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The role of microplastics and bacteria in host-pathogen interactions

Ana Margarida Rijo Vieira Fernandes

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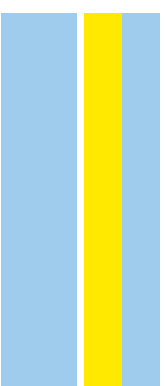The role of microplastics and bacteria in host-pathogen interactions
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The role of microplastics and bacteria in host-pathogen interactions

Dissertação de Candidatura ao grau de Mestre em Toxicologia e Contaminação Ambientais submetida ao Instituto de Ciências Biomédicas de Abel Salazar da Universidade do Porto.

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Acknowledgments

Thanks to every single person listed. Each one of you were remarkable and I wish you all the best! You deserve it!

This thesis is dedicated to my parents and my two brothers.

Thank you for your patience, your sacrifices and for all hours spend on the phone.

Words will never be enough to describe everything you've done for me.

Abstract

Plastics entered our life with a massification of both their utilities and their production, leading to an increase of these particles in the environment and in organisms. The breakdown of these plastics in the environment results in microplastics (MPs), small pieces of plastics with size between 1 µm - 5 mm. Smaller plastic are called nanoplastics. These micro- and nanoplastics have potential negative effects by themselves but also in association with organic contaminants, bacteria, heavy metals and additives incorporated during their production. In fish*, Photobacterium damselae piscicida (Phdp*) causes a bacterial septicemia disease called photobacteriosis, responsible for important economic losses in fish farming.

The main objective of this study was to evaluate the effects of MPs (0.184 mg/l and 0.216 mg/l) colonized with the bacteria *Phdp* (1×10^8 cfu/ml) on the European seabass (*Dicentrarchus labrax*).

In a first preliminary trial, the colonization of MPs by *Phdp* was investigated using bacteria growth media (TSB), natural seawater and artificial seawater as aqueous media. The bacteria were able to colonize the MPs. MPs bacterial colonization was higher, take more time to reach the pike of colonization, and the colonization time was higher in TSB than in the other tested media.

After the first trial, a seven-day *in vivo* bioassay to investigate the effects of MPs colonized by the bacteria on *D. labrax* was carried out. Groups of fish were exposed through feed to the following treatments: **i)** Control, **ii)** Bacteria, **iii)** Low MPs concentration, **iv)** High MPs concentration, **v)** Low MPs concentration with bacteria and **vi)** High MPs concentration with bacteria. In relation to the control group, fishes exposed to MPs colonized with the bacteria presented a possible interference with the vitamin B12 metabolism, increased mean corpuscular haemoglobin, reduction of red blood cell number, and increased haemoglobin concentration. The innate immune response was also activated after exposure to colonized MPs with an increase of white blood cell counts that may be linked to an immunity response to the live pathogen in the gut in some groups, and in others, microplastics may cause toxicity and probably inflammation. Regarding innate immune parameters, proteases were increased in fishes exposed to MPs colonized with bacteria when compared to the other groups, indicating some degree of infection. Anti-proteases did not present variations when compared to the control group. Regarding oxidative stress biomarkers, the activity of catalase and glutathione-S-transferases in liver showed no significant differences among fish exposed to different

treatments; however, fish exposed to colonized MPs had increased lipid peroxidation levels indicating lipid oxidative damage.

Overall, this study contributed to understand the interactive effects of MPs and pathogenic bacteria in a carnivorous fish as host.

Resumo

Os plásticos entraram em força na nossa vida, devido às suas utilidades levando a um aumento da sua produção e consequente entrada destas partículas no ambiente e nos organismos. A quebra desses plásticos no ambiente resulta em microplásticos (MPs), pequenos pedaços de plástico com tamanho entre 1 µm - 5 mm. Fragmentos menores do que estes são chamados de nanoplásticos. Estes micro e nanoplásticos têm potencialmente efeitos negativos por si mesmos, mas também em associação com contaminantes orgânicos, bactérias, metais pesados e aditivos incorporados durante a sua produção. Em peixes, a *Photobacterium damselae piscicida* (Phdp) causa uma doença de septicemia bacteriana chamada fotobacteriose, responsável por importantes perdas econômicas em pisciculturas.

O principal objetivo deste estudo foi avaliar os efeitos dos microplasticos (0,184 mg/l e 0,216 mg/l) colonizados com a bactéria Phdp ($1x10^8$ cfu/ml) no robalo (*Dicentrarchus labrax*).

Antes do bioensaio foi necessário um estudo preliminar, analisando a colonização dos microplásticos pela bactéria, usando três meios, o meio de crescimento bacteriano (TSB), a água do mar natural e a água do mar artificial. As bactérias foram capazes de colonizar os MPs em todos os meios estudados. Verificou-se que a colonização bacteriana dos microplásticos foi maior, demorou mais para atingir o pico de colonização e o tempo de colonização foi maior no meio TDS em comparação com os demais meios testados.

Após este primeiro ensaio, conduziu-se um bioensaio de sete dias in vivo para investigar os efeitos de MPs colonizados pelas bactérias em *D. labrax*. Onde foram utilizados 24 peixes por tratamento. Estes peixes foram expostos através da comida, a sete tratamentos: i) grupo controlo, em que os peixes são alimentados com ração comercial , ii) grupo alimentado com ração contaminada com bactérias, iii) grupo alimentado com ração contaminada com a menor concentração de MPs, iv) grupo alimentado com ração contaminada com a maior concentração de MPs, v)) grupo alimentado com ração contaminada com a menor concentração de MPs colonizados com as bactérias e vi)) grupo alimentado com ração contaminada com a maior concentração de MPs colonizados com as bactérias. Em relação ao grupo controlo, os peixes expostos aos microplásticos colonizados pela bactéria apresentaram uma possível interferência no metabolismo da vitamina B12, levando a um aumento da média da hemoglobina corpuscular, redução do número de hemácias e aumento da concentração de hemoglobina. A resposta imune inata também foi ativada após a exposição a MPs

colonizados, com um aumento na contagem de leucócitos que pode estar ligada a uma resposta imunitária ao patogénio vivo no intestino podendo causar toxicidade e provavelmente inflamação. Em relação aos parâmetros imunes inatos, verificou-se um aumento das proteases nos peixes expostos aos MP colonizados por bactérias quando comparados aos demais grupos, indicando uma possível infeção. As anti-proteases por sua vez não apresentaram variações quando comparadas ao grupo controlo. Quanto aos biomarcadores de stresse oxidativo analisados no fígado, tanto a catalase como a glutationa S-transferase não apresentou diferenças significativas, no entanto, peixes expostos a MPs colonizados tiveram um aumento dos níveis de peroxidação lipídica, indicando possíveis danos oxidativos.

Em geral, este estudo contribuiu para entender os efeitos da interação dos MPs e das bactérias patogénicas.

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Abbreviation Index

[ ]	Concentration
°C	Celsius degree
µm	Micrometer
AChE	Acetylcholinesterase
amp	Ampicillin
ara	Arabinose
ASW	Artificial seawater
BSA	Diluted albumin standards
CaCl$_2$	Calcium chloride
CAT	Catalase enzyme
cc	Cubic centimeter
cfu	Colony forming units
cm	Centimeters
DGAV	Direção-Geral de Alimentação e Veterinária
EU	European union
FAO	Food and Agriculture Organization of the United Nations
g	Gram
GFP	Green fluorescent protein
GST	Glutathione *S*-transferase activity
h	Hours
H$_2$O	Water
LPO	Lipid peroxidation
M	Molar
MCH	Mean corpuscular haemoglobin
min	Minutes
ml	Milliliter
mm	Millimeter
MPs	Microplastics
NaCl	Sodium chloride
NaH$_2$PO$_4$	Monosodium phosphate
NaHCO$_3$	Sodium bicarbonate
NaOH	Sodium hydroxide
nm	Nanometer
nmol	Nanomole
NP	Nanoplastics

OD	Optical density
PBS	Phosphate buffer
Phdp	*Photobacterium damselae piscicida*
PlasticsEurope	European Association of Plastics Producers
ppm	Part per million
RBC	Erythrocytes
rpm	Rotations per minute
SD	Standard deviation
SEM	Standard error of the mean
SW	Natural seawater
TBA	2- thiobarbituric acid
TBARS	Thiobarbituric acid reactive substances
TCA	Trichloroacetic acid
TSA	Tryptic soy agar
TSB	Tryptic soy broth
UV	Ultraviolet light
w/v	Weight / volume
WBC	Leucocytes
X g	G force
µl	Microliter

1. Introduction

Plastics entered our life in a relatively short time, with a massification of both their uses (medical instruments, smartphones, clothes) and their production (Luís et al. 2015). This massification occurs because they are very cost-effective, strong, durable, corrosion-resistant and water-resistant, chemically inert and exhibit good thermal and electrical insulating properties (Cole et al. 2011; Derraik 2002; Espinosa, Esteban, and Cuesta 2016; LI, TSE, and FOK 2016; Thompson et al. 2009). According to the European Association of Plastics Producers (PlasticsEurope) global plastics production in 2015 was 322 million tons of which about 270 million relate to polymers (PlasticsEurope 2016). The most commonly polymers used as plastic materials are polyethylene, polypropylene, polystyrene, polyvinyl chloride and polyethylene terephthalate, which on the whole represent approximately 90% of total world plastic production, being also the polymers with more scientific studies (Andrady and Neal 2009; Espinosa, Esteban, and Cuesta 2016).

Plastics are fragmented into smaller plastics by chemical, physical and biological processes. When the particles are smaller than 5 mm are called microplastics (MPs) and vary in size, shape, colour, chemical composition and density (Barnes et al., 2009; Corcoran et al., 2009). If smaller, are called nanoplastics (NPs), but its size is not consensual [< 1 µm (Mark A. Browne, Galloway, and Thompson 2007; Cole and Galloway 2015), 1 nm-1 µm (Rist and Hartmann 2018), 1-100 nm (European Commission, 2011) and also < 100 nm (Horton et al. 2017; Sharma and Chatterjee 2017)].

In the last decades, plastic pollution has been globally recognized as a critical hazard for aquatic ecosystems. The degradation of macroplastic debris in the oceans, the beach litter, estuaries and the bodies of freshwater into MPs and NPs has recently become a significant concern and an increasingly important area of research (Andrady 2011). Due to the widespread use of plastics, as well as their persistence in the environment and the resistance to degradation by many plastic materials lead to their accumulation in the aquatic environment, constituting a threat to organisms (Mattsson et al. 2015). . In addition, they also have a potential to bioaccumulate and be transferee along the trophic chain. MPs have been found to accumulate in zooplankton (Cole et al. 2011), and NPs have been shown to pass from algae to zooplankton and fish (Mattsson et al. 2015).

Although the macroplastics are the most widely studied, MPs and NPs may actually be more pervasive by number in marine and freshwater environments (Cozar et al. 2014). To date, there is increasing evidence that the MPs can be taken up via ingestion into aquatic organisms. In the same line, NPs are the least well-studied form of plastic debris with little

information about their environmental concentrations, bioaccumulation potential, toxicity or biological effects (Pitt et al. 2018). Several authors have reported that MPs can be ingested by different marine and freshwater organisms, including polychaetes, crustacean, bivalves and echinoderms (Avio et al. 2015). Nevertheless, one of the main hazards of MPs is due to their properties, since they can also adsorb several classes of organic pollutants (Mark Anthony Browne et al. 2011), heavy metals (Barboza et al. 2018; Karami, Golieskardi, Ho, et al. 2017; Luís et al. 2015) and/or pathogen bacteria (Harrison et al. 2014; Kirstein et al. 2016; Viršek et al. 2017) as well as metals which may be transferred to organisms and enter the food webs. Nonetheless, the quantification of NPs in aquatic systems has been hampered by the limited availability of standardized protocols and technical difficulties because the necessary analytical methods are still under development (Koelmans, Besseling, and Shim 2015).

1.1. Microplastics

Microplastics can be divided between primary and secondary according to their usage and source (Espinosa, Esteban, and Cuesta 2016; Sharma and Chatterjee 2017). Primary MPs are manufactured to have a micro or lower range of size and enter into the environment already as MPs (Espinosa, Esteban, and Cuesta 2016; Sharma and Chatterjee 2017). They are used in the production of polymer consumer products, cosmetics and personal care products (such as toothpastes, exfoliating creams), synthetic textiles (a single machine wash can release about 1900 fibers), and are used in several other industries (Espinosa, Esteban, and Cuesta 2016; Sharma and Chatterjee 2017). Usually they are released directly into the sea or other water bodies such as lakes and rivers because they are not filtered during wastewater treatment (Auta, Emenike, and Fauziah 2017; Mark Anthony Browne et al. 2011; Espinosa, Esteban, and Cuesta 2016; Wardrop et al. 2016). Secondary MPs result from the environmental breakdown of larger plastic material, such as plastic debris (fishing nets, ropes and plastic bags) into smaller fragments by the action of water, ultraviolet lights (UV), time and temperature (LI, TSE, and FOK 2016; Moore 2008). Estimates indicate that 50% of plastic products are intended to be disposable, belonging to the so-called secondary MPs (Espinosa, Esteban, and Cuesta 2016; Hopewell, Dvorak, and Kosior 2009; LI, TSE, and FOK 2016).

The entry of MPs into the aquatic ecosystem is worrying because they can be ingested by organisms, including fishes (Alomar et al. 2017; Bellas et al. 2016; Güven et al. 2017; Lusher, McHugh, and Thompson 2013; Neves et al. 2015) and accumulate in several organs such as gills (Lu et al. 2016), gastrointestinal tract (Grigorakis, Mason, and Drouillard 2017; Jabeen et al. 2017; Lu et al. 2016), liver (Avio, Gorbi, and Regoli 2015)

and muscle (Akhbarizadeh, Moore, and Keshavarzi 2018; Karami, Golieskardi, Choo, et al. 2017). This accumulation can affect the fish organ and systems by association between MPs and other compounds, such as organic pollutants and heavy metals (Adeyemi, Adewale, and Oguma 2014; Brandao et al. 2015; Hermenean et al. 2015; Luís et al. 2015) or by itself (Chen et al. 2017; Espinosa, Cuesta, and Esteban 2017; Ferreira et al. 2016; Fonte, Ferreira, and Guilhermino 2016; Karami et al. 2016; Karami, Groman, et al. 2017; Lu et al. 2016; Luís et al. 2015). MPs can also be toxic because they contain additives added during their manufacture to increase processability, durability, elasticity, such as colorants, stabilizers, flame retardants, peroxide, antistatic agent, and plasticizers (Avio et al. 2015; Espinosa, Esteban, and Cuesta 2016; Karami, Groman, et al. 2017; LI, TSE, and FOK 2016).

MPs exposure affects the hepatic system elevating the degree of tissue change on the liver, some of the changes included necrosis, hemorrhaging, odemia, vacuolation and infiltration, but also inflammation and cellular stress (Espinosa et al., 2017; Ferreira et al., 2016; Fonte et al., 2016; Karami et al., 2017b, 2016; Lu et al., 2016; Luís et al., 2015). These changes may be investigated using histopathological methods but also using biomarkers like lipid peroxidation (LPO) levels. In the endocrine system, MPs can induce a down-regulation of vitellogenin, choriogenin and estrogenic receptor (Rochman et al., 2014). Regarding neurotoxicity, several MPs have been found to induce acetylcholinesterase (AChE) inhibition and increase lipid peroxidation levels in the brain (Barboza et al. 2018; Ferreira et al. 2016; Fonte, Ferreira, and Guilhermino 2016; Luís et al. 2015; Oliveira et al. 2013). The presence of MPs in the surrounding water also cause alterations in the predatory performance (Fonte, Ferreira, and Guilhermino 2016; Luís et al. 2015; de Sá, Luís, and Guilhermino 2015).

In the environment, MPs may be successively fragmented into NPs (Cozar et al. 2014). MPs and NPs started to be more studied because of their possible effects on the human health because of their ability to transfer along the food trophic chain, (see figure 1) (Chae et al. 2018; Farrell and Nelson 2013; Mattsson et al. 2015), and their presence on edible parts like muscle and dried fish muscle (Akhbarizadeh, Moore, and Keshavarzi 2018; Karami, Golieskardi, Choo, et al. 2017). Other major concern it´s the presence of MPs in fishes that may be used as food for aquaculture (such as sub products of fishing, guts, heads, bones and the fish remain of processing), indicating the possibility of MPs entry in aquaculture.
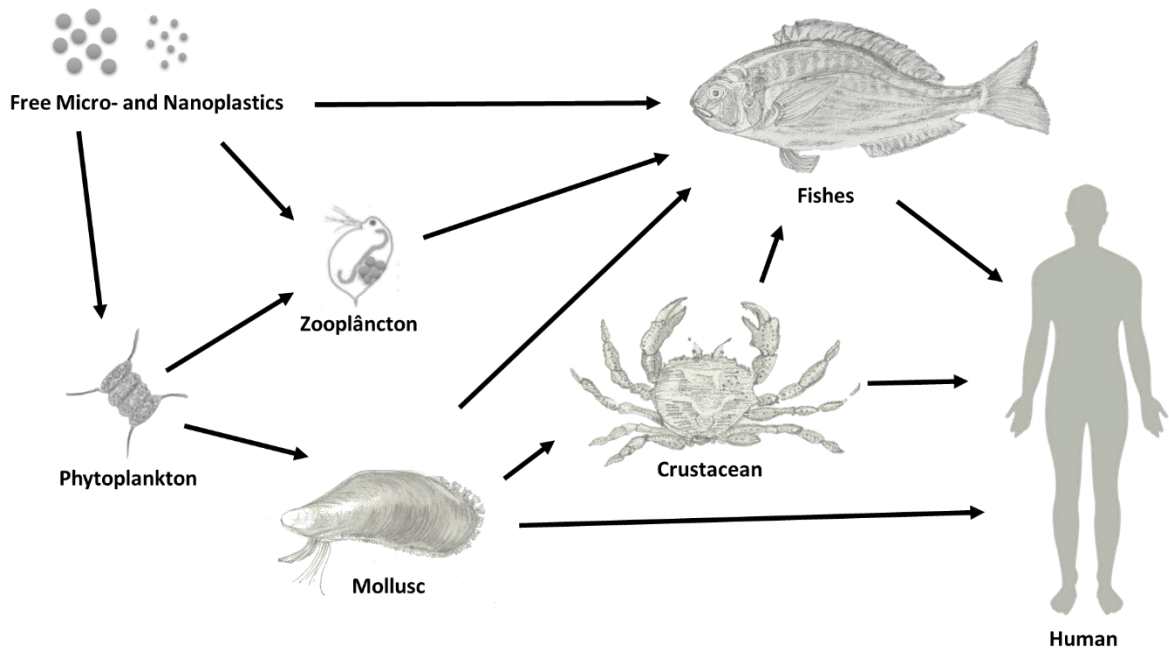
Figure 1. Bioaccumulation and transfer along the food chain of microplastics and nanoplastics.

1.2. *Photobacterium damselae piscicida*

Photobacterium damselae piscicida (Phdp) (Figure 2), causes a bacterial septicemia disease called pasteurellosis or photobacteriosis (Romalde 2002). This pathogen was first described after an epizootic outbreak in 1963 in the wild populations of Northern bass (*Morone americanus*) and striped bass (*Morone saxatilis*) in the USA (Chesapeake Bay) (Snieszko et al. 1964). Since then, it has been responsible for economic losses in Japan (Alicia E Toranzo, Magarinos, and Romalde 2005), Spain (Alicia E Toranzo et al. 1991), France (Baudin-Laurencin, Pepin, and Raymond 1991), Italy (Ceschia 1991), Greece (Bakopoulos, Adams, and Richards 1995) and Portugal (Baptista 1996).

Phdp strains are characterized by being gram negative, immobile and have coccobacillus forms depending on the culture time; this strains also have an elevated phenotypic and biochemical homogeneity (Thyssen et al. 1998). This pathology is temperature dependent and occurs when water temperatures rise above 20 °C; below 18 °C, fish can become vectors for long time periods and do not exhibit the disease (Romalde 2002). It is a commonly studied disease, is included in the list of diseases of mandatory declaration at Portuguese national level by the "Direção Geral de Alimentação e Veterinária (DGAV)", and is considered to be one of the most dangerous aquaculture diseases due to its high mortality rate, ubiquitous distribution and broad resistance to antibiotics (do Vale et al. 2005).

Phdp can be horizontally transmitted through the gills, gastrointestinal tract, by ingestion of contaminated foods or by the skin (Magariños et al. 1999; Magariños, Toranzo, and Romalde 1996). In the acute form of the disease, fish may not exhibit external clinical signs (Magariños, Toranzo, and Romalde 1996; Romalde 2002), but can present a large amount of hematopoietic stem cells in the gills and on the head, can also sometimes causes anemia (Liu, Lin, and Lee 2003; Alicia E Toranzo et al. 1991), the spleen, liver and kidney can be extended and have areas of multifocal necrosis and accumulation of bacteria (Magariños, Toranzo, and Romalde 1996; Romalde 2002; Alicia E Toranzo, Magarinos, and Romalde 2005). In the cchronic form, internal organs like the spleen and the liver, may present white nodules (0.5 to 3.5 mm) which are colonies of the bacterium (Magariños, Toranzo, and Romalde 1996; Romalde 2002; Alicia E Toranzo, Magarinos, and Romalde 2005).

Various vaccines against *Phdp* have been made, although only one enriched with extracellular products patented by the University of Santiago (Spain) has proved to be effective on larvae with only 50 days (A E Toranzo 2004). In the last years, antimicrobial agents have been widely used in the treatment of fish diseases, leading to the

appearance of strains resistant to these pharmaceuticals (Altinok and Kurt 2003). Bakopoulos et al. (1995) observed that European isolates of *Phdp* had a resistance to erythromycin, kanamycin and streptomycin. Japanese isolates of *Phdp* showed resistance to a greater number of antimicrobial agents including erythromycin, kanamycin, streptomycin, oxolinic acid, trimethoprim-sulfamethoxazole, cephaloridine and chloramphenicol (Bakopoulos, Adams, and Richards 1995). In addition to the acquisition of resistance to antibiotics, the ineffectiveness of antibiotic therapy may also be due to a period of intracellular parasitism (Romalde 2002).
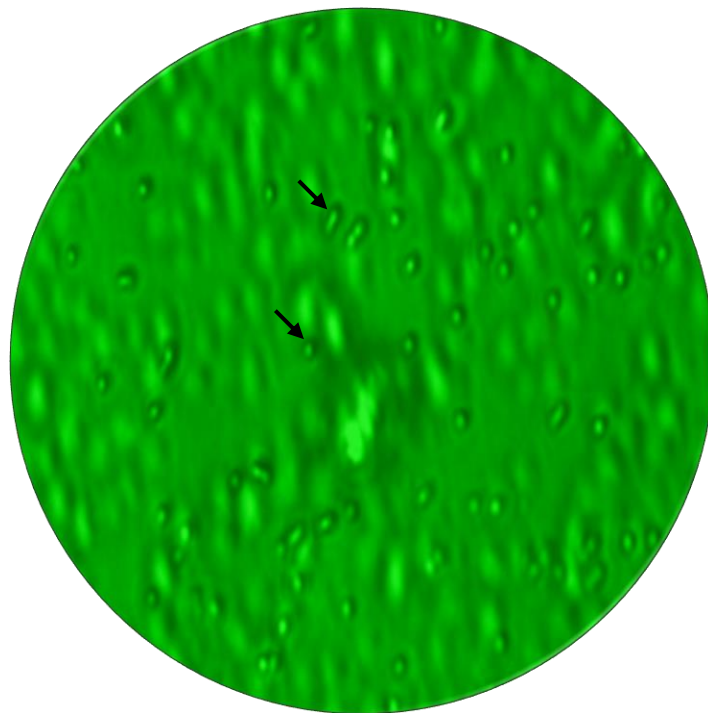
Figure 2. *Photobacterium damselae piscicida* (←) after being transformed with a fluorescent-label, that express green fluorescent protein (GFP) view using the fluorescence microscope using the FITC filter.

1.3. European seabass (*Dicentrarchus labrax)*

The European seabass *(Dicentrarchus labrax* Linnaeus, 1758) (Figure 3) presents a silvery grey to bluish on the back, silvery on the sides and the belly sometimes tinged with yellow. European seabass is an euryhaline (3‰ to full strength seawater) and eurythermic (2-32 °C) perciform fish, lives in coastal waters, up to a depth of 100 m, as well as in estuaries and coastal lagoons (at summer) and occasionally is also found in rivers. This species can be found in coastal waters of the Atlantic Ocean from the South of Norway to the Western Sahara and throughout the Mediterranean sea and the Black sea (Haffray et al. 2007). The European seabass is also a voracious predator, feeding on crustaceans, molluscs and small fish (Frimodt 1995). This species is also gonochoristic, females present a high fecundity (on average 200 000 eggs / kg of female) and start to reproduce in the wild life at different times. While in the Mediterranean sea, males attain the sexual maturity when they are 3 years old, females only attain it at four years old, however, in the Atlantic Ocean, the sexual maturity is attained at four and seven years for males and females, respectively (Allegrucci, Fortunato, and Sbordoni 1997; Haffray et al. 2007). Under farming conditions, male sexual maturity is reach at 2 years when they are about 200 g, whereas female sexual maturity is reached at 3 years old when they have a weight around 700g (Gorshkov et al. 2004).

Figure 3. European seabass (*Dicentrarchus labrax).*

This species is an economically important captured fish species in the Mediterranean coastal waters as is shown in the Figure 4. The excessive fishing effort became his main threat, being captured mainly with the troll, sport fishing line and gill nets. The increase in demand has led to a growing interest in aquaculture, as is shown in the Figure 5. The European seabass is the third most produced fish in aquaculture in Portugal, and as become one of the most studied species because they are subject to a wide range of

diseases leading to heavy losses in its production. Unfavourable environmental conditions and/or poor management practices may lead to stress leading to a reduction of growth rate and immune suppression resulting in increased susceptibility to an outbreak disease due to opportunistic pathogens (Afonso et al. 2005; Mauri et al. 2011).

Global Capture Production for species (tonnes)
Source: FAO FishStat
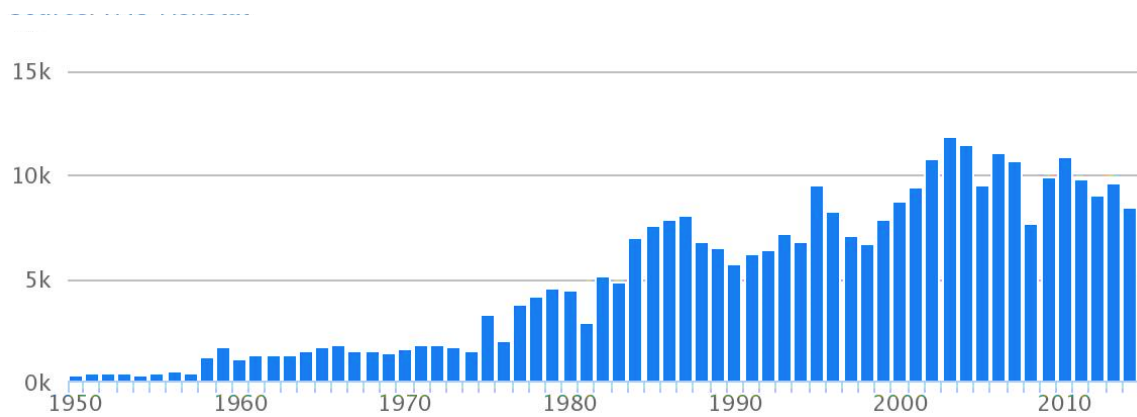
Figure 4. Global Capture Production of *Dicentrarchus labrax* until 2014. Source: FAO FishStat, 2018

Global Aquaculture Production for species (tonnes)
Source: FAO FishStat
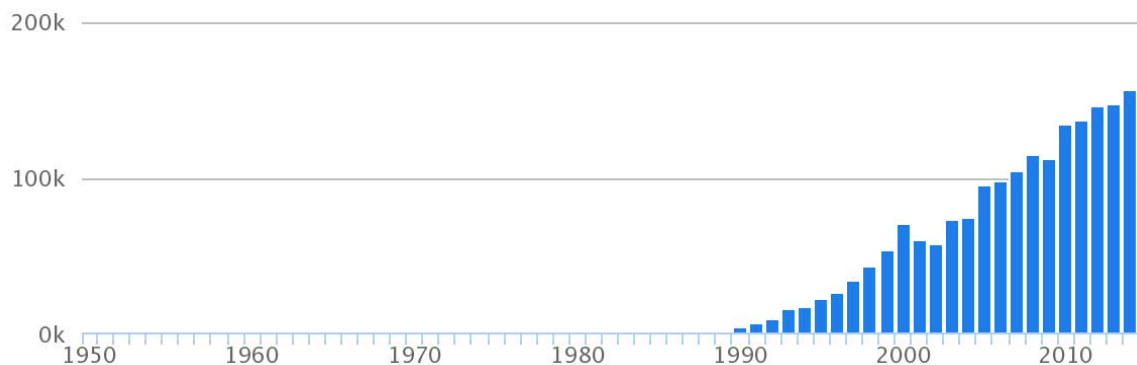
Figure 5. Global Aquaculture Production of *Dicentrarchus labrax* until 2014. Source: FAO FishStat, 2018

2. Objectives of Master Thesis

The main objective of this work was to evaluate the effects of the European seabass (*Dicentrarchus labrax*) after exposure to microplastics (0.184 mg/l and 0.216 mg/l) colonized with the bacteria *Photobacterium damselae piscicida* (1 x 10^8 cfu/ml). This species was selected for the present study because of it´s economic importance on Portuguese aquaculture.

3. Material and Methods

3.1. Ethical Issues

Fish collection and experimentation was directed supervised by trained scientists with accreditation to conduct animal experimentation (by DGAV, equivalent to FELASA category C) and was conducted according to the European Union Directive 2010/63/EU on the protection of animals for scientific purposes.

3.2. Fish

One hundred forty-four healthy European seabass were obtained from a local fish farm (Cantabria, Spain) and were placed in glass aquariums with 60L of capacity. Before the assay fishes were on a acclimatization period, for 2 weeks. Fish had a mean (± standard deviation – SD) of 32.2 ± 8.8 g body weight, a furcal length of 14.0 ± 1.3 cm and a total length of 14.4 ± 1.3 cm. Only healthy fish, as indicated by their activity and external appearance, were used in the experiment.

3.3. Microplastics and their decay

Red fluorescent MPs were purchased from Cospheric—Innovations in Microtechnology (USA). According to the manufacturer's information, MPs size was between 1–5 μm diameter, with a mean diameter of 2 μm, opaque, a density of 1.3 g/cc, excitation and emission wavelengths of 575 nm and 607 nm, respectively.

During the preliminary assay, it was necessary to measure MPs decay (percentage of MPs concentration decrease in test media over a 24h period of time).This measurement was directly assessed through fluorescence reading (Ferreira et al. 2016; Luís et al. 2015; Pacheco, Martins, and Guilhermino 2018) as: MP decay (%) = 100 − (F24×100/F0), where: F24 is the fluorescence measured 24 h after the preparation of the solutions, and F0 is the fluorescence measured immediately after the preparation of the solutions (Pacheco, Martins, and Guilhermino 2018).

3.4. Bacterial strain

Phdp, strain PP3, was kindly provided by Dr. Ana do Vale (Institute for Molecular and Cell Biology, University of Porto, Portugal) and was isolated from yellowtail (*Seriola quinqueradiata*; Japan) by Dr. Andrew C. Barnes (Marine Laboratory, Aberdeen, UK). Bacteria were routinely cultured at 22 ± 1 °C in tryptic soy broth (TSB) or tryptic soy agar (TSA) supplemented with 1.5% (w/v) NaCl (Costas et al. 2012).

To analyze the percentage of MPs colonization with the bacteria, a fluorescent-label was inserted on this bacterial strain, that express green fluorescent protein (GFP) if growth with arabinose (ara), that is the switch of the regulation system, this plasmid also confers resistance to ampicillin (amp). The plasmid pGLO, from Bio-Rad Laboratories was inserted by transformation using heat shock in accordance with the pGLO Bacterial Transformation Kit with a few adaptations. Briefly, *Phdp* were cultured for 48 h at 22 ºC on TSA, after that was necessary label one eppendorf with + pGLO (bacteria with the plasmid) and another with -pGLO (that will serve as a negative control), with a micropipette transfer 250 µl of the transformation solution (CaCl$_2$) to each tube and place them on ice (Bio-Rad Laboratories Inc 2014). With an inoculum loop, pick up a single colony of bacteria from the initial plate and place it in both tubes. Then immerse a sterilized inoculating loop into the tube with the plasmid pGLO, mix the loop plasmid into the cell suspension of the + pGLO tube. At this point it´s the thermal shock, + pGLO and - pGLO tubes are placed to a 42 °C water bath for an exact 50 seconds, after that, tubes will be back on ice for 2 min. Then it was added 250 µl broth nutrient broth to both tubes and incubate them for 10 min at room temperature. Finally using a sterile pipette, place 100 µl of the transformation and control suspensions on the appropriate plates (Plate 1: TSA / amp with + pGLO; Plate 2: TSA / amp / ara with + pGLO; Plate 3: TSA / amp with - pGLO; Plate 4: TSA with - pGLO). Plates were then placed in an oven at 25 ° C for 48h (Bio-Rad Laboratories Inc 2014). Bacteria growth in the plate 2 are the ones that internalize the plasmid and have the GFP active because of arabinose.

To reduce the experimental variation among assays, the same bacterial culture was divided into aliquots and these were stored at - 80 °C in TSB supplemented with 15% (v/v) glycerol for cryopreservation.

Transformed bacteria were cultured for 48 h at 22 ºC on TSA, and then inoculated into TSB and cultured overnight at the same temperature, with continuous shaking (100 rpm). Then, the concentration of bacteria was measured by spectrophotometry (625 nm) (Machado et al. 2015). Bacteria concentrations were adjusted to 1x10^8 cfu/ml according to the protocol of Costas et al. (2012) with adaptations protocol (Costas et al. 2012). Exponential growth bacteria were collected using centrifugation at 3500 x g for 10 min. Then this pellet was re-suspended in sterile TSB, natural seawater (Matosinhos, Portugal), artificial seawater or on all the previously listed medium´s already with MP (two concentrations 0.184 mg/l and 0.216 mg/l) for the *in vitro* assay and in sterile TSB or TSB with MP on the case of *in vivo* assay.

3.5. Colonization of microplastics by the bacteria

The colonization of microplastics by the *P. damselae piscicida* was based on Foulon et al. (2016) regarding the colonization of polystyrene MPs by a different bacteria (*Vibrio Crassostrea*) (Foulon et al. 2016) with some modifications. Briefly, transformed bacteria were centrifuged and their concentration adjusted to 1×10^8 cfu/ml (previously explained), then 15 ml of the suspension were incubated in 20ml pyrex glass test tubes with a cap done with cotton to allow bacteria breathing for 24h at 22°C with agitation (200rpm). This assay was done with 3 solutions, TSB, artificial seawater (ASW) (distilled water with 35g/L (w/v) of salt) and natural seawater (NSW) and two MPs concentrations (0.184 mg/l and 0.216 mg/l) and one bacterial concentration (1×10^8 cfu/ml). Solutions in triplicate were: (**i)** TSB with bacteria; **ii)** ASW with bacteria; **iii)** NSW with bacteria; **iv)** TSB lower MPs concentration with bacteria; **v)** ASW lower MPs concentration with bacteria; **vi)** NSW Lower concentration of MPs with bacteria; **vii)** TSB higher concentration of MPs with bacteria ; **viii)** ASW higher concentration of MPs with bacteria on; **ix)** NSW higher concentration of MPs with bacteria; as the experimental design described in Figure 6 shows.

This experiment was done with these solutions to verify if there were significant differences among them, to use the most benefit on the *in vivo* assay. To ensure that only the bacteria under study would grow, 10 mg/ml of ampicillin was placed in each test tube. Finally, to verify colonization 100 µl of the suspension every hour for 9h and after 24h, were put on a microscope slide and left to dry for 20 min thus being possible to perform the counts using the fluorescence microscope using the fluorescein (FITC) filter. While colonization was verified (each hour for 9 hours and after 24 hours), 100 µl of the suspension were measured by spectrophotometry (625 nm) to access bacterial growth along the assay.
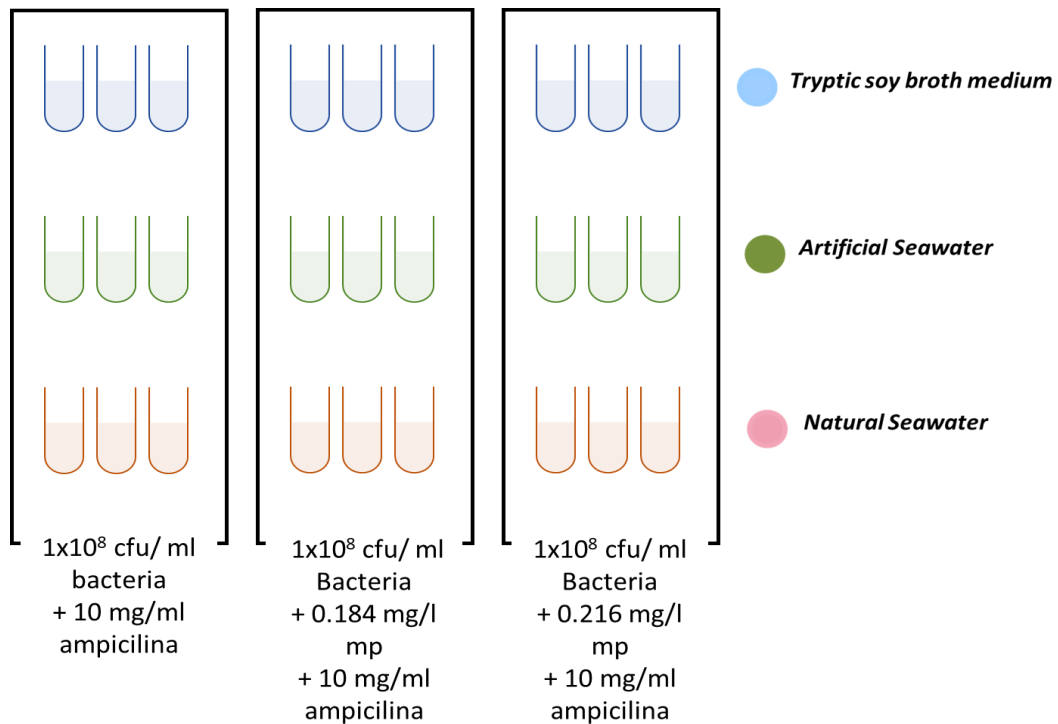
Figure 6. Experimental design of the preliminary assay. Solutions in triplicated were: **i)** TSB with bacteria; **ii)** ASW with bacteria; **iii)** NSW with bacteria; **iv)** TSB lower MPs concentration with bacteria; **v)** ASW lower MPs concentration with bacteria; **vi)** NSW Lower concentration of MPs with bacteria; **vii)** TSB higher concentration of MPs with bacteria; **viii)** ASW higher concentration of MPs with bacteria on; **ix)** NSW higher concentration of MPs with bacteria.

3.6. *In vivo* bioassay

European seabass specimens were weighed at the beginning and at the end of the bioassay, to determinate the feed ration (2% of the body weight) to provide and they were measured at the end of the bioassay. The bioassay was conducted in glass tanks (60L capacity), to minimize plastic exposure, filled to 50 L. Water quality was maintained with aeration and daily water changes of 20%. Photoperiod was 12h light/ 12h dark. Ammonia levels in the water were measured every day using commercial kits (Palintest Ltd, United Kingdom) and never exceeded 0.40 mg/l. During the trial, water temperature averaged 20.4 ± 0.8 ºC, salinity remained constant at 35 g/L and pH average was 7.9 ± 1.0.

Fish were exposed to the bacteria, MPs, or MPs colonized by the bacteria through the food. Treatments consisted of six groups of 24 European seabass each (12 fish per tank - duplicate tanks per group): (i) control group exposed to commercial food only; (ii) group of

exposed to a 1x 10^8 cfu/ml of transformed *Phdp.*: (iii) group exposed to 0.184 mg/l of MPs; (iv) group exposed to 0.216 mg/l of MPs; (v) group exposed to 0.184 mg/l of MPs colonized with the bacteria; and (vi) group exposed to 0.216 mg/l of MPs colonized with the bacteria (Figure 7).

To contaminate the food, first, it was necessary to know how many pellets were in 2% of feed, then 5 µl per pellet of the suspension (sterile TSB, TSB with bacteria, TSB with both concentrations of MP and TSB with the mixtures) were taken and placed on top of each pellet. Then, these pellets were placed and dried in the oven for 45 min at 30 ºC. Afterwards, fish were fed with these contaminated pellets, which were administered slowly and ensuring all were eaten fast, decreasing a possible decay of MPs in water. The colonization of microplastics by *Phdp* was done on the day of the exposer.

The assay lasted for 7 days. On the first day, fish were fed with 2% of their body weight of contaminated feed and were sampled after 24h (four fishes from each thank) to verify effects produced by MPs colonized with bacteria. Thereafter, the remaining fish were fed with non-contaminated feed for 3 days. On the fourth day, four fishes from each thank were sampled to verify effects from mp colonized with bacteria and after decolonization. Then the remaining fish received a second exposure of contaminated feed. After that day and until the last one of the assays, fish were fed with non-contaminated feed. On the last day (7 days after) took place the last sampling, as the scheme on figure 8 shows.
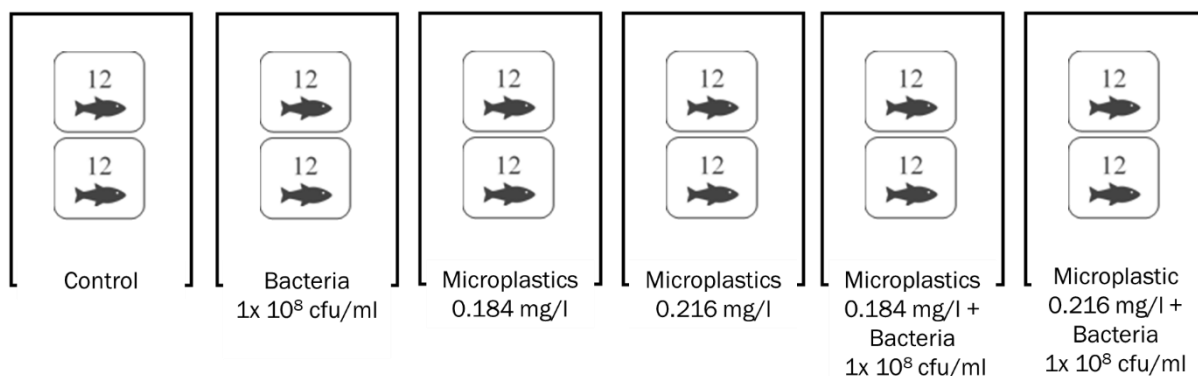
Figure 7. Experimental design of the *in vivo bioassay* with six experimental groups(i) control group exposed to commercial food only; (ii) group of exposed to a 1x 108 cfu/ml of transformed Phdp.: (iii) group exposed to 0.184 mg/l of MPs; (iv) group exposed to 0.216 mg/l of MPs; (v) group exposed to 0.184 mg/l of MPs colonized with the bacteria; and (vi) group exposed to 0.216 mg/l, as previously described.
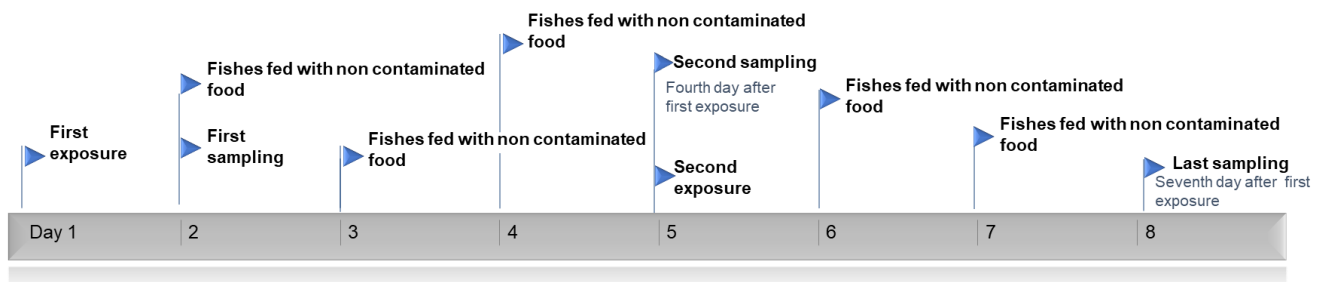
Figure 8. Experimental *in vivo* time line, control group were always fed with commercial feed without microplastics and/or bacteria

3.7. Sampling

At each sampling, 4 fishes from each thank (8 from each experimental group), were sacrificed with an overdose of anesthetic, 0.3 ml/l ethylene glycol monophenyl ether (1,000 ppm; Sigma-Aldrich, Steinheim, Germany) for blood and tissues sampling. Blood samples were withdrawn from the caudal vessel with heparinized syringes, placed in heparinized eppendorf tubes and used to determine total erythrocytes (RBC) and leucocytes (WBC) counts and haemoglobin. The remaining blood was used to collect plasma, following centrifugation (10 000 × g, 5 min, at 4 °C). Plasma was then frozen in liquid nitrogen and stored at −80 °C until further analysis. Following blood collection, fish were dissected, and collect liver then each eppendorf with tissues were immediately frozen in liquid nitrogen.

3.8. Haematological procedures

The haematological profile consisted of total red (RBC) and white (WBC) blood cells counts, haemoglobin evaluation, as well as the mean corpuscular haemoglobin (MCH) was also calculated as follows: MCH (pg cell) = (Haemoglobin /RBC) x 10

Haemoglobin, was measured by the Drabkin colorimetric method (Van Kampen & Zijlstra, 1966) following the manufacturer's instructions (Hb; SPINREACT kit, ref. 1001230, Spain).

3.9. Humoral Immune parameters

The plasma collected was used to analyze two humoral innate immune parameters.

3.9.1. Protease activity

Protease activity was quantified using the azocasein hydrolysis assay according to the method of Guardiola et al. (2014) (Guardiola et al. 2014) Briefly, 10 µl of plasma was incubated with 100 µl of ammonium bicarbonate buffer and 125 µl of azocasein (Sigma) for 24 h at room temperature in orbital shaker (100 rpm). The reaction was stopped by adding 250 µl of 4.6% trichloro acetic acid (TCA) and the mixture centrifuged (10,000 x g for 5 min). The supernatants were transferred to a 96-well plate in duplicate containing 100 µl of 1N NaOH, and the optical density (OD) read at 450 nm using a plate reader. Plasma were replaced by trypsin (5 mg/l Sigma), as positive control, corresponding to 100% of protease activity, or by buffer, as negative controls equivalent to 0% activity.

3.9.2. Antiprotease activity

Antiprotease activity was determined according to the method described by (Machado et al. 2015). Briefly, 10 µl of plasma samples were incubated with the same volume of a trypsin solution (5 mg/ml in $NaHCO_3$, 5 mg/ml, pH 8.3) for 10 min at 22 ºC. To the incubation mixture, 100 µl of phosphate buffer (PBS) (NaH_2PO_4, 13.9 mg/ml, pH 7.0) and 125 µl of azocasein (20 mg/ml in $NaHCO_3$, 5 mg/ml, pH 8.3) were added and incubated for 1 h at 22 ºC. Lastly, 250 µl of 10% TCA were added to each microtube and incubated for 30 min at 22 ºC. The mixture was centrifuged at 10 000 x g for 5 min at room temperature. Afterwards, 100 µl of the supernatant was transferred to a 96-well plate in duplicate containing 100 µl of 1 M NaOH per well. The OD was read at 450 nm in a microplate reader (Synergy HT). PBS instead of plasma and trypsin served as blank. Then the percentage inhibition of trypsin activity compared to the reference sample was calculated.

3.10. Biomarkers

The liver collected were homogenized and placed into aliquot to minimize contaminations and degradation, and were used to analyze three biomarkers, catalase (CAT), lipid peroxidation (LPO) and Glutathione S-Transferase activity (GST), and to determinate protein quantification.

3.10.1.   Liver homogenization

Liver homogenization followed the procedures described in Fernandes et al. (2017) with minor modifications. Briefly, samples of liver with 76.2 ± 9.5 mg were homogenized with 1 600 µl of ultrapure H_2O on a tissue homogenizer (Precellys 24 homogenizer, Bertin) for 2 cycles of 15 seconds each at 6 000 x g. Then were made four aliquots, one for LPO on a 2 ml eppendorf with 200 µl of homogenize liver and 4 µl of 4% BHT (2,6-Di-tert-butyl-4-methyl phenol, dissolved in methanol. To make the other three aliquots, to oxidative stress biomarkers, first on a 2 ml eppendorf was add 700 µl of homogenize liver with 700 µl of K-phosphate buffer (0.2M, pH 7.4). then were centrifuge for 20 min at 10 000 x g (4 ºC). To make each aliquot from the supernatant was taken 250 µl to GST and 100 µl for catalase and protein quantification. Aliquots were immediately frozen at -80 ºC, until necessary again

3.10.2. Protein quantification

Protein quantification of liver samples were done based on the protocol of Pierce ® BCA Protein Assay Kit (Scientific 2015). Briefly, is necessary prepare diluted albumin standards (BSA): i) 0 mg/ml, ii) 0.025 mg/ml, iii) 0.25 mg/ml, iv) 0.5 mg/ml, v) 0.75 mg/ml, vi) 1 mg/ml, vii) 1.5 mg/ml, viii) 2 mg/ml. Pipette 25 µl of each standard or sample in duplicate into a 96-well plate, then add 200 µl of the reaction reagent to each well and mix plate thoroughly on a plate shaker for 30 seconds. Cover the plate and incubate at 37 °C for 30 minutes. Finally cool the plate at room temperature and measure the absorbance at 562 nm.

3.10.3. Catalase (CAT)

CAT activity was measured following the method described by Oliveira et al. 2010 (Oliveira et al. 2010). The degradation rate of the substrate H_2O_2, monitored at 240 nm for 2 minutes (each read every 15 seconds interval). The reaction mixture consisted of 248 µl of 30% H_2O_2 (substrate), 30 ml of 0.05 M K-phosphate buffer, pH=7.0 and 10 µl of each sample. Enzymatic activities were determined in triplicated.

3.10.4. Lipid peroxidation (LPO)

LPO levels were determined following the method described by Oliveira et al. 2015 (Oliveira et al. 2015). Briefly, 100 µl of cold TCA 100% where added to each LPO sample and vortex, then 1ml of TBA 0.73% were added to all samples, blanks and vortex. After that samples where incubated four 1 hour at 100 ºC in an oven, and then centrifuge for 5minutes at 11500 rpm. Finally, the supernatant was pipette to a microplate (200 µl) in triplicate. The absorbance was measured at 535 nm and LPO was expressed as nmol of thiobarbituric acid reactive substances (TBARS) formed per mg of protein (Oliveira et al. 2015).

3.10.5. Glutathione-S-transferase activity (GST)

GST activity were determined following the method described by Oliveira et al. 2015 with adaptations (Oliveira et al., 2015). This activity was measured at 340 nm, each 20 seconds for 5 minutes. Briefly, the reaction mixture consisted of 250 µl of reaction solution (CDNB solution 60 mM, GSH solution 10 mM and phosphate buffer 0.2 M, pH=6.5) and 50 µl of each sample. GST activity was determined in duplicate for the European seabass (Oliveira et al., 2015).

4. Results

4.1. Colonization

Regarding the three control solutions [(i) TSB with bacteria; ii) ASW with bacteria; iii) NSW with bacteria] were used to verify the bacterial growth along the experimental time as is shown in the Figure 9. Time zero corresponds to the moment when the in vitro experiment started (point when all the solutions were prepared). The results showed that in TSB solution the maximum growth occurred 6 h after the beginning (2.82 x 108 cfu/ml) of the experiment, decreasing to 2.16 x 108 cfu/ml at the end of the trial (24h). Regarding ASW solution, the maximum growth was observed at 3h reaching 1.42 x 108 cfu/ml and then decreasing to 1.40 x 108 cfu/ml at 9h and remaining unchanged until 24h.

Lastly, the Phdp bacteria had a maximum growth of 1.40 x 108 cfu/ml after 2h in the NSW solution whilst the growth of this bacterium decreased to 1.26 x 108 cfu/ml at 8h of trial, remaining unchanged until the end of the experiment (24h).
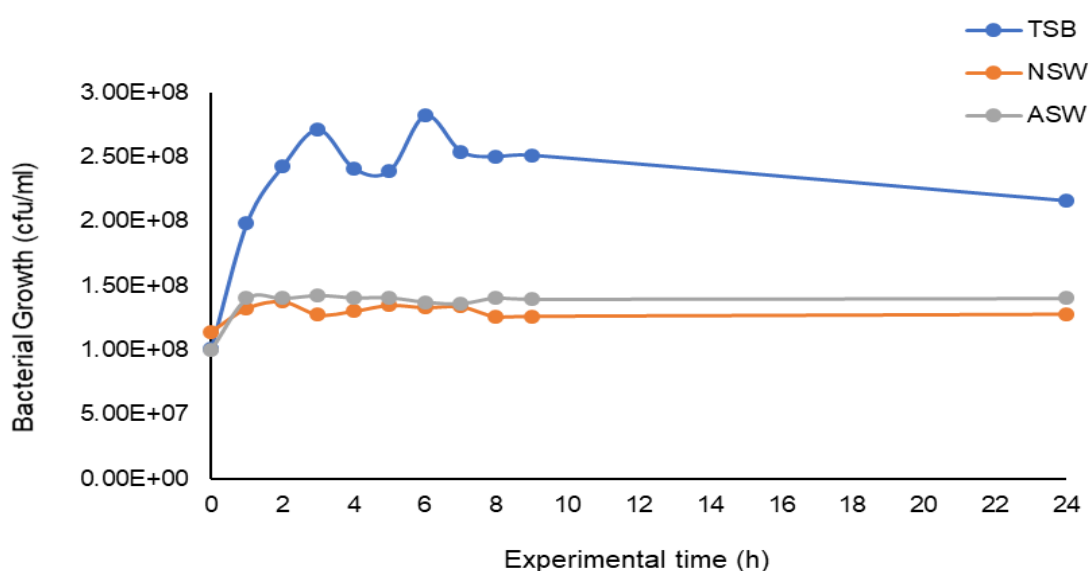
Figure 9. Transformed *Photobacterium damselae piscicida* growth (cfu/ml) in control solutions: TSB (●, blue), ASW (●, grey) and NSW (●, orange) for 24 hours. These data were based on duplicated measurements, for each sampling interval, 100 μl from the suspension were transferred to a 96-well plate, and OD measured at 625 nm, each hour until 9h and then after 24h.

Interestingly, a two-phase dynamic was observed in all control solutions. However, with the lowest concentration of MPs on the TSB solution the colonization phase occurred between 1 and 8 hours reaching 85.4% of colonization. On the other hand, the colonization phase in NSW and ASW solutions was observed between 1 and 4 hours reaching 60.5 and 71.4%, respectively. The highest MPs concentration reported different

results. In this case, the colonization phase was observed between 1 and 6 hours reaching 93.7% of colonization in the TSB solution, whilst the colonization reached 54% and 76.8% between 1 and 4 hours in NSW and ASW solutions, respectively. At the end of experiment (24 hours), the colonization percentage in all experimental solutions was 0%.
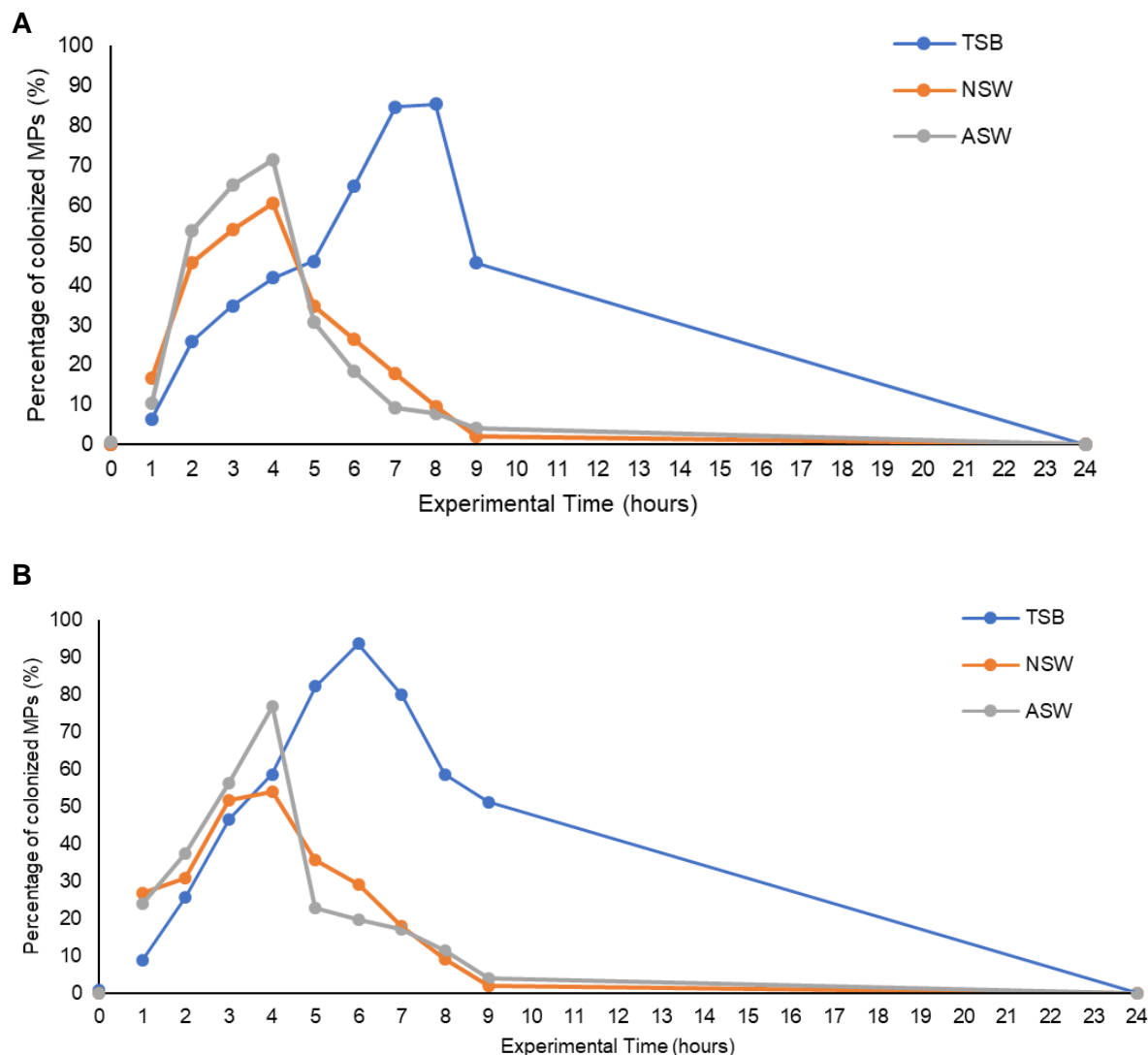
Figure 10. Percentage (%) of colonized MPs by transformed *Photobacterium damselae piscicida* in TSB (●, blue), ASW (●, grey) and NSW (●, orange) solutions for 24 hours. These data were based on triplicate independent experiments. Graphic A corresponds to the lowest MPs concentration (0.184 mg/l) and B to the highest one (0.216 mg/l).

MPs decay is described in the Table 1. The highest decay was verified in NSW, with a decay of almost 17% on both MPs concentrations (0.184 mg/l and 0.216 mg/l). Contrarily, the lowest decay was observed in the TSB solution in both MPs concentration. Finally, the results showed that when compared both MPs concentrations, a larger decay was reported in the highest concentration in comparison to the lowest one.

Table 1. Two MPs concentrations (0.184 mg/l and 0.216 mg/l) decay at the three different solutions tested (TSB, ASW and NSW) after 24 hours. These data were based on duplicated measurements directly measure with fluorescence reading from three independent experiments.

MPs concentration		0.184 mg/l			0.216 mg/l		
Solutions		**TSB**	**ASW**	**NSW**	**TSB**	**ASW**	**NSW**
Experimental Time (hours)	**0**	74.19 ± 0.43	59.17 ± 0.42	47.39 ± 0.09	136.28 ± 0.25	147.21 ± 4.45	118.49 ± 1.60
	24	71.19 ± 0.52	54.96 ± 0.44	39.35 ± 0.30	126.69 ± 0.40	134.57 ± 4.06	98.61 ± 1.71
Decay (%)		4.05	7.11	16.97	7.04	8.59	16.78

4.2. Total red blood cells and white blood cells

Total RBC and WBC count are presented in the Figure 11 and Figure 12, respectively.

Total RBC count presented significant differences between experimental groups at the same sampling point (Figure 11). After 24h from the first exposure, the total RBC count significantly decreased in all groups except on the lower MPs concentration when compared to the control group. When compared to the bacterial group, the results showed a significant decrease of the count on fishes from the higher MPs concentration with bacteria. Regarding to the lower MPs concentration, a significant decrease was also observed in fishes from the higher and lower MPs concentrations with bacteria. Finally, a significant decrease on RCB count was found in fish from higher MPs concentration with bacteria group compared to the higher MPs concentration without bacteria.

After 4 days of trial, the results showed a significant decrease on the count of RBC from the higher MPs concentration with bacteria when compared to the control group and when compared to the bacterial group. Contrarily, no significant differences were observed between experimental groups at the end of the experiment (7 days).
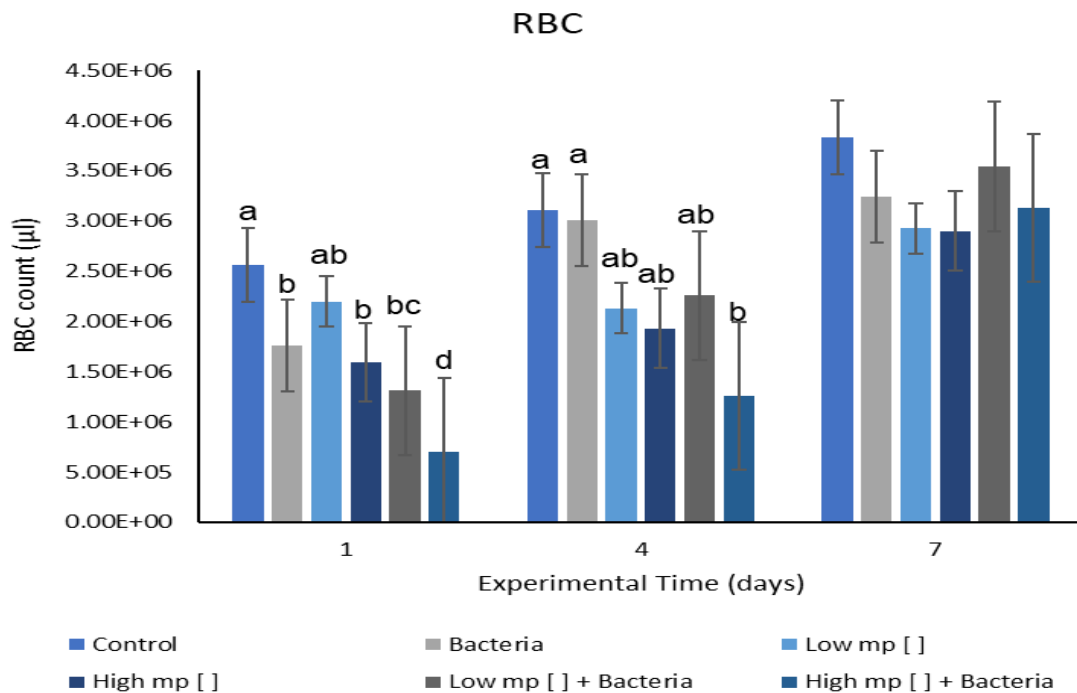
Figure 11. Total red blood cells (RBC) count (cell unit) in blood from fishes from six experimental groups: **i)** Control (●), **ii)** Bacteria (●), **iii)** Low MPs concentration (●), **iv)** High MPs concentration (●), **v)** Low MPs concentration + Bacteria (●), **vi)** High MPs concentration + Bacteria (●), at 1, 4 and 7 days after the first exposure. Bars represent the

mean counts ± SEM. Different letters indicate significant differences between experimental groups in each sampling time (one-way ANOVA; P ≤ 0.05).

Regarding time factor (Table 2), the results showed that the RBC number was significantly higher on the control group from the last sampling point (7 days) when compared to the first one, after 1 day. A significant increase was also shown between the first sampling point to the second and to the last one. When compared the RBC count from fishes from the higher concentration of MPs groups, the one from the last sampling point showed a significant increase when compared to the first one. Fishes from both experimental groups with MPs and bacteria presented a significant increase at end of the trial compared to the first and second sampling. Fish from the lower MPs concentration with bacteria also presented a significant increase from the first to the second sampling.

Table 2. Significant differences of RBC total counts (cell unit) in blood of fish from each experimental group: **i)** Control, **ii)** Bacteria, **iii)** Low MPs concentration, **iv)** High MPs concentration, **v)** Low MPs concentration + Bacteria, **vi)** High MPs concentration + Bacteria, regarding time (day 1, 4 and 7). Different letters indicate significant differences between the same experimental group in each sampling point (two-way ANOVA; P ≤ 0.05).

Time(days) Group	Day 1	Day 4	Day 7
Control	a	ab	b
Bacteria	a	b	b
Low MP [ ]	a	a	a
High MP [ ]	a	ab	b
Low MP [ ] + Bacteria	a	b	c
High MP [ ] + Bacteria	a	a	b

The mean corpuscular haemoglobin presented significant differences between experimental groups at the same sampling point (Figure 12). After 24h and 96h from the first exposure, the MCH significantly increase on fish from the higher MPs concentrations with bacteria when compared to fish from all the other groups. After 7 days was also found a significantly increase on fish from the higher MPs concentrations with bacteria when compared to fish from the control and bacteria group.
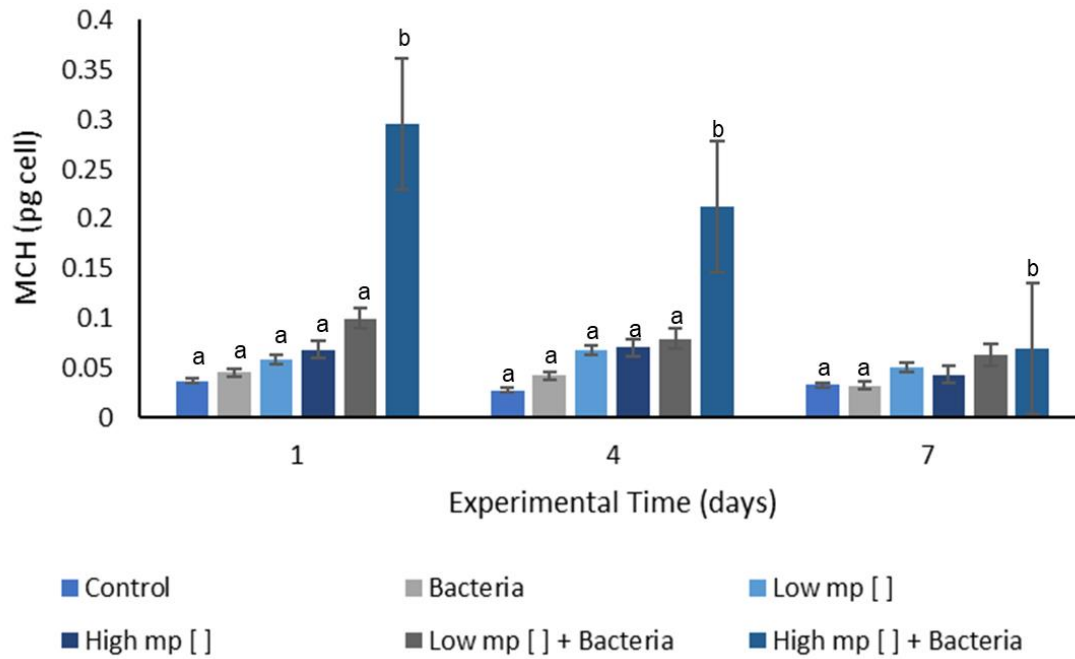
Figure 12. The mean corpuscular haemoglobin (MCH) (pg cell) in blood from fishes from six experimental groups: **i)** Control (●), **ii)** Bacteria (●), **iii)** Low MPs concentration (●), **iv)** High MPs concentration (●), **v)** Low MPs concentration + Bacteria (●), **vi)** High MPs concentration + Bacteria (●), at 1, 4 and 7 days after the first exposure. Bars represent the mean counts ± SEM. Different letters indicate significant differences between experimental groups in each sampling point (one-way ANOVA; $P \leq 0.05$).

Regarding time factor (Table 3), the results showed a significant increase on fishes from the higher concentration of MPs with bacteria group from de seventh day when compared to the first.

Table 3. The mean corpuscular haemoglobin (MCH) (pg cell) in blood of fish from each experimental group: **i)** Control, **ii)** Bacteria, **iii)** Low MPs concentration, **iv)** High MPs concentration, **v)** Low MPs concentration + Bacteria, **vi)** High MPs concentration + Bacteria, regarding time (day 1, 4 and 7). Different letters indicate significant differences between the same experimental group in each sampling point (two-way ANOVA; $P \leq$ 0.05).

Time(days) / Group	Day 1	Day 4	Day 7
Control	a	a	a
Bacteria	a	a	a
Low MP [ ]	a	a	a
High MP [ ]	a	a	a
Low MP [ ] + Bacteria	a	a	a
High MP [ ] + Bacteria	a	a	b

On the other hand, total WBC count presented significant differences between experimental groups from the same sampling points (Figure 13). After 24h from the first exposure, no significant differences were observed between experimental groups. However, after 4 days fishes exposed to the higher MPs concentration, the lower MPs concentration with bacteria and the higher MPs concentration with bacteria groups presented a significant decrease when compared to the bacteria and to the lower MPs concentration group. After 7 days, the only significant difference observed was a lower number of WBC count on fishes exposed to the higher MPs concentration with bacteria group when compared to the ones from the higher MPs concentration without bacteria.
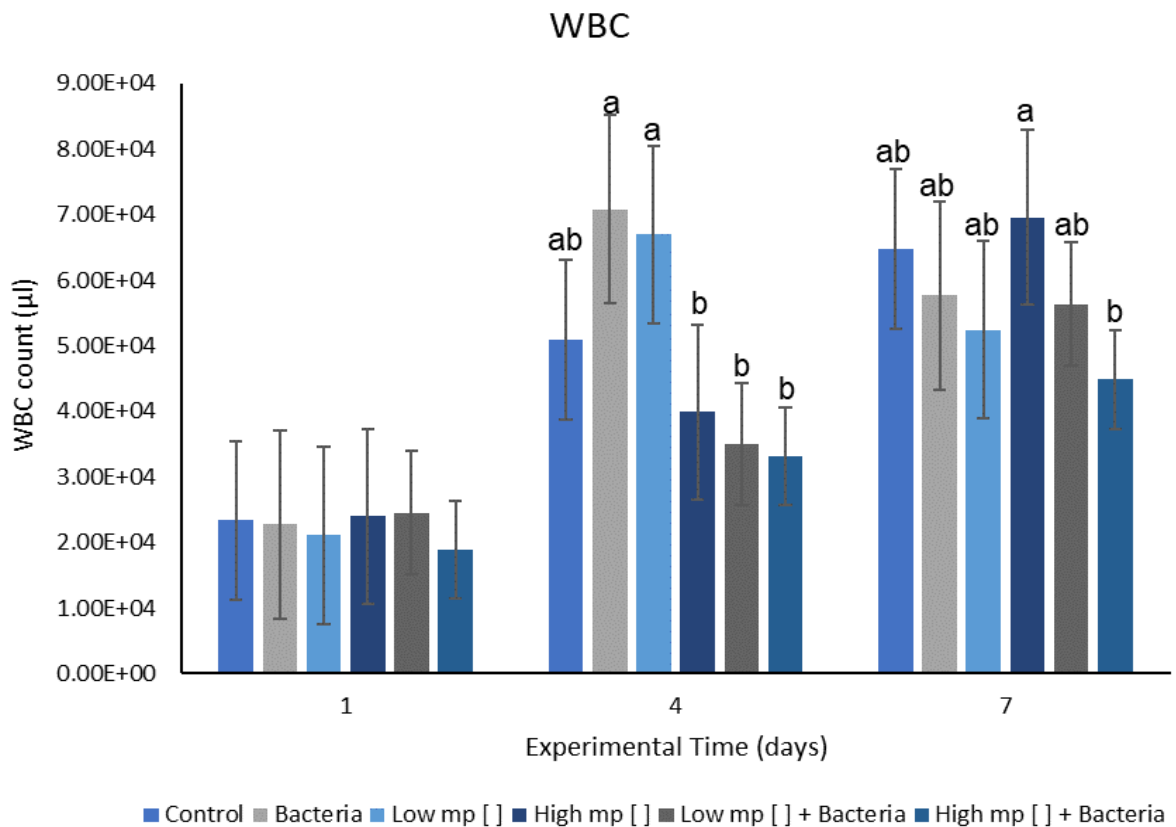
Figure 13. Total white blood cells (WBC) count (cell unit) in blood from fishes from six experimental groups: **i)** Control (●), **ii)** Bacteria (●), **iii)** Low MPs concentration (●), **iv)** High MPs concentration (●), **v)** Low MPs concentration + Bacteria (●), **vi)** High MPs concentration + Bacteria (●), at 1, 4 and 7 days after the first exposure. Bars represent the mean counts ± SEM. Different letters indicate significant differences between experimental groups in each sampling point (one-way ANOVA; P ≤ 0.05).

Regarding time (Table 4), the WBC number. Analyses show that the WBC total count were significantly higher in fish from the control group from the second and last sampling point when compared to the ones from the first. Data from the last sampling point, also has a higher count when compared to the second one.

Fishes exposed to bacteria and fishes from the lower MP concentration group presented a significantly higher WBC count on the second and last sampling when compared to the first one. Finally, the count from fishes exposed to MPs with bacteria (higher and lower MPs concentration) where significantly higher after 7 days when compared to the data from the first and also from the fourth day. The higher MPs concentration with bacteria also presented a higher count after 7 days than after 1.

Table 4. Significant differences of WBC total counts (cell unit) in blood of fish from each experimental group: **i)** Control, **ii)** Bacteria, **iii)** Low MPs concentration, **iv)** High MPs concentration, **v)** Low MPs concentration + Bacteria, **vi)** High MPs concentration + Bacteria, regarding time (day 1, 4 and 7). Different letters indicate significant differences between the same experimental group in each sampling point (two-way ANOVA; P ≤ 0.05).

Time(days) \ Group	Day 1	Day 4	Day 7
Control	a	b	c
Bacteria	a	b	b
Low MP [ ]	a	b	b
High MP [ ]	a	b	c
Low MP [ ] + Bacteria	a	a	b
High MP [ ] + Bacteria	a	a	b

4.3. Haemoglobin

Haemoglobin levels are presented in the Figure 14. After 24 h from the first exposure, an increase of haemoglobin levels were observed in the fish exposed to the higher MPs concentration with bacteria when compared to fishes from the control, the bacteria, and the higher MP concentration group. After 4 days of trial, the results showed a significant increase of haemoglobin levels on fishes from the higher MPs concentration with bacteria group when compared to the control and to the bacteria group. At the end of the experiment (7 days), the values of haemoglobin in fish blood increased on fishes from both higher MPs concentration with bacteria group and lower MPs concentration with bacteria group when compared to the bacteria group and to the higher MPs concentration group
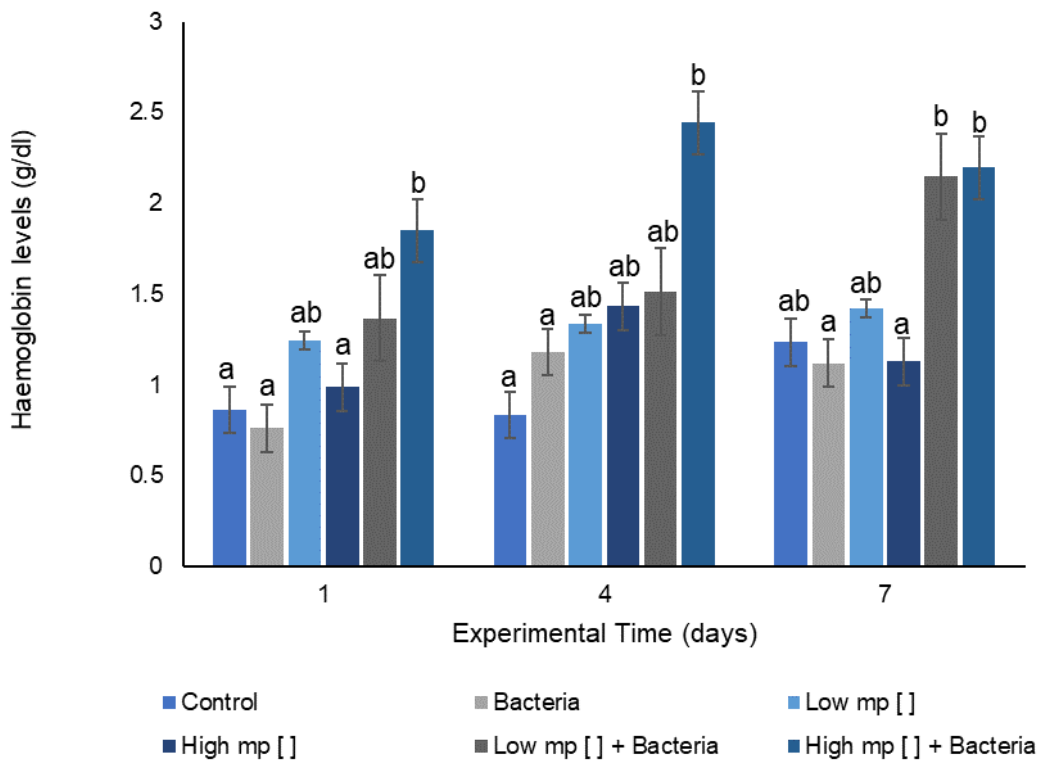
Figure 14. Hemoglobin levels (mg/ml) of fishes from six experimental groups: **i)** Control (●), **ii)** Bacteria (●), **iii)** Low MPs concentration (●), **iv)** High MPs concentration (●), **v)** Low MPs concentration + Bacteria (●), **vi)** High MPs concentration + Bacteria (●), at 1, 4 and 7 days after the first exposure. Bars represent the mean counts ± SEM. Different letters indicate significant differences between experimental groups in each sampling point (one-way ANOVA; P ≤ 0.05).

Regarding time factor (Table 5), the results only showed a significant increase in the hemoglobin levels in blood of fish from the control group of day 7 when compared to day 4.

Table 5. Significant differences of haemoglobin levels (mg/ml) in blood of fish from each experimental group: [**i)** Control, **ii)** Bacteria, **iii)** Low MPs concentration, **iv)** High MPs concentration, **v)** Low MPs concentration + Bacteria, **vi)** High MPs concentration + Bacteria, regarding time (day 1, 4 and 7). Different letters indicate significant differences between the same experimental group in each sampling point (two-way ANOVA; P ≤ 0.05).

Time (days) / Group	Day 1	Day 4	Day 7
Control	ab	a	b
Bacteria	a	a	a
Low MP [ ]	a	a	a
High MP [ ]	a	a	a
Low MP [ ] + Bacteria	a	a	a
High MP [ ] + Bacteria	a	a	a

4.4. Immune parameters measured in plasma of trial's fishes

4.4.1. Protease

Results of protease activity measured in the plasma of fish from experimental groups are shown in the Figure 15. After 24 hours from the first exposure, an increase of the protease activity was observed in the fish from the higher MPs concentration with bacteria group when compared to the bacteria, higher MPs concentration without bacteria and lower MPs concentration with bacteria group. Contrarily, after 4 and 7 days no significant differences were observed in the values of protease activity.
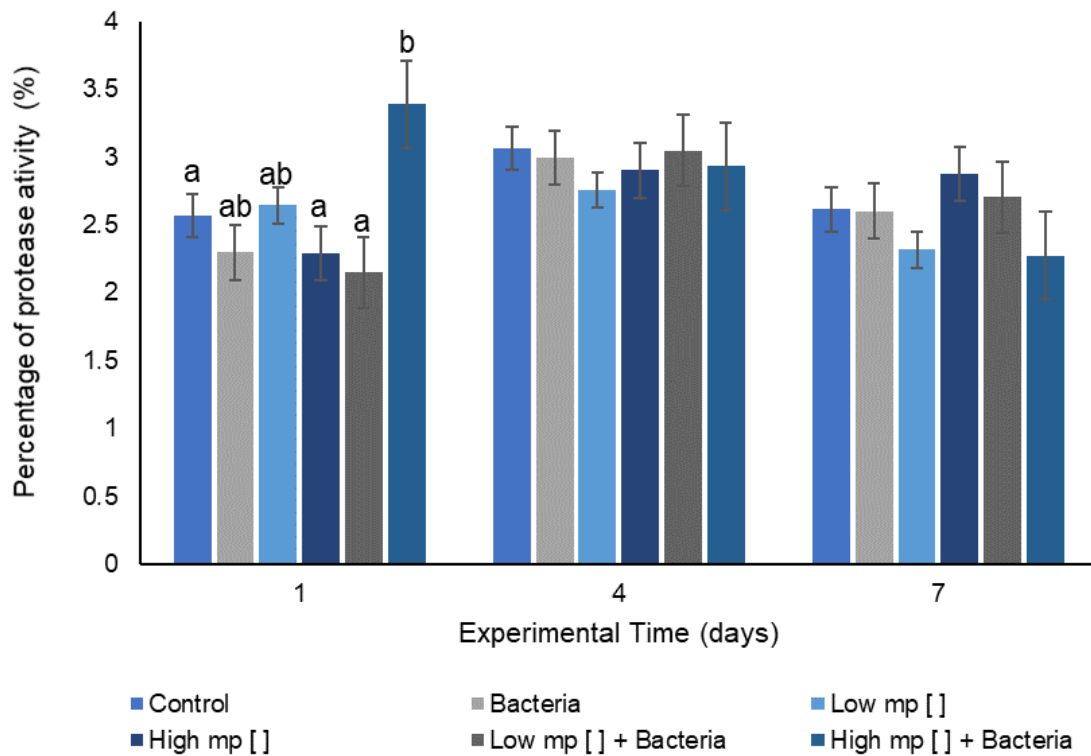
Figure 15. Protease activity (%) of fishes from six experimental groups: **i)** Control (●), **ii)** Bacteria (●), **iii)** Low MPs concentration (●), **iv)** High MPs concentration (●), **v)** Low MPs concentration + Bacteria (●), **vi)** High MPs concentration + Bacteria (●), at 1, 4 and 7 days after the first exposure. Bars represent the mean counts ± SEM. Different letters indicate significant differences between experimental groups in each sampling point (one-way ANOVA; P ≤ 0.05).

Considering the time factor (Table 6), the results only showed a significant decrease in the protease activity from plasma of fishes from the higher MPs concentration with bacteria group of day 7 when compared to day 1.

Table 6. Significant differences of protease activity (%) of plasma fish from each experimental group: **i)** Control, **ii)** Bacteria, **iii)** Low MPs concentration, **iv)** High MPs concentration, **v)** Low MPs concentration + Bacteria, **vi)** High MPs concentration + Bacteria, regarding time (day 1, 4 and 7). Different letters indicate significant differences between the same experimental group in each sampling point (two-way ANOVA; P ≤ 0.05).

Time (days) \ Group	Day 1	Day 4	Day 7
Control	a	a	a
Bacteria	a	a	a
Low MP [ ]	a	a	a
High MP [ ]	a	a	a
Low MP [ ] + Bacteria	a	a	a
High MP [ ] + Bacteria	a	a	b

4.4.2. Anti-protease

In the case of anti-protease activity, no variations were recorded in fish from any experimental group during the trial (Figure 16).

Figure 16. Anti-protease activity (%) of fishes from six experimental groups: **i)** Control (●), **ii)** Bacteria (●), **iii)** Low MPs concentration (●), **iv)** High MPs concentration (●), **v)** Low MPs concentration + Bacteria (●), **vi)** High MPs concentration + Bacteria (●), at 1, 4 and 7 days after the first exposure. Bars represent the mean counts ± SEM. Different letters indicate significant differences between experimental groups in each sampling point (one-way ANOVA; P ≤ 0.05).

Regarding time (Table 7), the results only showed a significant decrease in the anti-protease activity from plasma of fishes from the lower MPs concentration group of day 7 when compared to day 4.

Table 7. Significant differences of anti-protease activity (%) of plasma fish from each experimental group: **i)** Control, **ii)** Bacteria, **iii)** Low MPs concentration, **iv)** High MPs concentration, **v)** Low MPs concentration + Bacteria, **vi)** High MPs concentration + Bacteria, regarding time (day 1, 4 and 7). Different letters indicate significant differences between the same experimental group in each sampling point (two-way ANOVA; P ≤ 0.05).

Time (days) / Group	Day 1	Day 4	Day 7
Control	a	a	a
Bacteria	a	a	a
Low MP [ ]	a	a	a
High MP [ ]	a	a	a
Low MP [ ] + Bacteria	a	a	b
High MP [ ] + Bacteria	a	a	a

4.5. Biomarkers measured in liver from trial's fishes

Protein concentrations measured in the liver from trial's fishes. These values were used to correct CAT and GST enzymatic activities.

4.5.1. Lipid peroxidation activity

LPO activity, significant differences between groups are showed in the graph from Figure 17. After 24 h from the first exposure, an increase of LPO was observed in fish from the higher MPs concentration with bacteria group when compared to the control, bacteria, lower and higher MPs concentration. Contrarily, no significant differences were observed in any experimental group after 4 and 7 days.
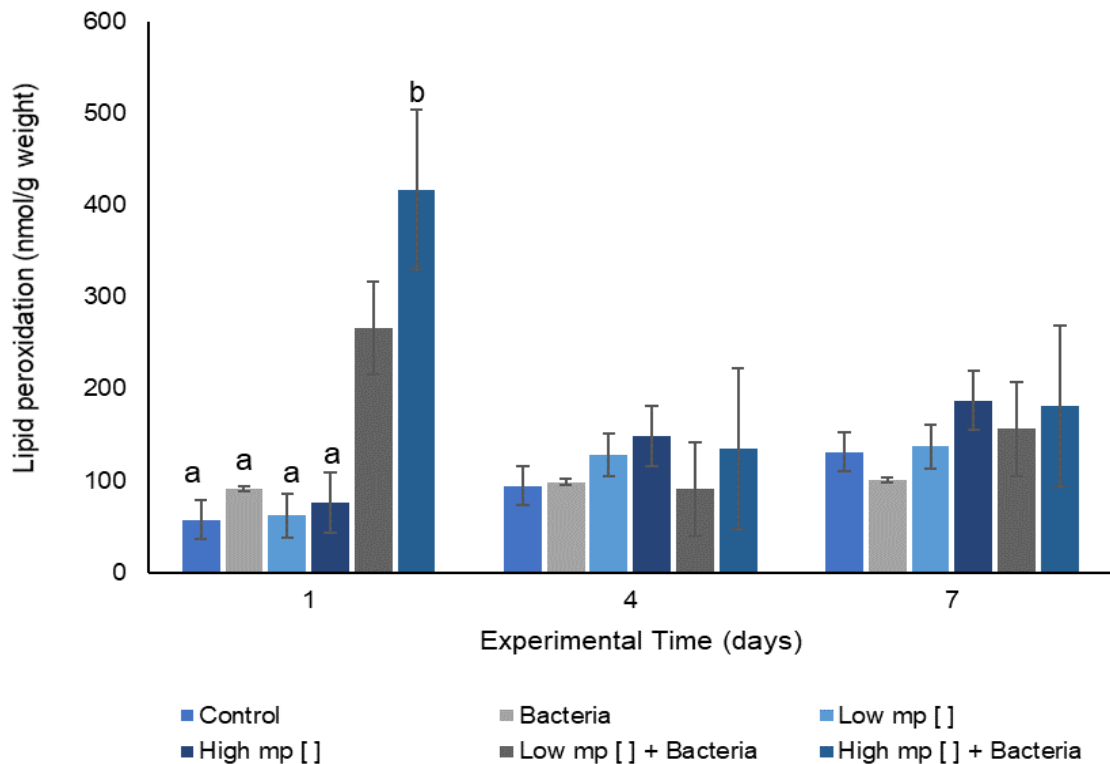
Figure 17. Lipid peroxidation activity (nmol/g weight) of fishes from six experimental groups: **i)** Control (●), **ii)** Bacteria (●), **iii)** Low MPs concentration (●), **iv)** High MPs concentration (●), **v)** Low MPs concentration + Bacteria (●), **vi)** High MPs concentration + Bacteria (●), at 1, 4 and 7 days after the first exposure. Bars represent the mean counts ± SEM. Different letters indicate significant differences between experimental groups in each sampling point (one-way ANOVA; $P \leq 0.05$).

Considering the time factor (Table 8), LPO activity increased in control fish at the end of trial (7 days) compared to values found after 1 day of exposure. In fish exposed to the lowest MPs concentration an LPO decrease was observed on day 4 and 7 when compared to the ones from day 1. Fish from the lower MPs concentration with bacteria presented a decrease on day 4 when compared to day 1.

Table 8. Significant differences of lipid peroxidation on liver of fish from each experimental group: **i)** Control, **ii)** Bacteria, **iii)** Low MPs concentration, **iv)** High MPs concentration, **v)** Low MPs concentration + Bacteria, **vi)** High MPs concentration + Bacteria, regarding time (day 1, 4 and 7). Different letters indicate significant differences between the same experimental group in each sampling point (two-way ANOVA; P ≤ 0.05).

Time (days) Group	Day 1	Day 4	Day 7
Control	a	a	b
Bacteria	a	a	a
Low MP [ ]	a	b	b
High MP [ ]	a	a	a
Low MP [ ] + Bacteria	a	b	a
High MP [ ] + Bacteria	a	a	a

4.5.2. Catalase activity

Values of catalase activity not showed variations in liver of fish from any experimental group at any sampling time (Figure 18).
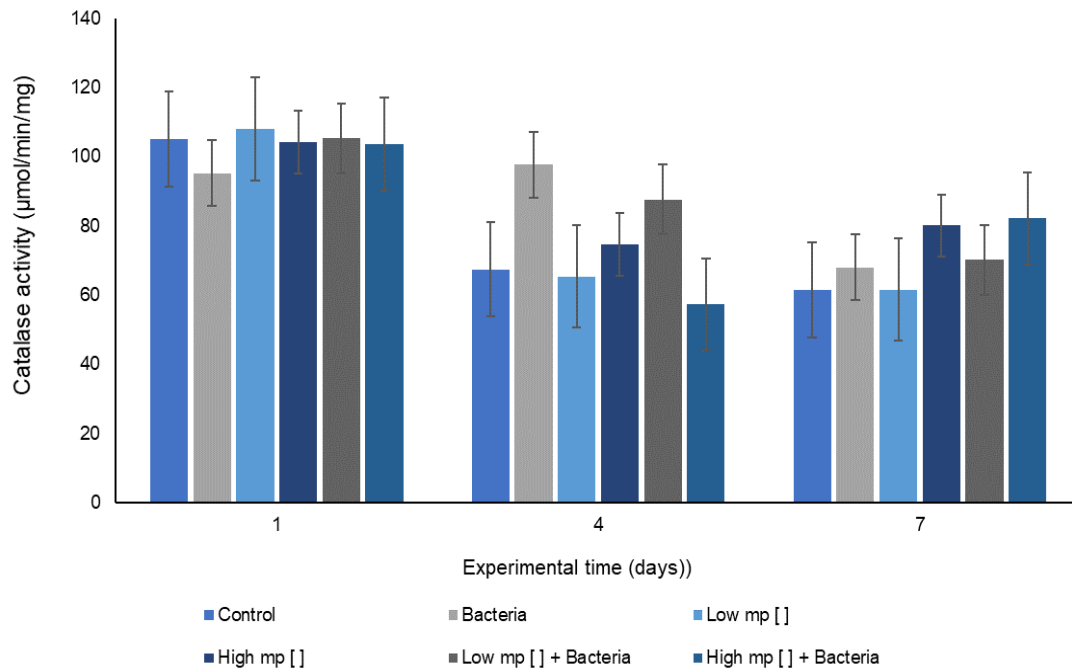
Figure 18. Catalase activity (µmol/min/mg) of fishes from six experimental groups: **i)** Control (●), **ii)** Bacteria (●), **iii)** Low MPs concentration (●), **iv)** High MPs concentration (●), **v)** Low MPs concentration + Bacteria (●), **vi)** High MPs concentration + Bacteria (●), at 1, 4 and 7 days after the first exposure. Bars represent the mean counts ± SEM. Different letters indicate significant differences between experimental groups in each sampling point (one-way ANOVA; P ≤ 0.05).

Considering the time factor (Table 9), CAT activity decreases in fishes from the lower MPs concentration group at the end of trial (7 days) compared to values found after 1 and 4 days of exposure.
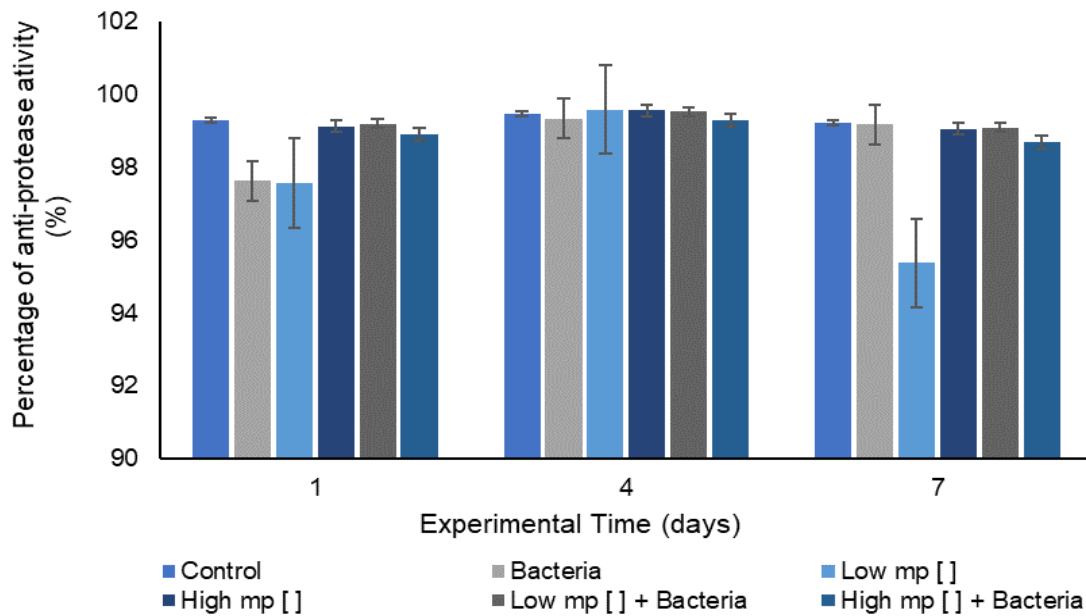
Table 9. Significant differences of catalase activity on liver of fish from each experimental group: **i)** Control, **ii)** Bacteria, **iii)** Low MPs concentration, **iv)** High MPs concentration, **v)** Low MPs concentration + Bacteria, **vi)** High MPs concentration + Bacteria, regarding time (day 1, 4 and 7). Different letters indicate significant differences between the same experimental group in each sampling point (two-way ANOVA; P ≤ 0.05).

Time (days) / Group	Day 1	Day 4	Day 7
Control	a	a	a
Bacteria	a	a	a
Low MP [ ]	a	a	a
High MP [ ]	a	a	a
Low MP [ ] + Bacteria	a	b	b
High MP [ ] + Bacteria	a	a	a

4.5.3. Glutathione-S-transferase activity

Values of GST activity not showed variations in liver of fish from any experimental group at any sampling time (Figure 19), and between sampling points.
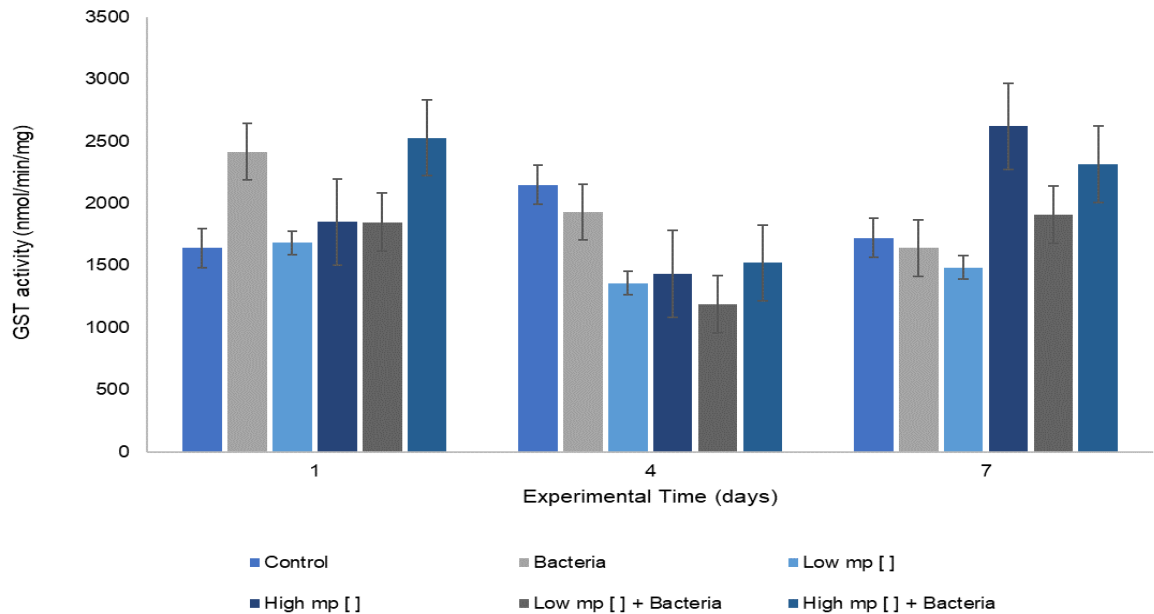
Figure 19. GST activity (µmol/min/mg) of fishes from six experimental groups: **i)** Control (●), **ii)** Bacteria (●), **iii)** Low MPs concentration (●), **iv)** High MPs concentration (●), **v)** Low MPs concentration + Bacteria (●), **vi)** High MPs concentration + Bacteria (●), at 1, 4 and 7 days after the first exposure. Bars represent the mean counts ± SEM. Different letters indicate significant differences between experimental groups in each sampling point (one-way ANOVA; P ≤ 0.05).

5. Discussion

As a preliminary *in vitro* trial, MPs colonization was tested to verify the better solution (the one with the higher and longer colonization percentage) to be used as vehicle to inoculate contaminated feed during the *in vivo* trial. These colonization dynamics were tested in three different solutions (i.e. TSB, ASW and NSW), two MPs concentrations (0.184 mg/l and 0.216 mg/l) and one bacterial concentration ($1x10^8$ cfu/ml), as previously described. The higher colonization percentage were on the TSB solution, than on artificial seawater solution and at last natural seawater, on both MPs concentrations. These results are concordant with the findings of Foulon et al. (2016). (Foulon et al. 2016) This author reported the same but with a different bacteria and bacterial medium, *Vibrio Crassostrea,* and Zobel medium (a bacterial medium solution), the method of colonization used on both studies were nearly the same.

Bacterial colonization was higher on the bacterial medium solution (TSB), and were slower than on seawater (toke more time to reach the colonization pike), and MPs stay more time colonized by the bacteria. With these findings, TSB was the chosen solution to carry out the *in vivo* experiment. In the present study, the percentage of *Phdp* colonization was higher than the values found by Foulon et al. (2016) to *V. Crassostrea*. (Foulon et al. 2016) In this assay were measured the bacterial growth and MPs decay to shed some light about this topic and provide more information than what was reported by Foulon et al. (2016). (Foulon et al. 2016) Bacterial growth was studied for 24 hours and the pike of growth of *Phdp* in TSB coincided with the colonization pike of colonization. Therefore, this fact could mean that when a higher concentration of bacteria is used, more colonization in TSB can be achieved. However, more studies are needed to confirm this hypothesis. On the other hand, the bacterial growth in both seawater (natural and artificial) did not show variations unlike Foulon et al. 2016 study, MPs decay were studied and by theory this decay may affect colonization. The biggest decay was on NSW on both MPs concentrations. Although the global decay was low, the colonization percentage after 24h were zero, so to access if the decay influences, MPs decay analyze should the done at each hour and not only after 24h.

After the preliminary study, the *in vivo* trial take place. After 1, 4 and 7 days of the first exposure, blood and tissue samples were collected.

In the case of haemoglobin levels, the results show that these values were significantly higher on fishes from the group exposed to higher MPs concentration with bacteria, on all experimental times. This increase may be linked to a higher necessity of

oxygen of fish after exposure to MPs colonized with bacteria. At the end of trial (7 days) and after a second MPs exposure, the fish exposed to the lowest concentration of MPs with bacteria also showed a significant increase in the hemoglobin values inside the same sampling point. These data could indicate that in the case of the combination of the lowest concentrations of MPs and bacteria, repeated exposure is required to produce effects, but the same cannot be assumed as true because although in this case a significant increase was presented within the sampling point, the same does not occur when compared to other experimental times.

The significant decrease in the number of RBC could mean that exposed fish at 24 hours of experiment, displays signs of stress. The largest decrease on RBC count was from fish in the group exposed to the highest concentration of MPs in association with bacteria, compared to the control group, bacteria and both groups with only MPs. On the fourth day after the first exposure there was an increase, although not significant (except in bacteria where it was significant), compared to the first day. At the fourth day, MPs and bacteria have already worked together (colonized MP with bacteria) and separated (after colonization). Within this experimental point, a significant decrease in counts was observed in fish from the group with higher concentration of MP and bacteria. At the end of trial (7 days) although there are no significant differences between experimental groups, there is an increase in counts compared to the data from the first sampling.

The increase in haemoglobin value (even if not significant in all groups) was coincident with a decrease in RBC values, and an increase of the MCH in fish blood. We can consider that the increase of MCH is the pathophysiological attempt to compensate the decreased RBC production due to reduced vitamin B12 availability. The still increased haemoglobin values can be explain with an increase in MCH that can balance the reduction of RBC number, when this increase cannot balance the total hemoglobin begins to decrease. These results can be explained with an interference on the vitamin B12 metabolism by the MPs when colonized with bacteria, being necessary's more studies to comprove this hypothesis. (Hamre et al. 1994)

WBC are responsible for the generalized response of the immune system, triggered by physiological stress (Tort 2011). After 4 days, unlike the first, there was a decrease in the counts of WBC from fish of the groups exposed to the mixtures (higher and lower MPs concentration with bacteria), as well as in fish from the group exposed to the lowest concentration of MPs compared to the ones from the group with only bacteria and the group exposed to the lowest concentration of MPs. This data may be connected to a cellular migration, for example to the intestine where the inflammatory process may be

occurring. In the last sampling time, a decrease in the number of WBC was observed in the fish from group exposed to the highest MPs concentration mixed with bacteria. Over the sampling time an increase in the amount of WBC can be observed. This increased may be linked to an immunity response to the live pathogen in the gut in some groups, and in others, microplastics that cause toxicity and probably inflammation (Ogier de Baulny et al. 1996).

Regarding immune-related enzymes studied in the present manuscript, proteases, which may play a protective role against pathogens by degrading pathogens and activating and enhancing the production of other innate immune components present in fish (Fernandes and Smith 2002). Percentage of protease activity presented a significant increase between fishes exposed to the highest MPs concentration and bacteria, on the first sampling point. This may be indicative of damage tissues, because as an inflammatory condition, proteases damage tissues (Ho, Pothoulakis, and Wai Koon 2013). This enzyme also showed a decrease in fish exposed to the higher MPs concentration with bacteria of day 7 when compared to day 1. This fact could be indicative of an infection.

Anti-proteases are protease inhibitors that contribute to the innate immunity of animals by inhibiting of the proteases released by the bacteria, limiting the digestion of proteins, source of amino acids (Ellis 2001).In the case of an inflammatory condition, proteases damage tissues while anti-protease stabilize tissue damage and help healing (Ho, Pothoulakis, and Wai Koon 2013).Contrarily, to the protease activity, the anti-protease activity did not show variations throughout the experiment.

At the end, to analyze oxidative stress, three biomarkers were tested, catalase, lipid peroxidation and glutathione s-transferase activity, in the liver of experimental fishes to verify oxidative stress.

The biomarker LPO is used as an indicator of lipid oxidative damage because can result in adverse effects of a wide range of physiological systems, including the hepatic system (Fonte, Ferreira, and Guilhermino 2016; Oliveira et al. 2013). In the present study, the LPO results presented significant differences between experimental groups. After 24 h from the first exposure, an increase of this parameter was shown on fishes from the higher MPs concentration with bacteria group when compared to the ones from the control, bacteria, lower and higher MPs concentration. Between sampling points were also a significant increase on fishes from the control group from the first to the seventh day. These results could indicate lipid oxidative damage in the liver of fish according to

previous studies (Ferreira et al. 2016; Fonte, Ferreira, and Guilhermino 2016; Luís et al. 2015). These authors reported that studies that exposed common goby fish (*Pomatoschistus microps*) studies with 0.216 and 0.184 mg/l and 1-5 µm MPs (polyethylene plastic microspheres) (Ferreira et al. 2016; Fonte, Ferreira, and Guilhermino 2016; Luís et al. 2015). At the lower MPs concentration and at the lower MPs concentration with bacteria group a decrease was verify from the first day to the fourth and to the seventh, indicating a possible diminution on lipid oxidative damage.

Oxidative stress was also verified measuring the activities of catalase and glutathione S-transferase in the fish liver. These enzymes are among the major antioxidant enzymes, protecting cells against peroxidation by decomposing reactive species and are commonly used as biomarkers of exposure to contaminants (Alomar et al. 2017; Chen et al. 2017; Karami, Groman, et al. 2017). The increment of the activity of these enzymes suggested the occurrence of oxidative stress (Chen et al. 2017). In this study, catalase and GST activity did not present significant differences between groups from the same sampling points, but at the lower MPs concentration group it was verified a decrease on day 7 and day 4 when compared to day 1. Catalase results are different from previous studies that indicate an increase of these biomarker at the presence of MPs in zebrafish (*Danio rerio*),, such as (Lu et al., 2016 study with zebrafish and with 5 µm polystyrene MPs with a concentration of 20 mg/l (Lu et al. 2016). GST results by its turn are concordant with previous studies (Ferreira et al., 2016; Luís et al., 2015; Oliveira et al., 2013). These authors exposed common goby (*Pomatoschistus microps*) to 0.216 and 0.184 mg/l polyethylene MPs (1-5 µm) and no significante changes were observed.

6. Further work

Further works are necessaries to complete this thesis. At the sampling, blood was also used to prepare blood smear preparations and it was also collected muscle, brain, head-kidney and intestine from the fishes. Then each eppendorf with tissues were immediately frozen on liquid nitrogen. Part of the intestine were also collected to histological analyses and other part were used to smear on a plate with TSA, to verify if *Phdp* reach this tissue.

7. Conclusions

About the preliminary trial, it is possible to deduce that MPs are colonized by *Phdp*. Bacterial colonization was higher on the bacterial medium solution (TSB), were slower (took more time to reach the colonization pike), and MPs stay more time colonized by the bacteria than on other tested solutions. Moreover, when a higher concentration of bacteria is used, a greater degree of colonization in TSB can be achieved. Regarding to the microplastics decay and its relationship with microplastics colonization by bacteria are still unclear, more studies are still needed.

About the live trial, fish when exposed to MPs colonized with bacteria presented a possible interference on the vitamin B12 metabolism, with an increase in MCH balancing the reduction of RBC number with the increased haemoglobin values. An increase of WBC values was also verified that may be linked to an immunity response to the live pathogen in the gut in some groups, and in others, microplastics that cause toxicity and probably inflammation. Regarding innate immune parameters, proteases presented an increase on fishes exposed to MPs colonized with bacteria when compared to the other groups, indicating some degree of infection and anti-proteases did not present variations when compared to the control group. Finally, about biomarkers only LPO were indicative of oxidative stress, because CAT and GST were inconclusive.

More analyzes are necessary to really understand all the implication of MPs colonized with bacteria exposure.

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