individually transferred to acrylic chambers with a total volume of 310 mL (300 mL empty chamber plus 10 mL of tubing), that were submerged in a 55L water bath maintained at a constant temperature of 18ºC throughout the experiment.
2- Each chamber was completely watertight and closed. The chambers were covered with opaque materials to avoid external disturbances and stimuli between individuals in the same trial.
3- Oxygen consumption begins to be recorded. Inside each chamber is a spot sensor (OXSP5, Pyroscience, Germany) which measures the oxygen consumption and sends this information to the equipment Firesting Optical Oxygen Meter (FireSting-O2-4C, Pyroscience, Germany) via a optic fiber cable (SPFIB-LNS, Pyroscience, Germany).
4- There are periods of water renewal and periods of oxygen consumption measurement, which is ensured by an automated system, in which after 10 minutes of measuring the oxygen consumption made by the fish inside the chamber, where there is no water renewal, a pump is switched on for 5 minutes to allow the water inside the chamber to be renewed and the oxygen levels to be restored to 100%. This 15-minute cycle (10 minutes of closed chamber/consumption and 5 minutes of renewal) was the result of preliminary tests with *S. aurata*, in order to ensure that O_2 levels inside the chamber do not drop below 80%, the threshold level to ensure the animal's well-being without inferring stress levels associated with oxygen deprivation.
5- The data collected by the O_2 sensor was recorded in the *Pyro Workbench* and analyzed in the *Pyro Workbench Data Inspector.*

The water was renewed inside each chamber through an automated flush pump that was inside a 250 L reservoir, with seawater at the same salinity and temperature as the water used in the aquariums of each

treatment, and oxygen levels at 100%, at a rate of approximately 135 ml/minute/chamber. The temperature and O_2 levels were maintained in the reservoir through refrigerators and O_2 aeration stones, respectively, and for the purpose of decreasing bacterial respiration, the reservoir also had a UV light to sterilize the water. It was used a Profilux controlling system (Profilux4, GHL, Germany), with a programmed timing sequence regulated by the GHL Control Center Software (version 1.1.4.4), to control the pumps (Figure 2.4).

The chambers were connected to a peristaltic pump that allowed water to circulate inside each chamber through a closed external gas-tight piping circuit, with a flow rate of 2 ml/min, to guarantee the mixing of water inside each chamber during measure.

Prior to each run, sensors underwent calibration, and chambers were cleaned using distilled water and 70% ethanol. Subsequently, the chambers were refilled with seawater.

A total of 26 fish were analyzed, 13 fish per day, randomly selected by treatment (3 per treatment/day), and this experiment took place over 2 days, with one measurement in the morning and another in the afternoon.

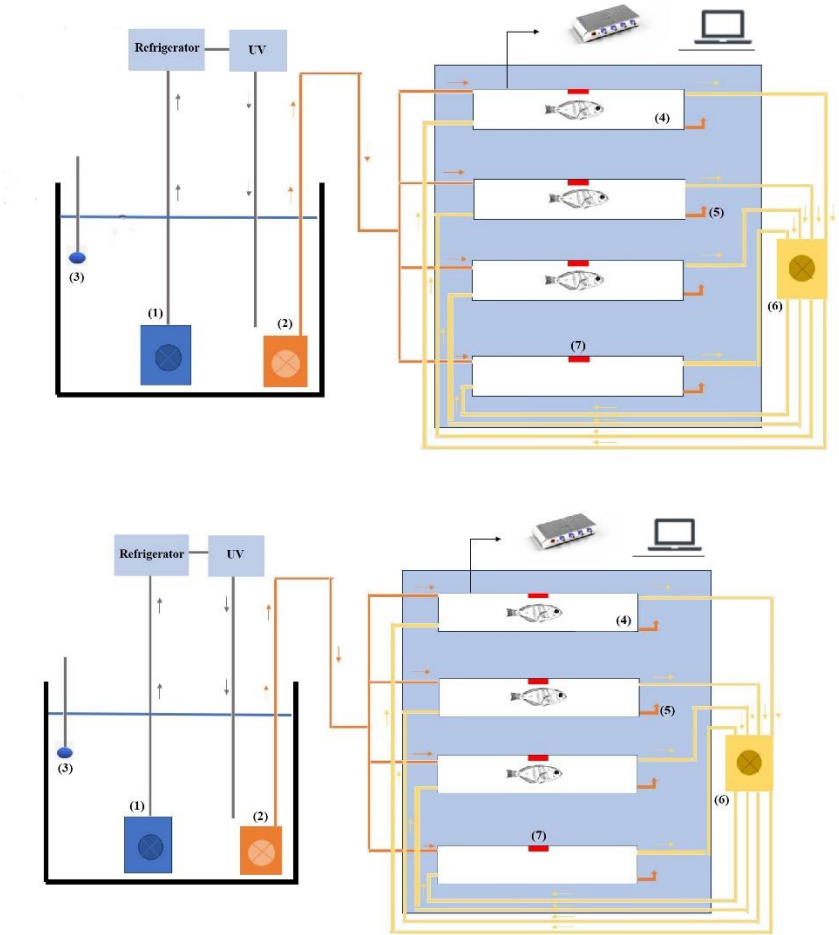


Figure 2.4. Scheme of the experimental set-up for the routine metabolic rate. (1) Pump that circulates water through both the refrigerator and the UV unit for sterilization purposes; (2) flush pump responsible for directing the water into the respirometry chambers during the flushing cycles; (3) air stone for aeration; (4) respirometry chambers; (5) non-return valve to prevent water from flowing back into the chambers; (6) peristaltic pump to ensure proper water mixing within the chambers; (7) sensor spot. (Adapted from: Almeida et al., 2024)

2.5. Preventive measures for quality assurance

To prevent cross-contamination between exposure treatments, several precautions were taken into consideration, including:

1. Dedicated material: using separate and dedicated material for each treatment group to prevent any potential transfer of contaminants between them. To each experimental treatment was allocated its dedicated set of materials (e.g. beaker, fish nets, specific cleaning material, among others) properly identified.
2. Proper cleaning: thoroughly cleaning and disinfecting all material and surfaces between each treatment to eliminate any residual traces of contaminants.
3. Timing and sequencing: carefully scheduling and sequency the handling of different treatments to avoid any accidental mixing or exposure.
4. Use of adequate wardrobe: researcher and users responsible for the experiment and animal welfare were equipped with a laboratory coat, gloves (changed between treatments), rubber boots, among others, to avoid cross-contamination between treatments and to guarantee the safety of users.
5. Use of glassware whenever possible to minimize the adsorption of contaminants to the materials used.

All of the 20L aquariums were disinfected before the experiment: firstly, soaked in a tank with sodium hypochlorite (ClNaO; CAS: 7681-52-9; 1.7 ml/l) and secondly, in a tank with sodium thiosulfate pentahydrate (Na2S2O3·5H2O; CAS: 10102-17-7; 0.8 g/l).

Water from the contaminated treatments was filtered with a mesh (for MPs exposure treatments) smaller than the size of the particle to retain any particles that may still be present in the water (i.e. particles that were not absorbed by the fish). Afterwards the mesh was discharged properly. For the chemical contaminant exposure treatments, water passed through activated carbon and was placed in a water treatment station with activated carbon for at least 24h prior disposal.

At the end of the exposure period, the 20L aquariums and all the experimental set-up were disinfected once again, following the same process.

2.6. Statistical Analyses

All statistical analyses were carried out using *Rstudio* software (R Core Team, 2023). To determine whether different treatments (i.e. Control, MPs, VFX and MPs_VFX) and periods (i.e. Exposure and Elimination) affected fish body condition, fish behavior and oxygen consumption, I used generalized linear mixed models using Template Model Builder, within the 'glmmTMB' package in R (Brooks et al., 2017). This approach allowed to integrate both the fixed effects of treatments and experimental periods with the random effects attributed to individual and replicate aquariums variability. After determining that the random effects only explained some variability of the data for the parameter weight, I proceeded on using generalized linear models for Fulton index, standard and total length. The parameter weight was analyzed through a Gaussian distribution, while Fulton index, standard and total length were analyzed through a Gaussian, with the logarithmic-link function. Chase, bite and stationary behaviors were analyzed through a Poisson distribution, while swimming was analyzed with a Gaussian, with the logarithmic-link function. Lastly, to analyze the metabolism data, RMR was determined by the mean MO_2 using the respR package in rstudio. The RMR was expressed in milligram of oxygen per kilogram per hour. RMR data was analysed using the gaussian family. Whenever significant differences

were detected, post hoc analyses were performed, using the 'emmeans' package, which enabled to pinpoint differences between specific treatment groups while duly adjusting for multiple comparisons. Model assumptions were checked using the package DHARMa (Hartig, 2022). *p*-values below 0.05 were considered significant.

3. Results

Throughout the 14-days exposure and the 7-days elimination periods, no mortalities were registered due to the treatment to which the fish were exposed to. Nonetheless, it is important to mention that, on day 11th of the exposure period, one of the replicate tanks from the control treatment was lost, due to technical problems in the air line supplying this aquarium, which unfortunately, caused the 8-fish of this replicate tank to die overnight.

3.1. Body condition

Fish weight (in grams) did not differ between treatments, with mean values ranging between 8.243 ± 1.103 g (lower value found for MPs treatment in the exposure period, supplemental table S2) to 10.204 ± 2.412 g (higher value found for the control treatment, supplemental table S2). However, significant differences were found between the exposure and elimination periods (p = 0.0122, supplemental table S3), in the control group (p = 0.0149, supplemental table S6, Figure 3.1).

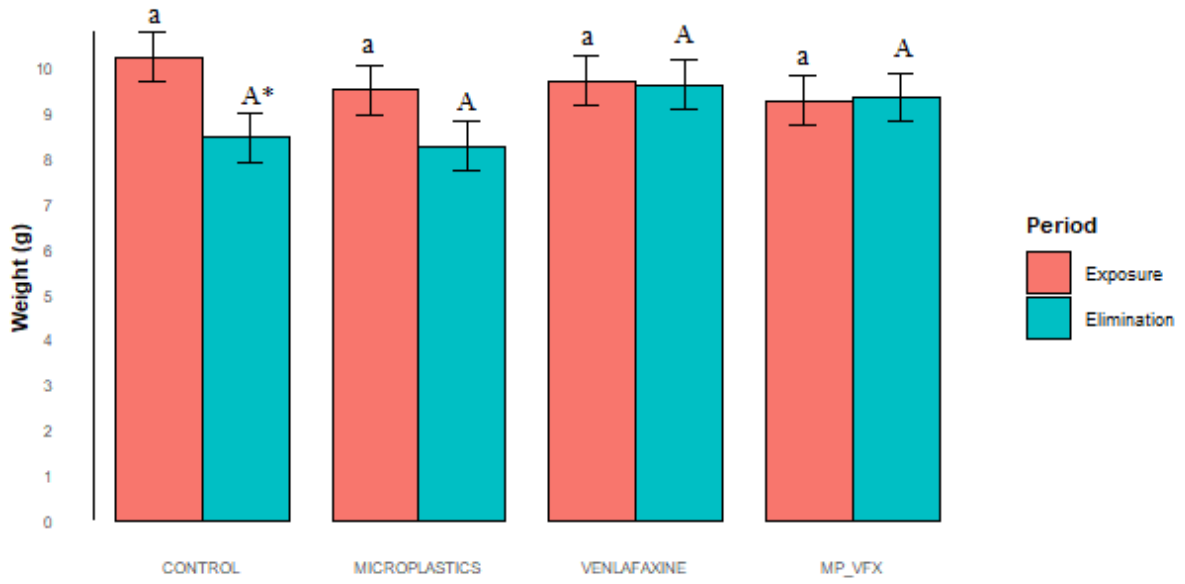


Figure 3.1. Weight (grams) of *S. aurata* juveniles per treatment in each period. Small and capital letters represent significant differences between treatments for the exposure and elimination periods, respectively. Values are represented as mean ± SE. Asterisks (*) represent differences between periods for each treatment.

Considering fish size (cm), standard length had a mean value of 7.29 ± 0.55 cm, and total length of 8.84 ± 0.63 cm (supplemental table S2). Both variables did not differ between treatments nor experimental periods (see supplemental tables S4 and S5).

As for the Fulton index, the post-hoc analyses showed a significant decrease between the control and the MPs treatments in the elimination period (p = 0.0313, supplemental table S8, Figure 3.2), and a

significant decrease for the MPs treatment between the exposure and elimination periods ($p = 0.000392$, supplemental table S9, Figure 3.2).

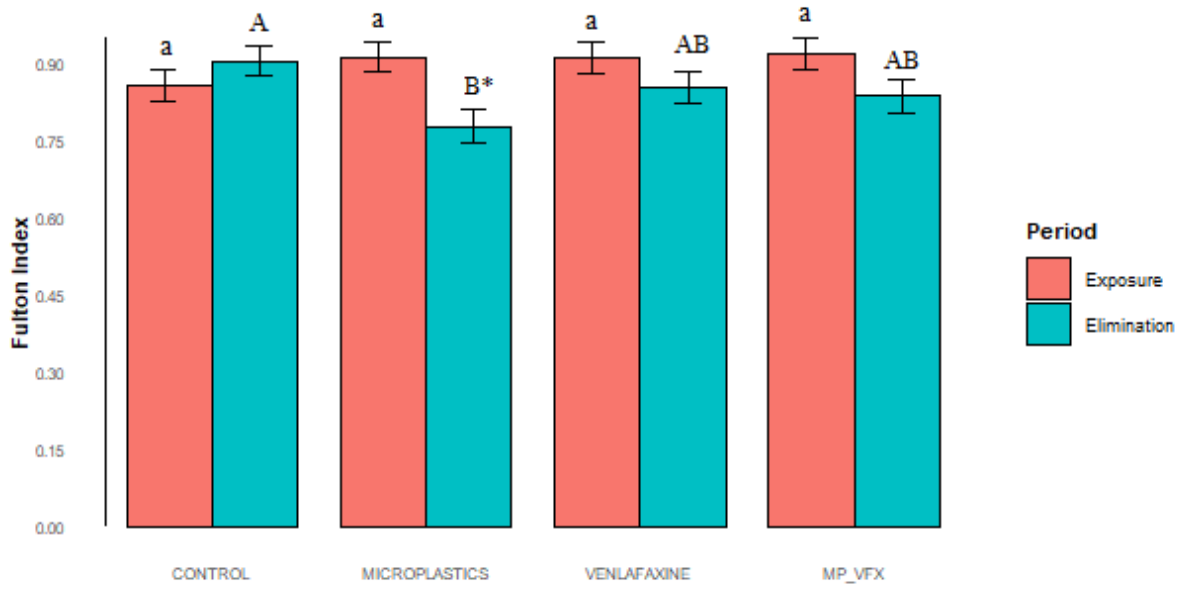


Figure 3.2. Fulton index of *S. aurata* juvenile fish for each treatment and period. Small and capital letters represent significant differences between treatments for the exposure and elimination periods, respectively. Values are represented as mean ± SE. Asterisks (*) represent differences between periods for each treatment.

3.2. Behavior

Regarding the effects of exposure treatments on fish behavior, individuals spent most of their time swimming, regardless of the treatment and period (Figure 3.3). Even though the glmmTMB showed a significant difference between the control and the MPs treatment ($p = 0.048$, supplemental table S10). The time spent swimming decreased from 119.30 ± 0.32 s in control to 116.54 ± 1.62 s in the MPs treatment, and 118.33 ± 1.55 s in control to 115.96 ± 3.00 s in the MPs treatment, in the exposure and elimination periods, respectively. However, these differences were not observed in the post-hoc tests, determined by the emmeans package (supplemental table S11).

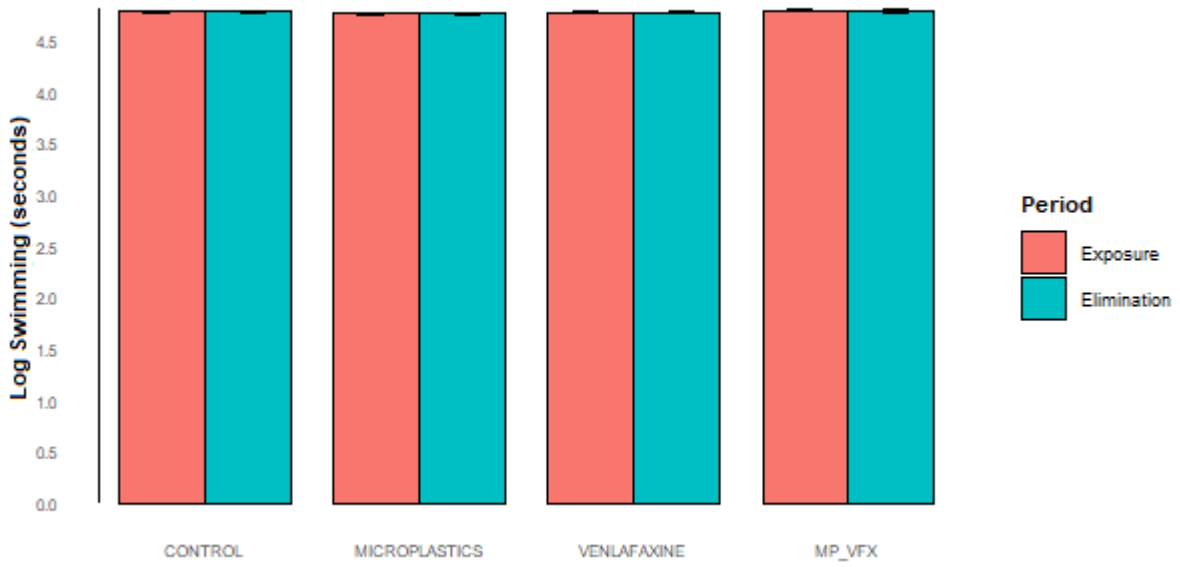


Figure 3.3. Log-transformation of time spent swimming (in seconds) by *S. aurata* juveniles in each treatment and period. Values are represented as mean ± SE.

As for the stationary behavior, juveniles exposed to MP ($p < 0.001$, supplementary table S13) and VFX ($p = 0.0074$, supplementary table S13) as isolated factors spent more time motionless, when compared to the control, in the exposure period, but no differences were found while exposed to the 2 contaminants simultaneously ($p = 0.388$, supplementary table S13, Figure 3.4). In fact, fish exposed to MPs and VFX alone, in the exposure period, spent 3-times (3.25 ± 1.65 s, $p = 0.0052$, supplementary table S14) and 2-times more immobile (2.23 ± 1.29 s, $p = 0.037$, supplementary table S14), respectively, when compared to control (0.52 ± 0.28 s). Nonetheless, in the elimination period, no significant differences were found between the exposure and the control treatments. Surprisingly, in the elimination period, juveniles from the MP and VFX spent more time motionless, than the ones exposed to both stressors simultaneously (MPs: 4 ± 3 s, $p = 0.0001$; VFX: 1.46 ± 1.10 s, $p = 0.021$, compared to 0.87 ± 0.52 s for MP_VFX, supplemental table S14).

Lastly, it was observed significant differences in time spent motionless between the periods of exposure and elimination for all treatments except for control. In fact, fish from MP ($p = 0.018$), VFX ($p = 0.042$), and both contaminants simultaneously ($p = 0.025$) spent more time motionless during the exposure than the elimination period (supplemental table S15).

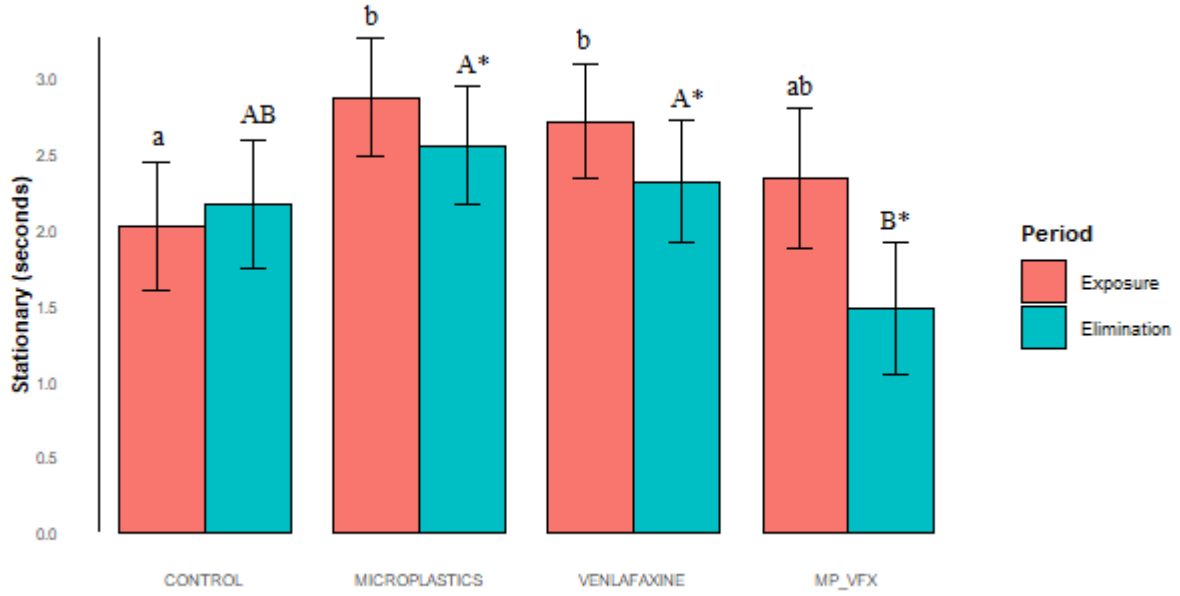


Figure 3.4. Time spent stationary (in seconds) by *S. aurata* juveniles for each treatment, in each period. Small and capital letters represent significant differences between treatments for the exposure and elimination periods, respectively. Values are represented as mean ± SE. Asterisks (*) represent differences between periods for each treatment.

Despite the observed differences for the time spent stationary, no significant differences were found in the frequency of chases, described as the frequency a fish attacks and pursues a conspecific, between treatments or periods, with the frequency of chases ranging between 1.33 ± 0.28 – 1.80 ± 0.26 (Figure 3.5, supplementary table S16). However, the frequency of bites, characterized as the frequency of bites towards a conspecific following a pursue, was found to be statistically different for the VFX and MP_VFX treatments (Figure 3.6). In fact, a smaller number of bites was registered in the VFX (1.03 ± 0.29; $p = 0.015$, supplementary table S18), and the 2 contaminants combined treatments (1.00 ± 0.29; $p = 0.0053$, supplementary table S18), when compared to the MP treatment (1.41 ± 0.29). No differences were found between periods, despite the tendency in all treatments to bite less in the elimination period (supplementary table S17, Figure 3.6).

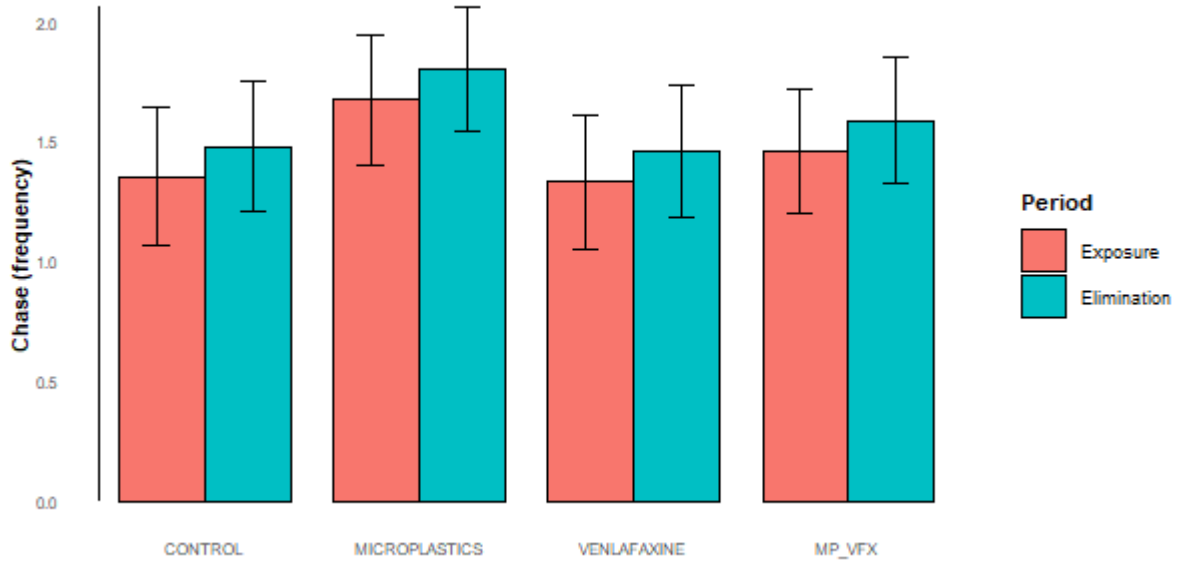


Figure 3.5. Frequency of chases during the exposure and elimination periods, according to each treatment. Values are represented as mean ± SE.

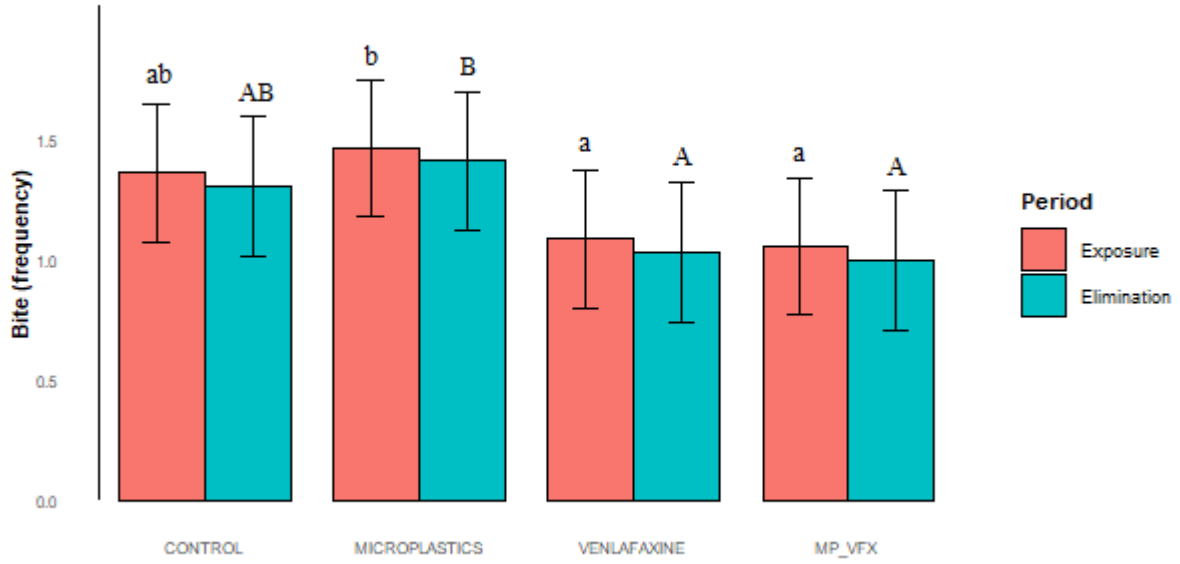


Figure 3.6. Frequency of bites during the exposure and elimination periods, according to each treatment. Values are represented as mean ± SE. Small and capital letters represent significant differences between treatments for the exposure and elimination periods, respectively.

3.3. Routine metabolic rate (RMR)

At the end of the exposure period, oxygen consumption was registered and RMR accounted for each treatment. Fish under all treatments showed a decrease in RMR when compared to the control, with the

MPs and MPs_VFX treatments the ones with the higher decrease in oxygen consumption (Figure 3.7). However, these changes were not statistically significant neither in the glm (supplementary table S19), neither when the treatments were compared between each other (supplementary table S20).

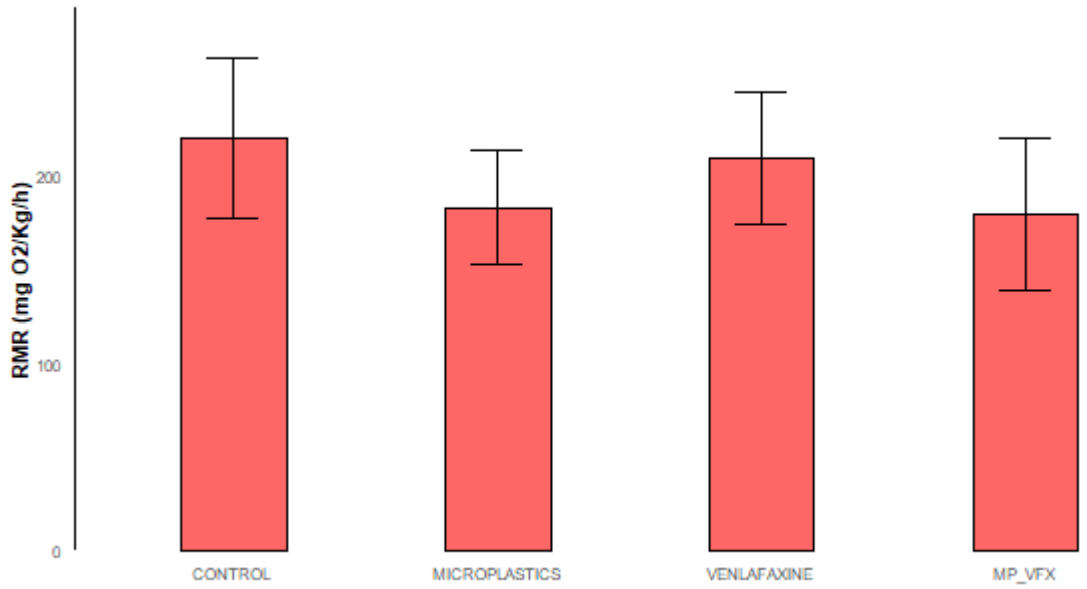


Figure 3.7. Routine Metabolic Rate (mg O_2/Kg/h) mean values ± SE. for each treatment.

4. Discussion

In this study, we investigated the impacts of MPs and VFX, both as sole and combined stressors, on the body condition, behavior and metabolism of juvenile *S. aurata*. Our findings revealed that while these contaminants induced behavioral changes in the fish, they did not significantly affect their body condition or oxygen consumption rate.

The exposure treatments did not significantly change the weight and length of *S. aurata* juveniles. However, a notable reduction in weight was observed in the control group between experimental periods, with slighter fish after the 7-day elimination period. This unexpected decrease lacks a clear explanation, as the amount of food per body weight was adjusted throughout the trial, and the sampling of individuals conducted randomly. Nonetheless, for reasons that remain unclear, the juveniles in the control group exhibited lower body mass at the end of the trial.

There was also a significant decrease in the Fulton index between the control and the MPs treatment during the elimination period, and between periods within the MPs treatment itself. The Fulton index is a measure used to assess the health condition of a fish, with higher value indicating better body condition (Nash et al., 2006). Therefore, our results suggest a decline in health condition of fish in the MPs treatment when the contamination ceased, and the water conditions were restored to control conditions (i.e. non-contaminated water). One possible explanation for this decline could be that the 14-day exposure period may have been too short for noticeable changes in body condition to manifest. Indeed, traces of MPs particles were still found in the fish digestive tract after the 7-day elimination period (data under analysis), suggesting that MPs might continue to affect the fish's body condition even after the contamination had stopped. This implies that the observed decline in the Fulton index may not be due to the elimination period itself, but rather the prolonged impact of the MPs. Our results align with those of Alomar et al. (2021), who also did not observe significant differences in *S. aurata* size between the control and the MPs treatments. However, they did notice a decrease in Fulton's index in the MPs treatment after one month of detoxification, but this was attributed to natural changes in water temperature, that decreased during the last months of the experiment, which did not occur in our experiment. Contrary to our findings, juvenile meagre *Argyrosomus regius* exposed to 3 μg/L of fluoxetine for 15 days exhibited a decrease in specific growth rate, weight and length, which was associated to serotonin-mediated appetite suppression. Although the Fulton's index decreased, this change was not statistically significant (Duarte et al., 2020). These differences may be linked to species-specific variations in antidepressant metabolism or differences in the antidepressants used (Smith et al., 2010). Likewise, Maulvault et al. (2018) found that juvenile fish *A. regius* exposed to VFX did not show significant differences in weight and length compared to the control group, which is consistent with our results.

Regarding fish behavior, we observed that juveniles exposed to either contaminant individually spent more time motionless compared to control group and exhibited reduced aggression, as evidenced by fewer bites towards conspecifics in VFX and co-exposure treatments, compared to the MPs group. Contrary to our findings, Schmieg et al. (2022) did not observe differences in the stationary behavior of juvenile brown trout exposed to MPs. However, according to Yin et al. (2019) black rockfish exposed to MPs displayed a decrease in foraging and exploratory behavior, which could potentially explain the increase in time spent stationary. The sedative effect of VFX likely explains the freezing behavior observed, which is consistent with documented effects in other fish species. For instance, brown trout exposed to the antidepressant amitriptyline demonstrated decreased swimming velocity and distance covered. Like our results, brown trout did not show any significant changes when exposed to MPs and amitriptyline together (Schmieg et al., 2020). However, the MPs_VFX treatment resulted in a slight,

though not statistically significant, decrease in stationary behavior. This may be due to a high concentration of MPs reducing the bioavailability of VFX in the water, thereby diminishing its impacts on fish behavior. In a study with juvenile brown trout exposed to amitriptyline and MPs at different concentrations, it was observed that at lower MP concentrations, the fish exhibited the same reduced swimming activity and increased freezing behaviors as those exposed to the antidepressant alone. However, at higher MP concentrations the behavioral effect of amitriptyline was slightly diminished, supporting the idea that MPs can indeed adsorb the contaminant, thereby reducing their exposure to fish (Schmieg et al., 2022).

During the elimination period, no significant differences were observed between the control and any of the three exposure treatments. This could be attributed to the conditions being reverted to the same as experienced in the control group, suggesting that the effects observed in each different treatment, may have also been reverted to control-like patterns. This is supported by the post-hoc analysis, which revealed significant behavioral changes between the exposure and elimination periods for all three contaminant treatments, but not for the control.

In the present study, biting frequency decreased with exposure to VFX and the combination of VFX and MPs. Similarly, in a study on aggression and competitive territory contests between *Pimephales promelas* males, a slight, though not significant, decrease in biting behavior was observed with exposure to virgin MPs and MPs with low concentrations of 17-alpha ethinyl estradiol (EE2) (Swank et al., 2022). EE2 is a synthetic estrogen commonly found in oral contraceptive pills that can interfere with the endocrine system of marine organisms (Miyagawa, Sato and Iguchi, 2016). In contrast, a slight increase in biting behavior was observed in groups exposed to MPs with a high concentration of EE2 when compared to the control. However, as seen in our results, Rios-Fuster et al. (2021) reported that *S. aurata* juveniles exposed to MPs exhibited increased biting and chasing behaviors, though not statistically significant.

Serotonin (5-hydroxytryptamine, 5-HT) plays a key role in regulating several fundamental functions, including motor control, arousal, sleep, feeding, social behavior, learning, and memory (Bacqué-Cazenave et al., 2020). According to Kania and Wrońska (2015), 5-HT modulation is involved in aggression patterns towards conspecifics, with its concentration in the brain inversely proportional to aggression levels (i.e., a decrease in 5-HT in the brain results in increased aggression, while the increase of 5-HT reduces it). This may explain our findings, as VFX might increase 5-HT levels in juvenile *S. aurata*, thereby reducing aggressive behaviors such as biting and chasing. In Kania and Wrońska (2015) study, exposure to sertraline, an SSRI antidepressant, at concentrations of 0.4 µg/L and 4.0 µg/L, in adult male *Betta splendens* increased synaptic levels of 5-HT, leading to a reduction in aggressive behaviors, such as ruffing fins, frontal attacks, and biting the intruder.

However, VFX exposure has also been shown to increase aggressive behavior in fish. For instance, Parrott and Metcalfe (2018) found that adult male fathead minnows exposed to 75 µg/l VFX (the highest concentration) over a full life cycle (167–168 days) attacked a dummy intruder fish more frequently in defense of an empty nest compared to the control. These contradictory responses may stem from various factors, such as differences in species, age, sex, dose and type of antidepressant, which can influence the response of 5-HT levels to the antidepressant, in this case, VFX (Mennigen et al., 2011). For example, after 4 weeks of exposure to the antidepressant fluoxetine, the same species, fathead minnows, exhibited a significant increase in male-to-female aggression, resulting in 67% fatalities (Weinberger and Klaper, 2014). These observations, together with the fact that all the above-mentioned studies focused on adult individuals, highlight the importance of our study, especially in understanding the consequences for juveniles. This allows us to determine whether juveniles respond differently to the contaminants than adults.

Critchell and Hoogenboom (2018) suggest that activity and aggression are correlated, as their experiment demonstrated that juvenile *Acanthochromis polyacanthus* became more active in the aquariums due to ongoing aggressive interactions. However, MPs exposure did not have significant effects on the frequency of aggressive behavior between dominant and submissive individuals during feeding. As previously mentioned, MPs at high concentrations can reduce the bioavailability of antidepressants in the water, thereby diminishing their effect on the fish (Schmieg et al., 2022). Moreover, MPs have been observed to exert synergistic effects by amplifying the impacts of other contaminants (Lu et al., 2018; Haghi and Banaee, 2016). This hypothesis could explain the results observed in the fish bite behavior when exposed to a combination of MPs and VFX. Specially, the decrease in biting behavior, when compared to control, mirrors the slight reduction seen with VFX exposure alone, although this was not statistically significant. The bioaccumulation of VFX by MPs may have amplified the antidepressant's effects, leading to the observed decrease in biting behavior.

Fish under all treatments exhibited a decrease in RMR when compared to the control, with VFX showing the smallest decrease among all treatments. However, these changes were not statistically significant. This observation aligns with findings by Watts et al. (2016), who reported a decrease in the oxygen consumption in the shore crab *Carcinus maenas* following exposure to MPs, with levels returning to normal after 16 hours. The decrease in oxygen consumption was attributed to minor effects on ion exchange induced by the plastic exposure, which were subsequently mitigated by physiological regulation, proving their resilient. In contrast, fish may not exhibit the same level of resilience as shore crabs, potentially being less capable of regulating ion exchange changes by the inhalation of MPs into the gills. This could explain the decrease in RMR observed in our study. In contrast, MPs exposure has also been correlated with increased oxygen consumption in juvenile black rockfish after 14 days of exposure, with a slight recovery after 7 days of depuration. This finding suggests that MPs induce respiratory stress in fish, which Yin et al. (2019) attribute to a possible defense mechanism involving the skin, respiratory system and metabolism.

As previously discussed, 5-HT regulates vital functions, including behavior, locomotion and cardio-ventilatory activity. It has been observed that an injection of 5-HT in the shore crab *Carcinus maenas* caused an immediate increase in both cardiac and ventilatory activity. Similarly, exposure to the antidepressant fluoxetine resulted in increased cardio-ventilatory activity, confirming that fluoxetine increases 5 HT levels in synapses (Robert et al., 2019). A similar response would be expected in our study, given that VFX increases 5-HT levels, however, our results showed a slight decrease in RMR following exposure to VFX. This may be explained by the anxiolytic effect of VFX in fish, which keeps them in a calmer state and could potentially reduce their metabolism and oxygen consumption (Simmons et al., 2017, Ziegler et al., 2021). The decrease in RMR observed during the simultaneous exposure to MP and VFX, though not significant, could also be explained by this anxiolytic effect of VFX, together with the bioaccumulation of VFX by MPs in fish (Qu et al., 2019). This accumulation might amplify the anxiolytic effects of VFX, potentially resulting in a much lower oxygen consumption.

5. Conclusion

Estimating the energy requirements for fish growth and survival relies on understanding their metabolism, which is closely linked to respiration (Jobling, 1982). Oxygen consumption, herein measured as RMR, serves as a reliable indicator of metabolic activity and overall fish health. Any diversion of energy towards coping with environmental contaminants, such as MPs and VFX, could impair key metabolic or physiological processes. On another hand, complex behaviors adopted by fish are what sustain aquatic communities and the structure of fish populations. These behaviors serve as a link between the physiological and ecological processes, serving as an early indicator of stress or health issues within fish populations (Kasumyan, 2001; Scott and Sloman, 2004). Therefore, observing fish behaviors allows us to detect if they are experiencing stress or adverse conditions, often evidenced by changes in social and feeding behavior (Correia et al., 2007). Disruptions to these behaviors, whether in social hierarchy or prey-predator dynamics, can cascade through ecosystems, affecting the entire marine food web and ecosystem health. For example, impaired prey capture or predators' evasion may reduce individual growth rates and survival, ultimately influencing population fitness and ecosystem dynamics (Ferreira et al., 2016). This highlights the relevance of the present study in understanding the consequences that the increase of these contaminants (i.e. MPs and VFX) have in the individuals, their communities and the whole ecosystem (Mattsson et al., 2014; Prichard and Granek, 2016).

In our study, the contaminants caused significant changes in fish behaviors. In fact, fish spent more time static when exposed to the two contaminants isolated. They were also less aggressive when exposed to MPs and VFX combined, as indicated by fewer biting incidents. Additionally, fish under all treatments showed a non-significant reduction in oxygen consumption (RMR) compared to control, suggesting a potential shift in response to contaminants. Moreover, while comparing our results with published data, we found life-stage-dependent responses to VFX and MPs. While juveniles displayed decreased aggression, which could affect their development and social integration, adults, on the other hand, may exhibit exacerbated aggressive behavior under chronic contaminant exposure, particularly in reproductive contexts. These differences highlight the importance of considering life stage when assessing the impacts of contaminants such as VFX and MPs, suggesting that the effects observed in juveniles may not translate directly to adults, but still have the potential to influence population dynamics and ecosystem health.

It must be kept in mind several factors that might have influenced the study's results. First, the 14-day exposure period may have been insufficient to detect significant effects on fish behavior, metabolism and overall health condition. Extending the exposure duration could provide a clearer picture of the long-term impacts of MPs and VFX. Additionally, the reduction in number of individuals per aquarium between periods due to fish sampling for biomarkers analysis (data under treatment) may have influenced the hierarchical structure, thereby altering behavior. While sampling was balanced across treatments, it is important to acknowledge this factor when interpreting behavioral changes. However, the absence of differences in control treatments between periods suggests that the reduction in the number of individuals likely did not drive the observed effects.

It is crucial to continue investigating the effects of MPs and VFX on the overall health of fish. Future studies should focus on their cellular-level impacts, including how these contaminants influence oxidative stress and the activity of metabolic enzymes, as well as their potential for bioaccumulation in tissues. Special attention should be given to early life stages of commercially important species, as exposure to these contaminants could hinder their development, affecting their ability to reach adulthood and reproduce.

Understanding how contaminants like microplastics and venlafaxine affect fish behavior and metabolism is not just crucial for preserving aquatic life, but also for safeguarding the integrity and balance of entire ecosystems. As environmental contamination increases so too does the importance of recognizing the far-reaching consequences of these pollutants on marine diversity.

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7. Annex

Supplementary table S1. Water parameters (calculation of the mean and standard deviation of the 4 aquariums per treatment for the parameters temperature and salinity)

	Temperature		Salinity	
	Mean	SD	Mean	SD
Control	18.0	0.079	35.3	0.71
MP	18.1	0.15	35.4	0.95
VFX	18.0	0.04	35.7	0.69
MP+VFX	17.9	0.05	35.5	0.92

Supplementary table S2. Mean and standard deviation of fish weight (grams), standard (cm) and total length (cm).

	Exposure						**Elimination**					
Treatment	**weigth (g)**		**standard length (cm)**		**total length (cm)**		**weigth (g)**		**standard length (cm)**		**total length (cm)**	
	mean	DP	mean	DP	mean	DP	mean	DP	mean	DP	mean	DP
Control	8.43	1.93	6.97	0.64	8.68	0.68	10.20	2.41	7.53	0.62	9.11	0.70
MP	8.24	1.10	7.30	0.74	8.62	0.66	9.48	1.26	7.26	0.37	8.77	0.48
VFX	9.33	2.08	7.37	0.60	8.91	0.79	9.26	1.86	7.16	0.59	8.74	0.70
MP+VFX	9.59	1.34	7.42	0.43	8.90	0.68	9.69	1.25	7.30	0.34	8.96	0.40

Supplementary table S3. Weight glmmTMB summary (Significant differences codes: '***' 0.001; '**' 0.01; '*' 0.05; '.' 0.1)

Term	**Estimate**	**Std..Error**	**z.value**	**Pr...z..**	
(Intercept)	10.20	0.539	18.9	5.5E-80	
MICROPLASTICS	-0.720	0.708	-1.02	0.309	
VENLAFAXINE	-0.516	0.708	-0.729	0.466	
MP_VFX	-0.949	0.708	-1.34	0.180	
Elimination	-1.77	0.708	-2.51	0.0122	*
MICROPLASTICS:Elimination	0.533	1.001	0.533	0.594	
VENLAFAXINE:Elimination	1.68	1.001	1.68	0.0937	
MP_VFX:Elimination	1.85	1.001	1.84	0.0651	

Supplementary table S4. Total length glm summary

	Estimate	Std. Error	t value	Pr(>|t|)
(Intercept)	2.1921	0.0190	115.3116	8.77E-79
MICROPLASTICS	-0.0228	0.0244	-0.938	0.352
VENLAFAXINE	0.0036	0.0240	0.150	0.881
MP_VFX	-0.0077	0.0242	-0.321	0.750
Elimination	-0.0132	0.0171	-0.772	0.443

Supplementary table S5. Standard length glm summary (Significant differences codes: '***' 0.001; '**' 0.01; '*' 0.05; '.' 0.1)

	Estimate	Std. Error	t value	Pr(>|t|)
(Intercept)	1.98	0.0206	96.459	1.3E-73
MICROPLASTICS	0.0037	0.0260	0.142	0.888
VENLAFAXINE	0.0151	0.0259	0.582	0.563
MP_VFX	0.0014	0.0261	0.0522	0.959
Elimination	-0.0064	0.0183	-0.347	0.730

Supplementary table S6. Post-hoc of weight (Significant differences codes: '***' 0.001; '**' 0.01; '*' 0.05; '.' 0.1)

contrast	Treatment	Estimate	SE	df	t.ratio	p.value	
Exposure - Elimination	CONTROL	1.77	0.708	61	2.51	0.0149	*
Exposure - Elimination	MICROPLASTICS	1.24	0.708	61	1.75	0.0845	
Exposure - Elimination	VENLAFAXINE	0.0967	0.708	61	0.137	0.8918	
Exposure - Elimination	MP_VFX	-0.0721	0.708	61	-0.102	0.9192	

Supplementary table S7. Fulton index glm summary (Significant differences codes: '***' 0.001; '**' 0.01; '*' 0.05; '.' 0.1)

	Estimate	Std. Error	t value	Pr(>|t|)	
(Intercept)	0.856	0.0312	27.455	4.05E-37	
MICROPLASTICS	0.0537	0.0430	1.25	0.216	
VENLAFAXINE	0.0527	0.0430	1.23	0.225	
MP_VFX	0.0598	0.0428	1.40	0.167	
PeriodElimination	0.0464	0.0431	1.08	0.285	
MICROPLASTICS:Elimination	-0.181	0.0622	-2.91	0.0050	**
VENLAFAXINE:Elimination	-0.105	0.0610	-1.72	0.0906	
MP_VFX:Elimination	-0.128	0.0611	-2.10	0.0401	*

Supplementary table S8. Post-hoc for treatments summary of Fulton index (Significant differences codes: '***' 0.001; '**' 0.01; '*' 0.05; '.' 0.1)

contrast	Period	Estimate	SE	df	t.ratio	p.value	
CONTROL - MICROPLASTICS	Exposure	-0.0537	0.0430	64	-1.249	0.598	
CONTROL - VENLAFAXINE	Exposure	-0.0527	0.0430	64	-1.225	0.613	
CONTROL - MP_VFX	Exposure	-0.0598	0.0428	64	-1.397	0.506	
MICROPLASTICS - VENLAFAXINE	Exposure	0.0010	0.0418	64	0.024	1.000	
MICROPLASTICS - MP_VFX	Exposure	-0.0062	0.0417	64	-0.149	0.999	
VENLAFAXINE - MP_VFX	Exposure	-0.0072	0.0417	64	-0.172	0.998	
CONTROL - MICROPLASTICS	Elimination	0.1272	0.0450	64	2.823	0.0313	*
CONTROL - VENLAFAXINE	Elimination	0.0521	0.0432	64	1.205	0.626	
CONTROL - MP_VFX	Elimination	0.0682	0.0436	64	1.565	0.406	
MICROPLASTICS - VENLAFAXINE	Elimination	-0.0751	0.0461	64	-1.629	0.370	
MICROPLASTICS - MP_VFX	Elimination	-0.0589	0.0465	64	-1.269	0.586	
VENLAFAXINE - MP_VFX	Elimination	0.0162	0.0447	64	0.361	0.984	

Supplementary table S9. Post-hoc for periods summary of Fulton index (Significant differences codes: '***' 0.001; '**' 0.01; '*' 0.05; '.' 0.1)

contrast	Treatment	Estimate	SE	df	t.ratio	p.value	
Exposure - Elimination	CONTROL	-0.0464	0.0431	64	-1.08	0.285	
Exposure - Elimination	MICROPLASTICS	0.134	0.0449	64	2.99	0.00392	**
Exposure - Elimination	VENLAFAXINE	0.0583	0.0431	64	1.35	0.181	
Exposure - Elimination	MP_VFX	0.0816	0.0433	64	1.88	0.0641	

Supplementary table S10. Swimming summary from GlmmTMB (Significant differences codes: '***' 0.001; '**' 0.01; '*' 0.05; '.' 0.1)

Term	Estimate	Std..Error	z.value	Pr...z..	
(Intercept)	4.7797	0.0098	488.992	0.000	***
MICROPLASTICS	-0.0225	0.0114	-1.975	0.048	*
VENLAFAXINE	-0.0102	0.0113	-0.900	0.368	
MP_VFX	0.0025	0.0113	0.224	0.823	
Elimination	-0.0015	0.0084	-0.184	0.854	

Supplementary table S11. Swimming post-hoc for treatments summary

contrast	Estimate	SE	df	t.ratio	p.value
CONTROL - MICROPLASTICS	0.0225	0.0114	270	1.98	0.200
CONTROL - VENLAFAXINE	0.0102	0.0113	270	0.90	0.805
CONTROL - MP_VFX	-0.0025	0.0113	270	-0.22	0.996
MICROPLASTICS - VENLAFAXINE	-0.0123	0.0110	270	-1.12	0.678
MICROPLASTICS - MP_VFX	-0.0250	0.0109	270	-2.29	0.103
VENLAFAXINE - MP_VFX	-0.0127	0.0109	270	-1.17	0.646

Supplementary table S12. Swimming post-hoc for periods summary

contrast	Estimate	SE	df	t.ratio	p.value
Exposure - Elimination	0.0015	0.0084	270	0.184	0.854

Supplementary table S13. Stationary summary from GlmmTMB with interactions between treatments and periods (Significant differences codes: '***' 0.001; '**' 0.01; '*' 0.05; '.' 0.1)

Term	Estimate	Std..Error	z.value	Pr...z..	
(Intercept)	2.01	0.424	4.75	2.04E-06	***
MICROPLASTICS	0.858	0.259	3.31	0.00093	***
VENLAFAXINE	0.700	0.261	2.68	0.0074	**
MP_VFX	0.324	0.376	0.863	0.388	
Elimination	0.153	0.315	0.484	0.628	
MICROPLASTICS:Elimination	-0.470	0.340	-1.38	0.166	
VENLAFAXINE:Elimination	-0.550	0.375	-1.47	0.142	
MP_VFX:Elimination	-1.01	0.485	-2.09	0.037	*

Supplementary table S14. Stationary post-hoc for treatments summary

contrast	PERIOD	estimate	SE	df	z.ratio	p.value	
CONTROL - MICROPLASTICS	Exposure	-0.858	0.259	Inf	-3.31	0.0052	*
CONTROL - VENLAFAXINE	Exposure	-0.700	0.261	Inf	-2.68	0.037	*
CONTROL - MP_VFX	Exposure	-0.324	0.376	Inf	-0.86	0.824	
MICROPLASTICS - VENLAFAXINE	Exposure	0.158	0.168	Inf	0.94	0.783	
MICROPLASTICS - MP_VFX	Exposure	0.534	0.322	Inf	1.66	0.347	
VENLAFAXINE - MP_VFX	Exposure	0.376	0.304	Inf	1.23	0.605	
CONTROL - MICROPLASTICS	Elimination	-0.388	0.214	Inf	-1.82	0.266	
CONTROL - VENLAFAXINE	Elimination	-0.150	0.288	Inf	-0.52	0.954	
CONTROL - MP_VFX	Elimination	0.689	0.298	Inf	2.31	0.095	
MICROPLASTICS - VENLAFAXINE	Elimination	0.238	0.226	Inf	1.05	0.719	
MICROPLASTICS - MP_VFX	Elimination	1.077	0.252	Inf	4.28	0.00011	*
VENLAFAXINE - MP_VFX	Elimination	0.839	0.291	Inf	2.88	0.021	*

Supplementary table S15. Stationary post-hoc for periods summary

contrast	TREATMENT	estimate	SE	df	z.ratio	p.value	
Exposure - Elimination	CONTROL	-0.153	0.315	Inf	-0.484	0.628	
Exposure - Elimination	MICROPLASTICS	0.318	0.135	Inf	2.36	0.018	*
Exposure - Elimination	VENLAFAXINE	0.398	0.196	Inf	2.03	0.042	*
Exposure - Elimination	MP_VFX	0.861	0.383	Inf	2.25	0.025	*

Supplementary table S16. Chase summary from GlmmTMB

Term	Estimate	Std..Error	z.value	Pr...z..
(Intercept)	1.35	0.29	4.69	2.67E-06
MICROPLASTICS	0.32	0.30	1.06	0.29
VENLAFAXINE	-0.021	0.31	-0.069	0.95
MP_VFX	0.11	0.31	0.36	0.72
Elimination	0.13	0.23	0.56	0.58

Supplementary table S17. Bite summary from GlmmTMB (Significant differences codes: '***' 0.001; '**' 0.01; '*' 0.05; '.' 0.1)

Term	Estimate	Std..Error	z.value	Pr...z..	
(Intercept)	1.36	0.288	4.73	2.19E-06	***
MICROPLASTICS	0.107	0.144	0.738	0.461	
VENLAFAXINE	-0.275	0.156	-1.77	0.0768	
MP_VFX	-0.305	0.152	-2.00	0.0454	*
Elimination	-0.058	0.097	-0.594	0.553	

Supplementary table S18. Bite post-hoc summary (Significant differences codes: '***' 0.001; '**' 0.01; '*' 0.05; '.' 0.1)

contrast	estimate	SE	df	z.ratio	p.value	
CONTROL - MICROPLASTICS	-0.107	0.144	Inf	-0.738	0.882	
CONTROL - VENLAFAXINE	0.275	0.156	Inf	1.77	0.288	
CONTROL - MP_VFX	0.305	0.152	Inf	2.00	0.188	
MICROPLASTICS - VENLAFAXINE	0.382	0.128	Inf	2.98	0.015	*
MICROPLASTICS - MP_VFX	0.411	0.124	Inf	3.30	0.0053	**
VENLAFAXINE - MP_VFX	0.029	0.135	Inf	0.218	0.996	

Supplementary table S19. RMR summary from glm

Term	Estimate	Std..Error	z.value	Pr...z..
(Intercept)	220.1	35.50	6.20	5.67E-10
MICROPLASTICS	-37.10	48.39	-0.767	0.443
VENLAFAXINE	-10.44	48.39	-0.216	0.829
MP_VFX	-40.63	50.21	-0.809	0.418

Supplementary table S20. RMR post-hoc summary ("diff" shows the average difference between treatments, "lwr" and "upr" show the lower and upper limits of the 95% confidence interval for each difference and "p adj" shows the adjusted p-values for multiple comparisons)

Treatment	diff	lwr	upr	p adj
CONTROL - MP	-37.10	-183.16	108.96	0.894
CONTROL - VFX	-10.44	-156.50	135.62	0.997
CONTROL - MP_VFX	-40.63	-192.20	110.95	0.878
MP - VFX	26.66	-113.68	166.99	0.952
MP - MP_VFX	-3.53	-149.59	142.53	0.9999
VFX - MP_VFX	-30.18	-176.25	115.88	0.939