PhD in Aquatic Biosciences // No. 41 - 2021

Environmental impact of microplastics in relation to Atlantic salmon farming

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FACULTY OF BIOSCIENCES AND AQUACULTURE

NORD University

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A thesis for the degree of
Philosophiae Doctor (PhD)

PhD in Aquatic Biosciences no. 41 (2021)
Faculty of Biosciences and Aquaculture

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ISBN: 978-82-93165-40-8

Print: Trykkeriet NORD

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Preface

This thesis is submitted in fulfilment of the requirements for the degree of Philosophiae Doctor (PhD) at the faculty of Biosciences and Aquaculture (FBA), Nord University, Bodø. Norway. The presented original research was performed as part of the Stipendiatprogram Nordland. The studies carried out were financially supported by Nord University and the Norwegian Institute of Marine Research (IMR).

The project team consisted of the following members:

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Kiron Viswanath, Professor, FBA, Nord University: Co-supervisor

Torstein Kristensen, Associated Professor, FBA, Nord University: Co-supervisor

Alessio Gomiero, Senior Researcher, NORCE, Randaberg: Co-supervisor

Tanja Kögel, Researcher, IMR, Bergen: Collaborator

Isabel S. Abihssira-García

Bodø, June 2021

To my parents and sister,
for their wholehearted love and support

Acknowledgements

After this long journey that is the PhD, filled with ups and downs, beauty but also roughness, I must thank all the people that stood by my side and have made the accomplishment of this thesis possible, starting from my supervisors and colleagues, and finishing by my friends and family.

First, I want to express my full gratitude to my supervisor Pål A. Olsvik. Thank you for giving me the opportunity to do this PhD. When I applied for this job, I remember thinking "this is the perfect job for me, describes exactly my professional experience", but I never actually thought I would get in. You opened a door for me to a professional and personal growth, and to the beautiful country of Norway. I enjoyed every meeting and talk together, where I felt free to speak what is on my mind and after which I was filled with motivation. You gave me freedom in many aspects of the PhD, for instance in carrying out the experiments partly my way, which has made me a more independent researcher. I also highly appreciate that you listened patiently to any problem and helped me in any way possible. I really appreciate everything you have done for me, and I will always remember all your little funny jokes/comments, they made this journey a bit easier.

I also would like to thank my co-supervisors Torstein Kristensen, Kiron Viswanath and Alessio Gomiero. I am really grateful for all your efforts to make this PhD possible and successful.

Torstein, you were, along with Pål, the first to guide me around the university and introduce me to the Norwegian culture. We drove around looking for blue mussels for my studies, which I remember as a great touristic experience, seeing all the beautiful landscapes. Lastly, you gave me the opportunity to work in Scale AQ, for which I am very thankful.

Kiron, you gave me the opportunity to work with IFC, and along with Park, which has been a wonderful professional experience. I really enjoyed the meetings the four of us, Park, Pål, you and I, discussing the experimental designs for the microplastics exposure

experiment. I am also very grateful for your help in organizing my stay in the CSIC in Vigo.

Alessio, your expertise in the Microplastic research field has introduced me to important new methods for studying microplastics, which has improved considerably my professional experience. In that aspect, I must also thank Kjell Birger Øysæd, who patiently explained to me how Pyr-GC-MS works. Although short, my visit to Stavanger was delightful and I am very thankful to both of you for your welcoming and friendliness towards me.

In addition, I would like to highlight the help of some colleagues that have been especially important during this PhD.

To Youngjin Park, my dear friend and partner for half of the thesis. One of the hardest worker I know, you have taught me so much during this journey. Always so patient, friendly and funny, working with you has been an amazing experience. I will always remember our working days under the sterile chamber and in front of the IFC, with music in the background and learning Korean and Spanish. I want to thank you with all my heart because you are one of the main persons that have made this possible, and I wish we will be able to work together in the future.

Tanja Kögel, your knowledge on the microplastic research field as well as on aquaculture has been of immense help for the accomplishment of this thesis. You gave very valuable input to the study and thoroughly checked all details, which improved the work considerably. I am very grateful for the tour around the new Microplastic lab in IMR Bergen, where I finally was able to see in person an µFTIR and where I got very excited to how much the Microplastic research field is growing. I really hope we will keep in touch for future collaborations.

To Deepti M. Patel, my first and dearest friend here in Norway, but also my research colleague. Part of some experiments, you patiently helped and taught me and my student in the lab. Working with you was always fun and made the work enjoyable. Your expertise helped during troubleshooting, for which I am grateful. Moreover, our own

research group “The Science Show” have been a great experience for me. I enjoyed each one of the meetings, were I felt free to speak my mind with no fear of judgement. I want to thank you for everything you have done to make my PhD a better experience since the first week, professionally but mainly personally.

Morten Krogstad, you have been a fundamental part of this PhD, and this could not have been done without you. You took care of important parts of my study: organized boat trips, ordered materials, and helped troubleshooting when my field experiment fell apart by the loss of the cages. I really appreciate how much concern you always showed and I want to thank you for all your time, effort and kindness.

Miia Häkkinen, my first student and a brilliant person. You have been an amazing student, smart, hard worker, curious, motivated and patient, and that is only as far as I can think. Thank you for this first experience as main supervisor, you made it so easy and I wish all the best in your future carrier. You helped me a lot during my experiments too, and you definitely had to be included in this special acknowledgments.

Bisa Viswanath, thank you for your wisdom, advise and nice discussions concerning the statistics of my studies. Your help has been very valuable and I am very thankful for it, for your patience and for your kindness.

I would like to extend my gratitude to Beatriz Novoa and her team at the Spanish Research Council of Vigo for giving me the opportunity to improve my knowledge on mussels. My stay with you considerably helped me accomplish this PhD.

I would also like to thank all the staff at Nord University; technicians, engineers, administration, PhD advisor, IT, etc. We are all part of the same team and none of this would be possible without each one of you. I would also like to express my gratitude to the staff of IMR Bergen, and Norce Stavanger. A special thanks to my PhD and post-doc colleagues for their advises and shared experiences, that have eased this journey considerably.

I would like to express my gratitude to the program Eurodyssée and “La Maison Wallonne de la Pêche” of Namur, Belgium, that were key for the start of this PhD. *Un*

grand merci à tous mes collègues de la Maison Wallonne de la Pêche, spécialement à Frederic Dumonceau et Julien Gilles, pour votre support professionnel et pour me montrer qu'une bonne équipe, comme la vôtre, est essentiel pour accomplir un bon travail.

Last, but definitely not least, I would like to thank my friends and family for their wholehearted support, without whom I would have never arrived this far.

To my dearest friends in Norway, who made my stay in Bodø an amazing journey. You made me discover a world of adventure, fun and beauty in this new country that was Norway. All the hikes, camping, and infinite moments and laughs together have given me strength to get to where I am today. I am really thankful to all of you that stand by my side when I needed it, for all the cheering and all the support during this almost four years. And among all these amazing people, I would like to dedicate a few special warm words to Èric Jordà Molina, who has been not only be a great friend and neighbour, but like a brother to me. Thank you for all the visits, the craziness, the tears of laughter, but most of all, thank you for your wholehearted support, love and kindness since the day we met.

To Christian Van der Berg, my beloved life partner. There is no actual words to express how thankful I am to you. Your constant support, cheering, motivational words, and, above everything else, all your love are the reason I got here today. You and your family, my current family now too, made of Norway another home for me. I am and will always be very grateful for all the warmth and love you gave me during this journey, and especially during the hardest times. Every moment and every adventure lived here became twice as wonderful with you by my side.

A special thanks to my family, especially my loving, caring and amazingly perfect parents that I love with all my heart and without whom I could have never reached to where I am today, and to my friends from Spain and Belgium. This section will be written in Spanish and French for them.

A mis padres, Bernard Abihssira y Maribel García Atenza, y a mi hermana Leaticia Abihssira García. No existen palabras para expresar lo mucho que os quiero y cuánto agradezco tener una familia como vosotros. Gracias por vuestro apoyo incondicional tanto en los momentos buenos como en los malos, gracias por siempre confiar en mí y no dudar de mis capacidades para llegar hasta aquí, pero sobre todo, gracias por todo el amor y cariño que siempre me habéis dado. Sois la base y la clave de mi éxito y nunca habría llegado hasta aquí si no fuera por vosotros.

A mi tío Antonio García Atenza, gracias por regalarme tu sabiduría, transmitirme tu calma y por todo el amor y cariño siempre dado. Tus palabras me han guiado y mantenido en pie en los momentos más difíciles.

A mis amigas, quienes han crecido conmigo y han determinado en gran parte quien soy, quienes han sabido impulsarme a lo alto con palabras de ánimo y quienes se han mantenido a mi lado pese a la distancia. Gracias por vuestro apoyo y por creer en mí.

Et bien sûre, merci à tous mes amis du programme Eurodyssée, ma famille belge. Grâce à vous j'ai eu le courage de recommencer une nouvelle vie dans un autre pays sans avoir peur d'être perdu ou seule. Un merci spécial à Benoît, tes conseils ont été fondamentaux pour obtenir ce travail, et à Concu, qui a été présente quand j'en es toujours eu besoin.

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Supplementary file

List of abbreviations

AFFC	Acoustic focusing flow cytometry
DDT	Dichlorodiphenyltrichloroethane
EQS	Environmental Quality Standards
EROD	Enzyme 7-ethoxy-resorufin-O-deethylase
EU	European Union
HDPE	High density polyethylene
IFC	Imaging flow cytometry
LDPE	Low density polyethylene
NO	Nitric oxide
PA	Polyamide
PAH	Polycyclic aromatic hydrocarbon
PBDE	Polybrominated diphenyl ethers
PCB	Polychlorinated biphenyl
PE	Polyethylene
PET	Polyethylene terephthalate
POP	Persistent organic pollutant
PP	Polypropylene
PPE	Personal protective equipment
PS	Polystyrene
PVC	Polyvinyl chloride
pyr-GCMS	Pyrolysis gas chromatography-mass spectrometry
TEQ	Toxic equivalent
µFTIR	Fourier transform infrared microspectroscopy

List of papers

Supplementary file	Polyethylene and polystyrene microplastic uptake by blue mussel haemocytes.

Paper I	Park, Y., Abihssira-García, I. S., Thalmann, S., Wiegertjes, G. F., Barreda, D. R., Olsvik, P. A., Kiron, V. (2020) Imaging flow cytometry protocols for examining phagocytosis of microplastics and bioparticles by immune cells of aquatic animals. Frontiers in immunology, 11: 203.
Paper II	Abihssira-García, I. S., Park, Y., Kiron, V., Olsvik, P. A. (2020) Fluorescent microplastic uptake by immune cells of Atlantic salmon (*Salmo salar* L.). Frontiers in Environmental Science, 8: 233.
Paper III	Abihssira-García, I. S., Kögel, T., Gomiero, A., Kristensen, T., Krogstad, M., Olsvik, P. A. Distinct polymer dependent sorption of persistent pollutants associated with Atlantic salmon farming to microplastics. Manuscript.

Abstract

Microplastic pollution is a global issue that has raised great concern to citizens of all countries. Microplastics are ubiquitous in the environment and have been found in all marine ecosystems, from the Arctic Ocean to the depth of the Mariana Trench. Despite the exponential increase of research on microplastic, many unknowns, such as environmental concentrations of small microplastics particles and toxicity of different microplastic types, still need to be defined. This thesis aims to provide new insight into the environmental impacts of microplastics focusing on Atlantic salmon aquaculture. For this purpose, novel imaging flow cytometry methodology was developed to study the impact of different microplastic types at the cell level by assessing cellular behaviour of several marine organisms, including salmon. Furthermore, the thesis increase our understanding about interactions between pollutants and microplastics in the marine environment, by studying the sorption of persistent organic pollutants (POPs) associated with Atlantic salmon farming by different microplastic polymers.

Imaging flow cytometry enabled us to study the response of immune cells from fish and mussels to microplastics as well as to characterize the size and agglomeration rates of the microplastic particles. Salmon and mussel immune cells showed distinct responses to different microplastic types. Salmon leucocytes had 10 times lower ability to uptake microplastics compared to mussel haemocytes, and cells phagocytosed polyethylene (PE) more efficiently than polystyrene (PS), unlike mussel haemocytes. In salmon, PE accumulated in the leucocytes over a period of 3 days, while PS appeared to be excreted by the cells. However, mussel haemocytes exposed to medium and low microplastic concentrations accumulated both PE and PS over time, while haemocytes exposed to high microplastic concentrations showed a decreased in the number of cells with microplastics. These results show that different microplastic types can induce diverse responses in distinct species.

The study of interactions between microplastics and POPs associated with salmon aquaculture showed that dioxins and polychlorinated biphenyls (PCBs) can bind in relatively high amount to various microplastic polymers when these are present near

fish farming sites. Sorption of such POPs was polymer dependent, and polyethylene terephthalate (PET) and polyvinyl chloride (PVC) showed high sorption of dioxins and PCBs compared to high density polyethylene (HDPE). These results suggest that microplastics are potential vectors of pollutants associated with fish farming, and should be considered in future risk assessments of marine aquaculture.

1. Introduction

Plastic is an integral part of modern society. Benefits of plastic are plenty, and its chemical stability, durability, strength, resistance, ductility and waterproofing potential makes it the idea choice for numerous purposes (Grumezescu and Grumezescu, 2019). However, these characteristics also make plastic a threat to the environment. Plastic is nowadays found everywhere on the globe, and accumulation of these synthetic materials in the marine environment is of special concern worldwide (Wabnitz and Nichols, 2010, Thushari and Senevirathna, 2020). In addition, a new source of plastic pollution has appeared from last year as a consequence of the COVID-19 pandemic. Single-use personal protective equipment (PPE), such as gloves, face masks and face shields, are primarily made of plastic (Ammendolia et al., 2021). Due to improper disposal and littering of PPE, these materials have found their way into beaches, rivers and coastlines, that is less than a year after the first wave of COVID-19 (Figure 1) (Iftimie et al., 2021, De-la-Torre and Aragaw, 2021). Yet, the magnitude of the environmental impact of this pandemic in terms of plastic pollution is still unknown and many questions are to be answered.

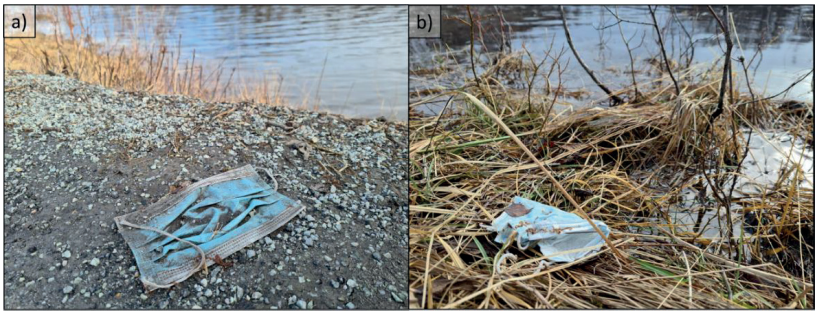

Figure 1: Single-use masks found along the bank of the river Futelva, Bodø, at approximately 2-3 km from reaching the sea. Plastic pollution in aquatic environments as consequence of the COVID-19 pandemic is also noticeable above the Arctic Circle. Pictures captured in April 2021.

1.1. Plastic and its history

Plastic is a material made from natural, semi-synthetic or synthetic polymers (Van der Vegt, 2006). However, today the term plastic mainly refers to synthetic polymers, as also referred to in this thesis.

The first recognized synthetic polymer, a thermosetting plastic called Bakelite, was created in 1907 in New York by the chemist Leo Baekeland (Baker, 2018). The formation of polystyrene (PS) was already known by that time. In 1839, Edward Simon found out that monomers of styrene obtained from the resin of the tree *Liquidambar orientalis* could be polymerized. However, styrene was not synthetised from petroleum-derived ethylbenzene until 1930. It is in this year that synthetic PS was created for the first time, and this thermoplastic polymer was not produced and commercialized until one year later, in 1931 (Boyer, 1981, Scheirs and Priddy, 2003). The first synthetic thermoplastic, polyvinyl chloride (PVC), was discovered in 1912, although it might have been synthetised for the first time earlier. Commercialization of PVC did not materialize until the early 1930s (Mulder and Knot, 2001). Similarly, although the polymerization of ethylene was already reported in 1930, it went unappreciated. The existence of a polymer of ethylene, or polyethylene (PE), was first acknowledged in 1933, after which PE was quickly commercialized due to wartime needs (Peacock, 2000). Polyethylene terephthalate (PET) and polypropylene (PP), two of the main thermoplastic polymers, were discovered later. PET was discovered and patented in the UK in 1941 (Whinfield and Dickson, 1941, Danso et al., 2018), and PP was synthetised for the first time in 1954 in Italy (Natta and Corradini, 1960) (Table 1).

Table 1: Chemical formulae of the synthetic polymers used in this thesis, listed by earlier to later official discovery year from top to bottom. Chemical formulae obtained from (Wang et al., 2020a).

Synthetic polymer	Discovery year	Chemical formula
Polyvinyl chloride (PVC)	1912	$(C_2H_3Cl)_n$
Polystyrene (PS)	1930	$(C_8H_8)_n$
High density polyethylene (HDPE)	1933	$(C_2H_4)_n$
Polyethylene terephthalate (PET)	1941	$(C_{10}H_8O_4)_n$
Polypropylene (PP)	1954	$(C_3H_6)_n$

Hardly 40 years after the synthesis and commercialization of plastics, microplastics were found in the marine environment for the first time (Carpenter and Smith Jr, 1972).

1.1.1. Plastic as a pollutant

Plastic is extensively used in the current society due the versatility and benefits of this material. Plastic can be classified either as thermosetting or thermoplastic based on its response to temperature (Grumezescu and Grumezescu, 2019). Thermosets are plastic polymers that undergo an irreversible process of solidification due to the formation of chemical bonds among their molecules. This network results in hard and rigid material whose melting temperatures surpasses the decomposition temperature and, consequently, such plastics cannot be recycled. Some examples of thermosetting plastic are formaldehyde-based thermosetting resins, mainly used as adhesives, polyurethanes, mainly used as foam in construction and furniture, and silicones and epoxies, used in a wide range of applications such as coating, paint, adhesive and composites (Dodiuk & Goodman, 2013). Thermoplastic polymers, on the other hand, can be remelted a limited number of times after solidification and can therefore be recycled. These polymers are not only strong and stable but also ductile, transparent and hydrophobic materials, explaining why they have been extensively used since they were developed. The main types of thermoplastic polymers, their main uses, and items they can be recycled into are listed in Table 2.

Table 2: Common thermoplastic polymer types, their applications, and items they can be recycled into. Information obtained from Alabi et al. (2019).

Symbol	Thermoplastic polymer	Common uses	Recycled into
	**Polyethylene terephthalate**	Soft drinks and water bottles, Salad domes, Biscuit trays, Salad and dressing containers	Pillow and sleeping bag filling, Clothing, Soft drink bottles, Carpeting, Building insulation
	**High density polyethylene**	Bags, Bottles of milk, juice, shampoo, chemicals and detergents, Ice cream containers, Buckets, Rigid agricultural pipe, Crates	Bins, Buckets, Detergent containers, Posts, Fencing, Pipes, Plastic timber
	**Unplasticized polyvinyl chloride (uPVC)**	Cosmetic containers, Electrical conduit, Plumbing pipes and Fittings, Blister packs, Wall cladding, Roof sheeting, Bottles	Flooring, Film and Sheets, Cables, Speed bumps, Packaging, Binders, Mud Flaps and Mats, New Gumboots and Shoes

	**Plasticized polyvinyl chloride (pPVC)**	Garden hoses, Shoes soles, Cable sheathing, Blood bags and tubing	Flooring, Film and Sheets, Cables, Speed bumps, Packaging, Binders, Mud Flaps and Mats, New gumboots and shoes
	**Low density polyethylene**	Cling wrap, Bags, Squeeze bottles, Irrigation tubing, Mulch Film, Refuse Bags	Bin liners, Pallet sheets
	**Polypropylene**	Bottles, Ice cream tubs, Potato chip bags, Straws, Microwave dishes, Kettles, Garden furniture, Lunch boxes, Packaging tape	Pegs, Bins, Pipes, Pallet sheets, Oil funnels, Car battery cases, Trays
	**Polystyrene**	CD and video cases, Plastic cutlery, Imitation glassware, Low-cost brittle toys	Coat hangers, Coasters, White ware components, Stationery trays and accessories, Picture frames, Seed trays, Building products
	**Expanded polystyrene**	Foamed cups, Takeaway clamshells, Foamed meat trays, Protective packaging, Building and food insulation	Coat hangers, Coasters, White ware components, Stationery trays and accessories, Picture frames, Seed trays, Building products
	**Styrene acrylonitrile (SAN), Acrylonitrile Butadiene Styrene (ABS), Polycarbonate (PC), Polyamides (PA)**	Automotive and appliance components, Computers, Electronics, Cooler bottles, Packaging	Automotive components, Plastic timber

Plastic production has increased exponentially since this material was first synthetised and commercialized (Figure 2). Its production went from 1.5 million tons in 1950 to 322 million tons in 2015, and the estimated worldwide consumption of plastic by 2025 is 400 million tons. Today, thermoplastics accounts for about 80% of the total plastic consumption in western Europe, and probably even more in North America (Biron, 2018). Consequently, thermoplastics are the main type of plastic polluting the environment. Thus, this thesis focuses on the impact of thermoplastics in the marine environment.

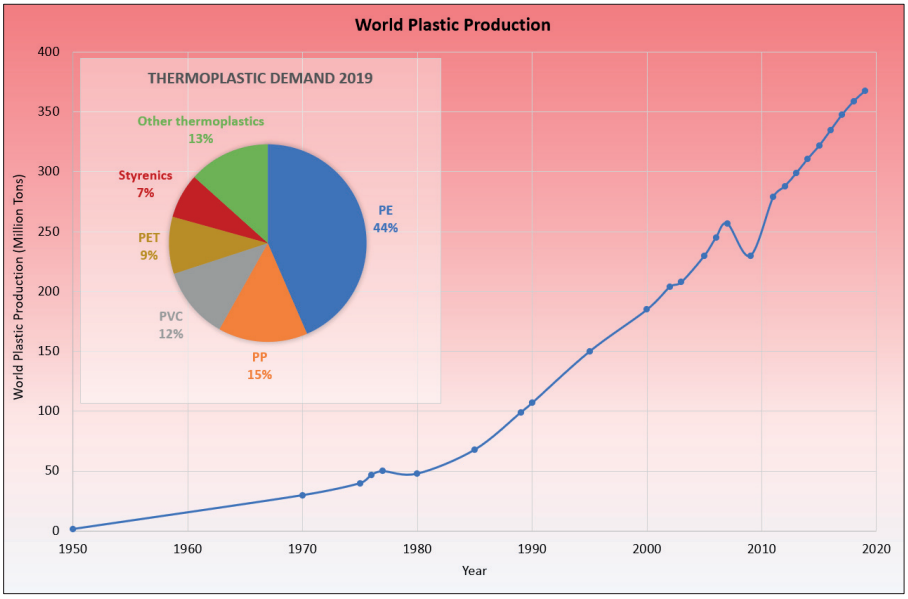

Figure 2: Global plastic production from 1950 to 2019. Pie chart shows the demand of thermoplastics in 2019 divided by polymer type. Data obtained from (PlasticsEurope, 2021) and (Biron, 2018).

Mismanaged waste, littering and wear and tear of diverse plastic items are some of the main causes of plastic in the environment. (Worm et al., 2017). In oceans, the major inputs of plastic is from land-based sources, which either originate from coastal anthropogenic activities or are transported by rivers to the marine environment. A recent study estimated that between 0.8 million and 2.7 million tonnes of plastic enters the oceans from rivers, and contrarily to what was previously estimated, more than 1600 rivers are responsible for 80% of those emissions (Meijer et al., 2021). These results suggest that an increased effort is needed to reduce emissions of plastics to the oceans. Plastic in the environment can take decades and even centuries to fully disintegrate due to the slow degradation of this man-made material. The presence of diverse plastic items in marine waters has therefore become a threat to many species. Entanglement of pinniped, seabirds, turtles and other marine organisms with macroplastics (Duncan et al., 2017, Ryan, 2018, Jepsen and de Bruyn, 2019), ingestion of macro-, meso- and microplastics, and the potential of plastic as vector of pollutants (Bråte et al., 2017) are some of the concerns that have been associated with plastic pollution.

Plastic as a pollutant in the environment is commonly classified by size considering the impact of each size-class on organisms. Size-based classification of plastic litter was first proposed in 1999 by Gregory, who introduced the terms micro-, meso-, macro- and megalitter (Gregory, 1999). How to divide plastic litter in size classes is still under debate and can vary depending on the literature. Generally, macroplastics are defined as plastic materials above 20 mm in size, mesoplastics as particles between 5 and 20 mm, microplastics as particles ranging 1 µm to 5 mm, and nanoplastics as plastic particles below 1 µm in size (Arthur et al., 2009, Hartmann et al., 2019). Due to the increasing global concern over microplastics, this thesis will focus on such plastics and their impact on the marine environment.

1.2. Microplastics

The presence of microplastics in the environment was recorded for the first time in surface waters of the Sargasso Sea in 1972 (Carpenter and Smith Jr, 1972). Plastic particles from 2.5 to 5 mm were collected during pelagic samplings with neuston nets. The same year, plastic particles identified as polystyrene and ranging in size from 0.1 to 2 mm were detected in coastal waters of southern New England, USA, in the bay surrounded by New York, Connecticut and Rhode Island (Carpenter et al., 1972). Soon, other scientists started investigating the presence of small plastic particles in the environment (Colton et al., 1974, Gregory, 1978, Moore et al., 2001). However, it wasn't until 2004 that the term "microplastic" was coined by Thompson et al. (2004).

In 2008, the upper size limit of microplastics (5 mm) was proposed by a group of environmental research scientists based on their possible ecological effects (Arthur et al., 2009). Recently, Hartmann et al. (2019) have proposed to redefine the term microplastic to adapt it to the international unit system and, therefore, define microplastics as particles between 1 µm and 1 mm. Until now, the minimum size of microplastics has been under debate and no clear consensus exists among the researchers in this field. Several scientists have defined microplastics as particles above 1 µm, while others have used 100 nm as the lower limit following the recommended definition of nanomaterials of the European Commission (EC, 2011, Mendoza et al.,

2018, Hartmann et al., 2019). However, nanomaterials are purposefully manufactured at the nanoscale (1 to 100 nm) due their technological advantages (Stone et al., 2017), while nanoplastics mainly come from the degradation of bigger-sized plastic particles and, therefore, do not necessarily have to follow such definition. Microplastics are, in turn, commonly subcategorised into large and small depending on their size, but the established threshold between the two categories, set at 1 mm, 500 µm, or at a smaller size, is highly variable among the literature (Ter Halle et al., 2017, Primpke et al., 2020). In this thesis, microplastics are defined as plastic particles ranging from 1 µm to 5 mm, and nanoplastics are defined as plastic particles below 1 µm in size.

The ubiquitous presence of microplastics in the environment is a well-known fact. Due to oceanic currents and atmospheric transport (Evangeliou et al., 2020), microplastics have been detected even in the most remote places such as mountain lakes in Mongolia (Free et al., 2014), Arctic sea ice (Peeken et al., 2018) and in Antarctic sediments (Cunningham et al., 2020). In the marine environment, microplastics have been found essentially everywhere: oceans, estuaries, fjords, beaches, coastal waters, surface waters, water column, marine sediment and even in the deepest waters of the Mariana Trench (Lusher, 2015, Andrady, 2017, Peng et al., 2018, Harris, 2020).

Microplastics in the environment mainly originate from the breakdown and weathering of larger plastic debris, which degrade as a consequence of physical, chemical and biological processes occurring in the environment. These are known as secondary microplastics and include particles such as microfibres and microplastic fragments. Another source of microplastics in the marine environment comes from the release of plastic microparticles manufactured as such for commercial purposes. These are called primary microplastics and include particles such as microbeads, used as abrasive in cosmetics and cleaning products, and pellets (also called mermaid tears) used in the production of larger plastic objects. Their release into the environment can be due to industrial spillage or due to the lack of proper sewage treatment (Cole et al., 2011, Thompson, 2015). However, microbeads account for a relatively low proportion of the microplastics in the marine environment (Lindeque et al., 2020). The major shapes of microplastics found in seawater are fibres, which mainly come from clothing of fishing

gear, and fragments, in that order (Barrows et al., 2018, Bikker et al., 2020, Huntington et al., 2020, Lindeque et al., 2020). In Norwegian waters, the main source of microplastics arise from tire abrasion (Bråte 2017). Equipment used in activities such as aquaculture can also be a source of these particles in the marine environment (Lusher et al., 2017a). It has recently been shown that feeding pipes used in Atlantic salmon farming, which are mainly made of HDPE, can release microplastics along with the feed pellets due to the pressure and temperature they are subjected to (Gomiero et al., 2020). The main microplastic polymers found in the marine environment are thermoplastics. PE has been widely reported to be the major microplastic polymer that pollutes marine waters, followed by PP (Enders et al., 2015, Suaria et al., 2016, Ter Halle et al., 2017). PE microplastics are the major polymer found in marine sediments in Norwegian fjords, followed by PVC and PET (Gomiero et al., 2019).

The concentration of microplastics recorded in marine waters varies considerably among locations, as shown in Figure 3, but also among studies. A recent study found that higher concentrations of microplastics above 100 µm in size were present in the Arctic Ocean (31.3 ± 6.5 particles/L), followed by the Southern (15.4 ± 8.1 particles/L), Atlantic (13.4 ± 0.9 particles/L), Pacific (7.0 ± 0.8 particles/L) and Indian (4.2 ± 1.2 particles/L) Oceans (Barrows et al., 2018). However, the concentration of microplastics in the marine environment has probably been underestimated from one to four orders of magnitude based on the adopted sampling methods (Covernton et al., 2019). Microplastics in the sea have mainly been sampled with towing nets of 250 to 333 µm mesh size, such as neuston nets and manta trawl, since they enable filtration of large amount of water (Arthur et al., 2009, Kang et al., 2015, Miller et al., 2017). It was recently shown that sampling microplastics with a 100 and 333 µm mesh net resulted in microplastic concentrations 10 and 2.5 times greater, respectively, than using a 500 µm mesh net (Lindeque et al., 2020). The use of nets to collect microplastics are not the only cause for underestimation of microplastic concentrations. The mesh size used to filter water samples collected with Niskin bottles or jars are also important to accurately quantify microplastics. A study found that seawater filtered through an 8 µm stainless steel mesh accounted for 8.5 times more microparticles than when using a 65 µm mesh

size (Covernton et al., 2019). Most of the available data of microplastic concentrations in seawater corresponds to the larger size fraction of microplastics. Fragments as small as 1.6 µm have been reported in the marine environment (Galgani et al., 2010), but lack of proper methods available for capturing and analysing particles below 10 µm have hindered the accurate quantification of microplastics in the environment. However, great efforts have been put in the recent years to improve the technology and protocols for measuring microplastics, and instruments such as Raman microspectroscopy (µ-Raman), Fourier transform infrared microspectroscopy (µFTIR) and pyrolysis gas chromatography-mass spectrometry (pyr-GCMS) can now detect particles below 10 µm (Müller et al., 2020). For instance, µ-Raman can detect particles down to 1 µm (Schymanski et al., 2018), µFTIR have been shown to detected particles down to 2.1 µm and pyr-GCMS can detect particles below 10 µm if he mass of plastic is large enough (Gomiero et al., 2020b). Furthermore, a new methodology using surface-enhanced Raman spectroscopy (SERS) with Klarite substrates have been shown to retain and detect sizes down to 360 nm (Xu et al., 2020).

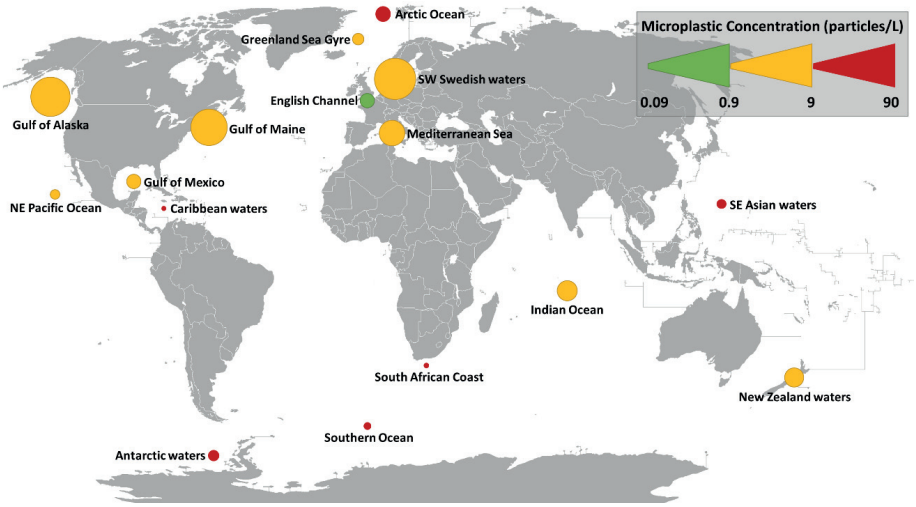

Figure 3: World map showing average microplastic concentration in different marine regions. Red dots show higher microplastic concentrations, followed by yellow and green dots. Dot sizes are proportional to the microplastic concentrations. Data obtained from (Desforges et al., 2014, Enders et al., 2015, Waller et al., 2017, Barrows et al., 2018, Jiang et al., 2020).

The fast-developing technology for analysing microplastics reflects the increasing concern over these particles. The number of publications on microplastics have increased exponentially in the last decade (Figure 4). More than 93% of the total literature on micro- and nanoplastic has been published after 2008 (Sorensen and Jovanović, 2021). One of the main concerns about microplastics is the ingestion of such particles by organisms. Intake of microplastics has been documented in over 2020 species, including fish and bivalves. A special focus has been placed on the impact of microplastics on marine life; more than 80% of the studies of biota interaction with microplastics have been done on marine organisms (Lusher et al., 2017b). The microplastic content in the digestive tract of fishes from several seas and oceans have been analysed, and 0.3 to 77% of sampled wild fish had ingested microplastics with up to 5.88 particles detected per fish in average (Jovanović, 2017). However, small microplastics were not assessed in these studies. A study carried out in coastal Portuguese waters analysed the stomach content of 26 fish species and found that 19.8% of the fish had microplastics inside them and average particle per fish was 1.40 ± 0.66. Plastic particles identified were all above 200 µm. Interestingly, 63.5% of the species that ingested microplastics were benthic, whereas 36.5% were pelagic. In accordance to the microplastic types found in the environment, the majority of the particles ingested were microfibers, identified as PE, PET and rayon, acrylic and nylon. On the other hand, the microplastic fragments were identified as PP, PE and alkyd resin, a polyester commonly used in paint (Neves et al., 2015). Similarly, over 90% of microplastics (100 µm to 5 mm) found in the digestive tracts of Chinook salmon (*Oncorhynchus tshawytscha*) juveniles captured nearshore Vancouver Island, Canada, were microfibers, and 59% of the fish sampled had ingested microplastics (Collicutt et al., 2019). Microplastics have also widely been found to be ingested by farmed and wild mussels all over the world, with microfibers as the dominant shape accumulated followed by fragments. The main polymers found in mussels are PE, PP, PS, PET, PA, PVC and cellophane. The abundance of microplastics in mussels varies among studies, ranging from 0.086 ± 0.031 to 259 ± 114 particles/g of wet weight (Li et al., 2019). Similarly to what was found in fish, a study carried out in China found that farmed

mussels (pelagic) contained less microplastics in their tissue compared to wild mussels (benthic). The most common shapes of microplastics (5 µm to 5 mm) detected in the mussels were microfibers followed by fragments (Li et al., 2016). Mussels (*Mytilus* spp.) sampled along the Norwegian coast, including in Bodø, have also been reported to have microplastics in their tissues. Mussels collected south from Sognefjord (North Sea), and in the north of Finnmark (Barents Sea) had higher concentrations of microplastics compared to mussels collected in middle Norway (Norwegian Sea) (Bråte et al., 2018).

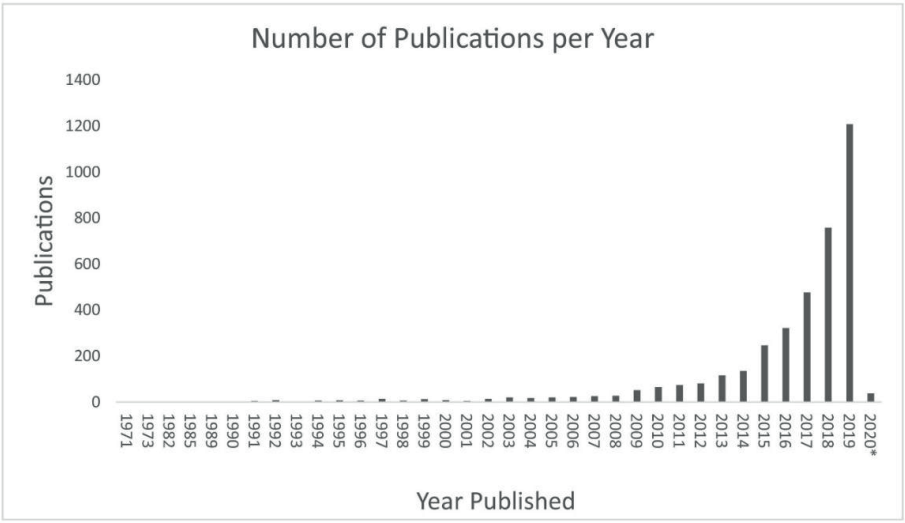

*Figure 4: Histogram showing the number of micro- and nanoplastic studies published each year. The bibliographic search was conducted from 1900 until the end of December 2019. Publications from 2020 were early accessed articles, as denoted by *. Figure reproduced from (Sorensen and Jovanović, 2021) with permission from Elsevier.*

Some of the concerns associated to microplastic ingestion are the potential translocation of these particles from the digestive tract to other organs and tissues (Browne et al., 2008, Wang et al., 2020b, Ma et al., 2020), their transfer through the trophic chain (Farrell and Nelson, 2013), and their toxicity.

1.2.1. Toxicokinetics of microplastics

Several studies have reported the presence of microplastics in internal tissues and organs of organisms (Ding et al., 2018, Zitouni et al., 2020, Gomiero et al., 2020a). This suggests that particles are translocated from external tissues or the digestive tract into

the body. Uptake and translocation of microplastics into the body can occur through different pathways; microplastics can enter through cells by endocytosis or can pass in between cells (Galloway, 2015). In invertebrates such as bivalves, microplastics uptake first occurs on the gills. Part of the microplastics filtered through the siphon might get trapped in the microvilli and be engulfed by the gill epithelial cells through endocytosis. Non-engulfed particles are then egested through pseudo-faeces or transported to the digestive tract, i.e. stomach, intestine and digestive tubules, where microplastics can be translocated by endocytosis or by passing in between cells. Non-translocated microplastics might accumulate in the digestive tract or be excreted through faeces (Von Moos et al., 2012, Woods et al., 2018). In vertebrates such as fish, translocation of microplastics into the body can also occur both in gills and the digestive tract, and microfold cells might play an important role in this process. Microfold cells, or M cells, are specialised epithelial cells present in the gut-associated lymphatic tissue (GALT) of mammals. These cells have shown the ability to take up macromolecules, bacteria and relatively large particles from the gut and translocate them to the lamina propria (LP). In fish, homologue of M cells have been detected in the posterior intestine and in gills of salmonids (Fuglem et al., 2010, Kato et al., 2018). Thus, M-type cells could potentially take up small microplastics from the digestive tract and gills of fish, and translocate them into the LP. The LP is a connective tissue that contains numerous immune cells such as macrophages and lymphocytes (Boudry et al., 2004, Park, 2021). Microplastics translocated to the LP could then be taken up by phagocytic immune cells, which could transfer such particles to other tissues and organs (Abihssira-García et al., 2020). The second entry pathway of microplastics to the body could be uptake through intercellular junctions between cells. This pathway is more likely to occur during pathologies that affect the digestive tract and that lead to a gap junction in the intestinal epithelial barrier (Amornphimoltham et al., 2019). In fish, enteritis can be caused by compounds present in the diet. For instance, saponins, a compound present in soya, are known to cause inflammation in the distal intestine of salmon (Krogdahl et al., 2015). Farmed salmon fed with feeds containing soya might therefore be at higher risk of getting microplastics translocated into the body through this pathway.

Microplastics translocated into the body might be partially excreted by immune cells. However, translocated particles might also be incorporated into tissues and accumulate for long time periods due to the lack of enzymatic pathways to degrade plastic in most of the species (Wright et al., 2013). Microplastics have recently been found in muscle and liver tissues from both wild and farmed salmonids (Gomiero et al., 2020a). Similarly, both wild and farmed blue mussels have been reported to contain microplastics (Li et al., 2016). Translocation and accumulation of microplastics in marine organisms poses an additional threat to marine ecosystems, since the probability of trophic transfer is increased. Moreover, accumulation of microplastics in consumed tissues of farmed species, such as salmon fillet, might present a potential risk for human food safety.

1.2.2. Toxicity of microplastics

Exposure to microplastics can have negative impact on organisms in several ways. Changes in feeding behaviour and disruption of the immune, endocrine, nervous and reproductive systems are some of the effects documented on organisms exposed to microplastics (Jovanović, 2017, Ma et al., 2020). Numerous studies have reported the exposure concentration to be one of the main factor influencing the toxicity of microplastics (Prokić et al., 2019). Nile tilapia (*Oreochromis niloticus*) exposed to high microplastics concentrations (100 mg/L) of PS had acute disruption of biochemical parameters involved in the kidney and liver function compared to tilapias exposed to lower concentration (1 and 10 mg/L). In addition, haematological parameters such as percentage of monocytes was significantly affected at high microplastics concentrations (Hamed et al., 2019). In fish larvae, exposure to high microplastic concentrations have been documented to significantly increase mortality rates exposed to high (Mazurais et al., 2015). In blue mussels (*Mytilus edulis*), relatively high concentrations of 4-6 µm HDPE microplastics (20 mg/L) have also been shown to induce greater alteration in the gut microbiota than environmental realistic concentrations (0.2 mg/L) (Li et al., 2020). However, high concentrations of microplastics can result in particles aggregation, which might reduce the negative impact at the cellular level compared to lower microplastics concentration. This has been observed in blue mussels exposed to three

environmentally relevant microplastic concentrations (0.008, 10, and 100 µg/L), where the two lower concentrations significantly increased the acid phosphatase (AcP) activity of the haemocytes, while the higher concentration did not (Revel et al., 2019). Thus, environmental realistic concentrations of microplastics could induce a significant toxic effect at a smaller scale compared to high concentrations. Exposure time is an important factor to assess in the toxicity of microplastics. Some studies have observed that organisms can adapt to microplastic exposure. Mediterranean mussels exposed for 24 h to 50 PS particles/mL (3 µm) had elevated amount of microplastics in the digestive glands, but such levels decreased at 48 h, suggesting that mussels excreted the particles after longer exposure times (Cappello et al., 2021). However, other studies have reported that negative effects of microplastics only appear after several weeks of exposure. For example, mussels (*Mytilus* spp.) exposed to PS microplastics only showed decreased byssus production after 36 weeks of exposure. In addition, the water clearance rate of the mussels, i.e. capacity to filter microalgae, declined after 18 weeks of exposure to high microplastic concentrations and decreased significantly after 36 weeks exposure (Hamm and Lenz, 2021). Prolonged exposure to microplastics might thus induce a chronic stress response resulting in higher toxicities than expected. Long exposure experiments are therefore needed to fully understand the negative impact of microplastics on organisms.

Physical and chemical properties of microplastics are two other main factors determining their toxicity (Ma et al., 2020) (Figure 5). Microfibers tend to be more ingested by both bivalves and fish in the marine environment compared to other shapes (Li et al., 2016, Wang et al., 2020b). However, size also plays an important role in the ingestion and impact of microplastics. The intake of microplastics by the Korean mussel (*Mytilus coruscus*) have been shown to be higher for particles of 10 µm in size than for particles of 100 µm (Wang et al., 2021a). In goldfish (*Carassius auratus*) exposed to 300 mg/L of PS micro- and nanoplastic, larger amounts of 0.25 µm particles accumulated in the intestine than 8 µm particles, while higher concentrations of 8 µm particles were found in the gills (Abarghouei et al., 2021). Both the size and shape can influence the toxicity of ingested microplastics. For instance, mortality of shrimps (*Palaemonetes*

pugio) exposed to microspheres and microfragments of around 35 µm was not affected, while a mortality of above 20 % was recorded for shrimps exposed to bigger spheres (≥75 µm) and fragments (93 µm). However, all microfiber sizes induced a mortality of 35 to 55% (Gray and Weinstein, 2017). This study suggested that the increased toxicity of microfibers could be due to the inability of the organisms to egest such particles, leading to a gut blockage and damage of internal structures and resulting in death. By contrast, a study carried out in mussels (*Mytilus* spp.) found that particle shape did not play a role in the toxicity of microplastics. Mussels exposed to microfibers (10 µm x 30 µm) and microspheres (20 µm) had similar levels of such particles in the digestive glands. Both exposures to microfibers and microspheres induced oxidative stress in the digestive glands of the mussels at 24 h, but the stress levels were back to normal after 7 days of exposure, indicating a temporary oxidative stress that is unlikely to have long-term effects in the mussels (Cole et al., 2020). Particle shape can therefore play a significant role in the toxicity of microplastics in some species, but might be less significant in others.

Particle size appears however to be an important factor to consider when examining the toxicity of microplastics in different organisms. As mentioned previously, bigger microplastic fragments and spheres increased the mortality in shrimp (Gray and Weinstein, 2017). The increased mortality observed in organisms exposed to larger particles could be related to a blockage of the digestive tract but also to internal damage. Histopathological analysis showed that European sea bass (*Dicentrarchus labrax*) chronically exposed to PVC microplastics (<300 µm) for 30 and 90 days had moderate and severe alterations, respectively, in the distal intestine (Pedà et al., 2016). In goldfish, larger particles of PS (8 µm) induced more severe lesions in the gills compared to smaller PS particles (0.25 µm). However, the smaller microplastic particles induced more histological lesions in the liver and intestine. Moreover, exposure to the smaller PS particles had a stronger effect on the activity of the catalase and superoxide dismutase (SOD), and the gene expression of catalase and heat shock protein (HSP70), suggesting that smaller particles induce oxidative and cellular stress more strongly than larger particles (Abarghouei et al., 2021). Similarly, smaller plastic particles have a stronger

impact on the immune system of mussels. Exposure to nanoplastics (50 nm) increased the granulocyte/hyalinocyte ratio at 24 h and 7 days, contrary to microfibers (10 µm x 30 µm) and microspheres (20 µm), which could be due to translocation of the nanoparticles to the haemolymph or inflammation caused by such particles. However, both micro- and nanoplastics induced oxidative stress in the mussels digestive glands and gills, respectively, at 24 h exposure, but levels went back to normal following 7 days exposures (Cole et al., 2020). In general, smaller microplastics have higher impact on cells. The mortality of human cells exposed to 1 g/L of small PP particles (approximately 6 µm) increased by 20%, while exposure to larger PP particles (approximately 30 µm) did not have cytotoxic effect on the cells (Hwang et al., 2019). In mouse macrophages, PS particles of 12.5 µm were not phagocytosed, while small PS particles (1.2 and 6.2 µm) were taken up by the immune cells and caused stronger inflammation and necrosis in tissues such as spleen, pancreas, and liver (Tomazic-Jezic et al., 2001). In addition, small microplastics are more likely to translocate from the digestive tract to other organs and tissues. Goldfish exposed to 300 mg/L of plastic for 168 h accumulated about twice as many 0.25 µm PS particles in the liver compared to 8 µm PS particles (Abarghouei et al., 2021). European sea bass fed for 5 days with feed containing microplastics (0.33 mg/g of feed) accumulated more particles ranging between 0.45 µm and 1.2 (2.83 ± 1.81 particles/g) in the liver than particles above 1.2 µm (Zitouni et al., 2021). Similarly, a study carried out in the Tunisian coast found higher abundance of microplastics smaller than 3 µm in size in the muscle of wild fish *Serranus scriba* compared to bigger-sized microplastics (Zitouni et al., 2020). Moreover, egestion of small microplastics take longer time; rotifers (*Brachionus koreanus*) exposed to micro- and nanoplastic showed faster egestion rates of 6 µm particles than of 0.5 and 0.05 µm particles (Jeong et al., 2016). In Mediterranean mussels (*Mytilus galloprovincialis*), 1 µm microplastics were detected in the faeces up to 50 days after exposure, while 90 µm microplastics were not detected after 28 days of the exposure, suggesting a higher depuration rate for the larger particles (Kinjo et al., 2019).

Polymer types can also impact marine organisms differently. Uptake rates of certain microplastic polymers by pelagic or demersal and benthic species might depend on the

density of the polymer type. For instance, low density microplastics tend to be more ingested by pelagic and planktonic organisms such as ichthyoplankton, leading to satiation and starvation, and reduced growth rate, fitness and predator avoidance (Jovanović, 2017). On the other hand, high density polymers, such as PET, tend to sink and be ingested by demersal fish, such as Atlantic cod (Bråte et al., 2016). In addition, diverse polymers can induce different toxicity in the organisms. Mediterranean mussels exposed to 1.5 g/L of PS microplastics (<100 μm) showed a decreased haemocyte lysosomal membrane stability and an increased level of lipofuscin in the haemocytes, while mussels exposed to PE microplastics had a lower granulocyte/hyalinocyte ratio compared to the control organisms due to a significant degranulation of the haemocytes (Avio et al., 2015). European sea bass fed with pellets containing microplastics showed different responses to PE and PVC. Fish exposed to PE had histopathological alterations related to mechanical abrasion, while fish exposed to PVC showed alterations normally related to chemical injuries. In head kidney leucocytes, oxidative stress increased by exposure to PE but was unaltered by PVC microplastics, while the phagocytic ability of head kidney leucocytes of fish exposed to PVC was increased but unaltered in fish exposed to PE microplastics (Espinosa et al., 2019). However, the average size of PE microplastics was slightly smaller than PVC microplastics, and this difference could have partially affected the fish responses to the two microplastic types.

The toxicity of microplastics can also be impacted by chemicals associated with them. During the manufacture of plastic materials, a range of additives, such as plasticisers, are added to improve the commercial properties of the products. These additives are added after polymerization and are not covalently bound to the plastic. Therefore, after ingestion of microplastic, these chemicals can leach and cause negative effects on organisms (Sjödin et al., 2003, Rist et al., 2018, Wang et al., 2020a). For instance, invertebrates exposed to bisphenol A (BPA), a plasticiser commonly used in polycarbonate plastics and epoxy resins, showed neurological disturbances and, at high concentrations, increased mortality rates, and fish exposed to BPA showed disrupted endocrine system, lower growth rates and abnormal behaviours (Naveira et al., 2021). Phthalates, commonly used as plasticiser in PVC, have been associated with allergies

and asthma in humans, and can cause reproduction and neurodevelopment anomalies (Mankidy et al., 2013, Bamai et al., 2014). In the marine environment, chemical toxicity associated with microplastics do not come exclusively from chemicals added during the manufacture of plastic, but can also stem from pollutants binding to such particles. Microplastics collected from beaches of three islands located in the Pacific Ocean were found to induce higher toxicity in Japanese medaka fish (*Oryzias latipes*) than virgin microplastics. Ingestion of environmental microplastics induced DNA damage, increased EROD activity, affected larval normal growth and increased fish mortality (Pannetier et al., 2020). In another study, PVC microplastics deployed for three months in a harbour in Italy induced more severe histopathological alterations in European sea bass distal intestine than virgin PVC microplastics after 30 and 60 days exposure (Pedà et al., 2016). Similarly, low density polyethylene (LDPE) deployed for 3 months in a harbour in San Diego Bay, California, induced severe liver histopathological alterations, including glycogen depletion, fatty vacuolation and cell necrosis, in medaka compared to virgin LDPE (Rochman et al., 2013b).

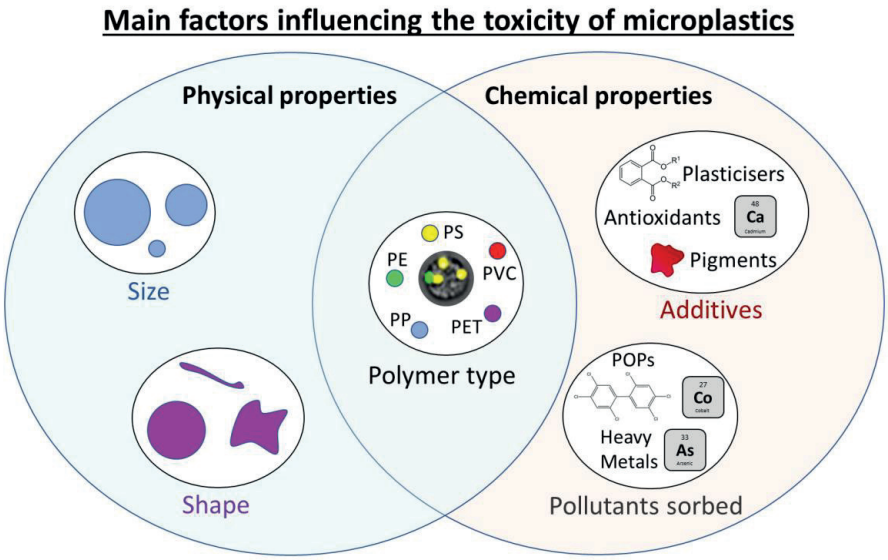

Figure 5: Graphic illustration showing the main properties of microplastics that can influence their toxicity on living organisms.

Several studies have shown that pollutants sorbed to microplastics can be transferred to the organisms. For instance, mussels and fish exposed to microplastics spiked with pyrene, benzo(a)pyrene, PCBs, PBDEs or triclosan, accumulated significant amounts of chemicals in their tissues, documenting that microplastics can act as vectors of adsorbed pollutants (Rochman et al., 2013b, Avio et al., 2015, Pittura et al., 2018, Webb et al., 2020). This could also have serious implications in the bioaccumulation of pollutants through the food web. However, the transfer of pollutants from microplastics to organisms might have an insignificant impact compared to the direct environmental exposure to the pollutants themself when present in the water (Koelmans, 2015).

1.2.3. Interaction between microplastics and pollutants

Microplastics are known to sorb pollutants in aquatic environments due to the hydrophobicity of such chemicals and the high surface/volume ratio of microplastics. Heavy metals and organic pollutants bound to microplastics have been reported worldwide (Guo and Wang, 2019). In this dissertation, I will focus on the interaction of organic pollutants with microplastics.

Persistent organic pollutants (POPs) have a relatively long half-life and can induce negative impacts on living beings. The hydrophobicity of these chemicals causes them to partition to solids when they are in aqueous environments and to accumulate in fatty tissues of organisms, making them very persistent in biota, and bioaccumulating and magnifying in the food chain. Their presence in marine organisms such as fish and mammals is well known. There are several classes of POPs, among which are the unintentionally produced types, such as polychlorinated dibenzo-p-dioxins and-furans (PCDD/Fs) —commonly referred as dioxins— and polycyclic aromatic hydrocarbons (PAHs), and the intentionally produced ones for commercial purposes, such as polychlorinated biphenyls (PCBs), brominated flame retardants (BFRs), and organochlorine pesticides (Jones and De Voogt, 1999, van der Gon et al., 2007).

Several POPs, such as PCBs, PBDEs, PAHs and pesticides, have been documented to bind to plastic in the marine environment, although there is a lack of information about dioxin (PCDD/F) levels sorbed to environmental microplastics (Figure 6). PE pellets

collected from waters in sandy beaches around the world were found to have quantifiable levels of PCBs, while organic pesticides were found only in some of the sites studied. The highest levels of PCBs in plastic were found in the US coast, followed by Japan and Western Europe (Ogata et al., 2009). Another study sampled plastic fragments along the north Pacific Ocean (east and west coast, and open ocean, including the North Pacific Gyre) and in the Caribbean Sea. PCBs, PAHs and PBDEs were detected in all fragments and sites, while levels of DDTs were more variable. PCBs and PAHs levels in plastics were higher in urban beaches than in remote beaches and open ocean, probably due to higher influence of industrial and anthropogenic activities. However, the amount of PBDEs in plastic did not vary depending on the anthropogenic proximity, which could be due to the fact that PBDEs are used in some countries as flame retardant in plastic. The levels of PBDEs detected in plastic could, therefore, be attributed to the PBDEs added during plastic production instead of the PBDEs bound to plastic in the marine environment. Levels of DDT in plastic varied among sites because it is used in some countries to control malaria (Hirai et al., 2011). A study carried out in the shore of Santos Bay, Brazil, found high amounts of PAHs in all microplastics (PE and PP) collected, especially darkened pellets, but difference in the sorption of this chemical among polymer types were not detected due to the sample variability (Fisner et al., 2017). However, five polymer pellets (HDPE, LDPE, PP, PVC and PET) deployed for several months in San Diego Bay, USA, showed different sorption rates to POPs. PCBs were found in higher concentration in LDPE, followed by HDPE and PP, after 12 months in the sea. Levels of PCBs in PVC and PET were one order of magnitude lower and reached the equilibrium concentration on PVC and PET microplastics approximately 6 months after their deployment. Similarly, PAHs bound in higher concentrations to HDPE and LDPE, followed by PP. PAH levels in such polymers reached the equilibrium concentration approximately after 6 months, while in PVC and PET equilibrium was reached at 3 months in the sea (Rochman et al., 2013b).

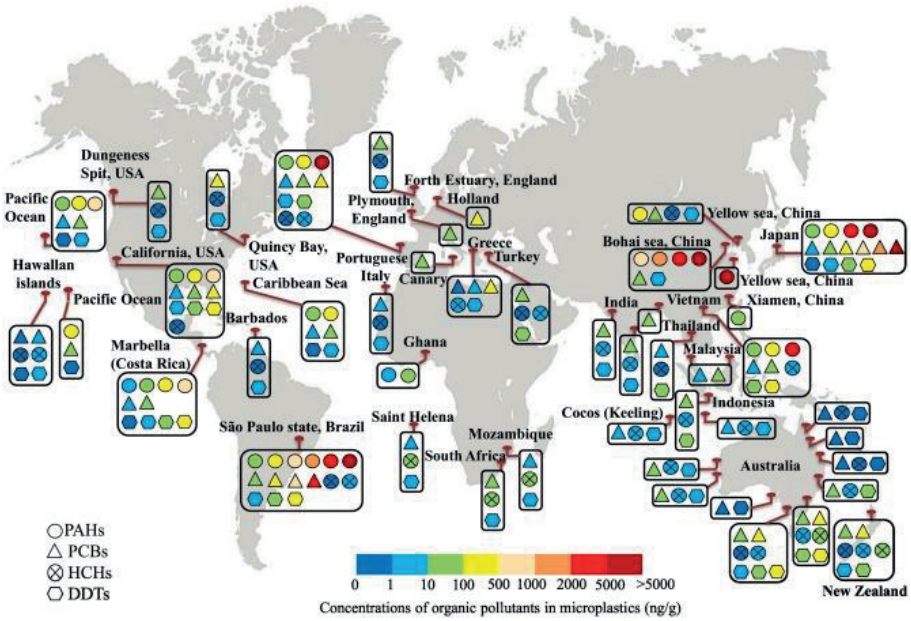

Figure 6: Average concentration ranges of organic pollutants bound to microplastics, reproduced from (Guo and Wang, 2019) with permission from Elsevier.

Sorption affinity of pollutants to diverse microplastic polymers mainly depends on the degree of crystallinity of the plastic. Nevertheless, polarity of polymers in seawater and biofilm formation around microplastics can also affect this parameter. In addition, levels of organic matter in water can decrease the sorption of pollutant to microplastics (Endo and Koelmans, 2016, Rummel et al., 2017, Guo and Wang, 2019). Crystalline regions of a polymer are characterised by well-ordered molecules that have restricted motion. On the contrary, amorphous regions of a polymer have more randomly ordered molecules characterised by a higher mobility and porosity. Chemicals require less interaction energy to bind to the amorphous regions of polymers compared to the crystalline regions and, hence, chemicals will bind in higher amounts to these polymer sites (Endo and Koelmans, 2016). Polymers contain both crystalline and amorphous regions, but depending on the degree of crystallinity, a polymer will be considered crystalline, semi-crystalline or amorphous (Table 3). For instance, PS and PVC are considered amorphous polymers, PET, LDPE and PP are semi-crystalline, and HDPE is crystalline (Wang et al.,

2020a). However, the degree of crystallinity of a polymer can vary considerably during production depending on the desired characteristics of the final product (Carraher Jr, 2003). Temperature can also affect the properties of plastic. Above the glass transition temperature (T_g), polymers tend to be rubbery and increase their chemical-binding capacity, while below T_g polymers are in a glassy state, with properties similar to crystalline polymers. Thus, higher temperatures reduce the crystallinity and glassy state of the polymers, but can also decrease the sorption coefficient of chemicals from water to plastic (K_{pw}) (Endo and Koelmans, 2016).

Table 3: Degree of crystallinity and glass transition temperature (T_g) of the polymers used in this thesis. Data obtained from (Khonakdar et al., 2007, Endo and Koelmans, 2016, Wang et al., 2020a)

Polymer	Crystallinity	Glass transition temperature (°C)
High density polyethylene (HDPE)	High crystallinity	-102
Polyethylene terephthalate (PET)	Semi-crystalline	69
Polypropylene (PP)	Semi-crystalline	-13
Polystyrene (PS)	Amorphous	100
Polyvinyl chloride (PVC)	Amorphous	81

** Both crystallinity and T_g of a polymer can vary depending on the production and properties desired of the final plastic.*

Microplastics tend to bind chemicals present in the water. In seawater, hydrophobic compounds, i.e. POPs, adsorbed to microplastics remain bound to these particles even when the concentration of such compounds decreases in the water. Hence, microplastics are potential vectors of pollutants from polluted waters to low-polluted environments. However, if microplastics loaded with pollutants pass through intermediate polluted waters, a displacement of the bound pollutants might occur, and a new equilibrium will be reached (Heinrich et al., 2020). In such cases, microplastics will still transport pollutants from the first polluted area to the final low-polluted waters, but in lower concentrations since part of the pollutants are displaced by the new pollutants (Figure 7). The ability of microplastics to transport POPs from one environment to another will therefore depend on the concentration and composition of pollutants in intermediate waters. If microplastics drift from highly polluted to non-

polluted waters, their ability to transport pollutants will be high, while if they pass through intermediate polluted waters, their vector capacity will be decreased.

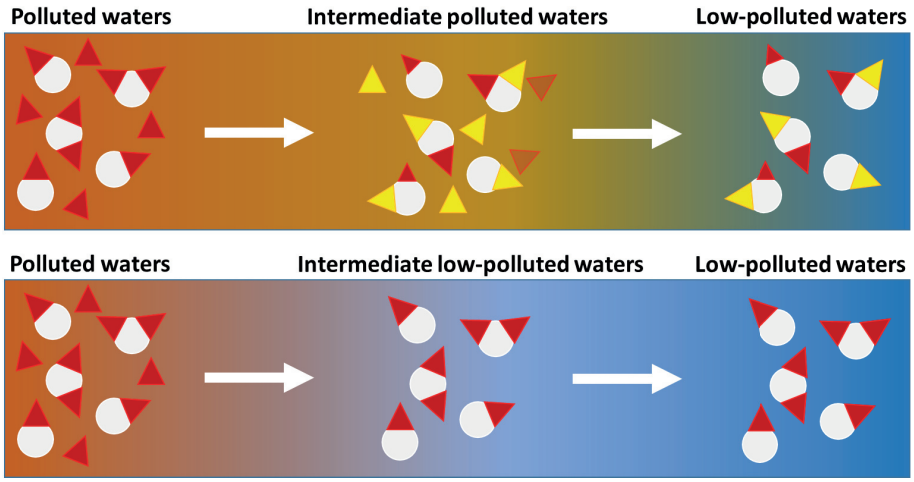

Figure 7: Role of microplastics (white spheres) as vectors of pollutants from polluted waters to low-polluted waters depending on the pollution levels of intermediate waters. On top, pollutants from polluted intermediate waters (yellow triangles) displace part of the pollutants from polluted waters (red triangles) bound to the microplastics. On the bottom, the vector capacity of microplastics is increased because pollutants from polluted waters are not displaced in intermediate low-polluted waters.

1.3. Immunity

Since their appearance on Earth, living beings have constantly been evolving and creating new physical and chemical defence systems to protect themselves from harmful agents. This defensive feature, known as immunity, has a very important role in the evolution and diversification of the species (Loker, 2012). A weakened immunity caused by factors such as pollution can be fatal both at individual and population levels.

The immune system consists of a set of cells and molecules responsible for recognizing and fighting infectious foreign substances that invade the host's body, although non-infectious foreign substances can also trigger immune responses (Abbas et al., 2014). The immune system can be divided into innate and adaptive. The innate immune system represents the front line defence against pathogens. It is non-specific, and contrary to the adaptive immune system, previous pathogen recognition is not required for its activation. The innate immune system triggers the adaptive immune response

(Medzhitov, 2001, Tort et al., 2003). The latter, only present in vertebrates, is stimulated by the contact of cells and molecules with the foreign agent and is characterized by its specificity and memory (Abbas et al., 2014, Costa, 2008). Nevertheless, lower vertebrates such as fishes predominantly depend on the innate immune system for their defence, while invertebrates rely only on this arm of the immune system (Tort et al., 2003).

1.3.1. Invertebrates

Invertebrates, which lack adaptive immune system, have a primeval immunity. Cells in charge of recognizing, fighting, and triggering further immune responses are the so-called haemocytes. In bivalves, haemocytes are especially present in the haemolymph but are generally found in all the tissues of the organism. Bivalves have an open circulatory system, which make them very susceptible to microbial infections. Haemolymph is therefore an important defensive interface for the innate immune system to fight against the invasive microorganisms (Bachère et al., 2015).

Haemocytes are commonly classified as granulocytes and hyalinocytes, although a third population a semi-granular cell type has been identified in several species (Wootton et al., 2003, Le Foll et al., 2010, Bachère et al., 2015). Granulocytes are considered to be the main cells involved in the immune defence of bivalves. These eosinophilic cells contain hydrolytic enzymes, such as lysozyme, and they can release antimicrobial peptides. Granulocytes are phagocytic cells and have the capacity to activate the respiratory burst and producing nitric oxide (NO). Hyalinocytes are basophilic agranular cells that lack some of the above mentioned functions, such as phagocytic activity, but are known to produce NO (Burgos-Aceves and Faggio, 2017).

The functional activity of haemocytes can be affected by xenobiotics. Microplastics, heavy metals and organic chemicals have been reported to disrupt phagocytosis, lysosomal membrane stability (LMS) and respiratory burst, besides inducing apoptosis in bivalve haemocytes (Burgos-Aceves and Faggio, 2017).

1.3.2. Vertebrates

In lower vertebrates, such as teleost fishes, the innate immune system is the main mechanism of defence against pathogens. Vertebrate immune cells are called leucocytes, and they are more differentiated than haemocytes. Leucocytes are generally divided into monocytes, neutrophils and T- and B-lymphocytes, the latter being normally considered as adaptive immune cells. All leucocytes have been shown to have phagocytic activity, but monocytes and neutrophils have long been recognized as the majors phagocytes of vertebrates (Nagasawa et al., 2014). Monocytes can differentiate into dendritic-like cells and macrophages, the latter being the phagosome par excellence (Aoki et al., 2008). In addition to their phagocytic activity, macrophages participate in inflammatory and microbicidal responses, and are involved in tissue repair and wound healing depending on their polarization state. Similar to haemocytes, macrophages can produce NO and reactive oxygen species (ROS) (Park, 2021).

The major lymphoid organs in teleosts are the kidney—especially head kidney—, spleen and thymus by order of development in marine species. Head kidney is also involved in antibody production and can induce a cascade of immune responses in the organism (Zapata et al., 2006, Whyte, 2007).

1.4. Atlantic salmon farming

Atlantic salmon (*Salmo salar*) is the most farmed marine fish species and one of the world most consumed fish, with Norway being the major producer (FAO, 2020). Atlantic salmon farming has economically become one of the main industries in Norway (Taranger et al., 2015). Approximately 1000 salmonid farms are distributed along the Norwegian coast (BarentsWatch, 2021). In 2019, Norwegian sales of Atlantic salmon exceeded 1.3 million tonnes, with a revenue of 68 000 million NOK, equivalent to approximately 3 times the sales and 7 times the value of 1999 (Directorate of Fisheries, 2020).

1.4.1. Pollutants associated to salmon farming

Atlantic salmon is an oily fish species that has high fat content (12 % approximately) in its tissues compared to other fishes such as cod (0.5 % approximately) (Gallart-Jornet et al., 2007). Consequently, the accumulation of POPs in salmon tissues can be relatively high. Farmed Atlantic salmon fed for 12 months with fish feed was shown to accumulate the following levels of POPs in fillets: 0.39 pgTEQ/g of dioxins, 2.1 pgTEQ/g of dioxin-like PCBs, 13 ng/g of PCB_6, 2.6 ng/g of $PBDE_7$, and 0.041 ng/g ww of 16 PAHs, while levels of organochlorine pesticides varied from non-quantifiable levels to 21 ng/g ww for the sum of DDT (Berntssen et al., 2010). Feed used in fish farming is known to contain quantifiable levels of POPs, such as dioxins, PCBs, PAHs and pesticides, and is the main origin of pollutants from the farms and to the salmons. In 2010, a study reported the following levels of POPs in traditional fish feed: 2.05 pgTEQ/g of dioxins, 6.08 pgTEQ/g of dioxin-like PCBs, 25.5 ng/g of PCB_6, 7.3 ng/g of $PBDE_7$, and 1.1 ng/g ww of 16 PAHs, while levels of organochlorine pesticides varied from non-quantifiable levels to 68 ng/g ww for the sum of DDT (Berntssen et al., 2010). The levels of POPs in fish feed have been, however, reduced considerably in the recent years. Analysis of fish feed in 2018 recorded levels substantially lower than the values reported in 2010: 0.33 pgTEQ/g of dioxins, 0.3 pgTEQ/g of dioxin-like PCBs, 3 ng/g of PCB_6, 0.38 ng/g of $PBDE_7$, and 4.9 ng/g of sum of DDT (Sele et al., 2019). Consequently, the level of POPs in farmed salmon have also decreased to levels even below those found in wild fish. In 2017, a study observed the following levels of POPs in fillet of farmed salmon: 0.23 ± 0.07 pgTEQ/g of dioxins, 0.29 ± 0.09 pgTEQ/g of dioxin-like PCBs, 4.0 ± 1.2 ng/g of PCB_6, 0.5 ± 0.4 ng/g of $PBDE_7$, and 5±1 ng/g of the sum of DDT (Lundebye et al., 2017). The production of Atlantic salmon in Norway is strictly controlled and, despite the quantifiable levels of POPs in salmon tissues, all reported levels are below the maximum permitted pollutants levels set by the EU legislation (EC, 2020).

1.4.2. Microplastics associated to fish farming

Aquaculture can be a source of microplastics to the marine environment. Marine fish farming uses an extensive amount of plastic materials, such as rings, nets and feeders.

Only in Norway, it has been estimated that 192 000 tonnes of plastic are used in aquaculture, and this activity generate approximately 30 000 tonnes of plastic waste per year of which only 8 000 tonnes are recycled (Hognes and Skaar, 2017, Syversen et al., 2020a). Plastic materials used in fish farming are placed for months or years in the sea and are subjected to weathering by biological, chemical and mechanical processes, potentially releasing microplastics to the surrounding waters. In addition, the loss of such plastic materials during, for instance storms, become an added source of plastic, and eventually of microplastics, to the environment (Lusher et al., 2017a). A study of litter along the Norwegian coastline found that up to 50% of waste collected at the shore originated from salmon farms, and the contribution of aquaculture to stranded litter was especially important in areas relatively far from big cities (Vangelsten et al., 2019). Recently, it has been shown that sediments collected in areas close to fish farms had higher concentrations of microplastics compared to areas further away from the farms and to areas not influenced by the farms (Krüger et al., 2020). Microplastics (<15 µm) have been shown to originate from feeder pipes used in fish farming sites, which are usually made of HDPE. These particles are released along with fish feed due to the high pressure- and temperature-caused abrasion of the pipes when the fish pellets go through (Gomiero et al., 2020b). Moreover, fisheries in Norway have been estimated to release tonnes of plastic fragments annually to the marine environment, mainly from ropes and nets as a consequence of abrasion cause by contact to the sea bottom (Syversen et al., 2020b). Fish farms use high amounts of ropes in seawater facilities which are subjected to wear and tear and might release microplastics, as shown in fisheries.

1.5. Mussels

Mussels (*Mytilus* spp.) are sessile invertebrate animals found in temperate seas all over the world. These medium-sized bivalves are filter-feeders and tend to grow in big groups in shallow waters and form beds (Figure 8). For these reasons, mussels are commonly used as sentinel species to monitor coastal pollution, since they accumulate pollutants from specific environments in their tissues and their sampling areas are

accessible, as shown in Figure 8 (Beyer et al., 2017). Mussels have been recently monitored along the Norwegian coast and levels of pollutants found in their soft tissues have been compared to the biota Environmental Quality Standards (EQS). In almost all sites sampled, including those from Bodø, PCB7 and PBDE levels were above the EQS (Green et al., 2020).

In 1975, mussels were first proposed as a tool to biomonitor marine pollution (Goldberg, 1975). Recently, it has been proposed to use these animals as bioindicators of microplastic pollution since a positive correlation between the amount of microplastics in mussel and the surrounding water has been observed (Li et al., 2019). This would be especially relevant for monitoring small microplastics in the marine environment. Mussels have been shown to efficiently ingest particles below 100 µm (Wang et al., 2021a). Moreover, smaller particles could translocate into the haemolymph and be taken up by the haemocytes, as well as be retained in the digestive glands. Food digestion in mussels is initiated at the rotating crystalline style, where food is physically homogenised. After extracellular digestion in the stomach, larger particles are sent to the intestine while smaller particles go into the digestive glands to undergo intracellular digestion. Such glands contain terminal epithelial tubules where small particles can get trapped (Cole et al., 2020).

Five species of *Mytilus* occur in the northern hemisphere: the blue mussel *(Mytilus edulis)*, the Mediterranean mussel *(Mytilus galloprovincialis),* the bay mussel *(Mytilus trossulus)*, the California mussel *(Mytilus californianus)* and the Korean mussel *(Mytilus coruscus)*. However, *Mytilus* species are known to interbreed and give fertile descendance. The occurrence of hybrids in the environment is very common, for which the term "*Mytilus edulis* complex" was coined to refer to all species of *Mytilus* including both hybrids and non-hybrids (Gaitán-Espitia et al., 2016, Beyer et al., 2017, Simon et al., 2020). In this thesis, the mussels employed for the studies are referred to as blue mussels, or *Mytilus edulis*, considering the geographic distribution of this species and for the sake of clarity.

Figure 8: Blue mussels growing in beds (blue-black patches) in a beach in Åselistraumen, Bodø, Norway (a, b). Mussels are normally submerged during high tide and become visible during low tide. Adult mussels (c, d) were collected from this low-polluted site for the studies carried out during this PhD.

2. Objectives

The aim of this thesis was to study the environmental impact of microplastics in relation to Atlantic salmon farming. For this purpose, a novel imaging flow cytometry (IFC) method was developed to study cellular responses to microplastics in different organisms. IFC was used to assess phagocytic activity in immune cells from fishes and mussels as well as to accurately determine cellular accumulation of microplastics by using cell images and by analysing particle sphericity and agglomeration rate. In addition, IFC was employed to evaluate distinct responses of immune cells to different microplastic types by labelling two polymers, PE and PS, with different fluorochromes. This was done to be able to compare how microplastic exposure affect the immune responses in two very diverse marine animals of ecological and economical importance, Atlantic salmon (vertebrate) and blue mussel (invertebrate). After studying the impact of microplastics on salmon and mussel immune cells, we wanted to understand whether microplastics can sorb pollutants associated with fish farming and potentially transfer them to marine organisms. For that purpose, we examined the interactions between microplastics and aquafeed pollutants. Microplastics were placed in the sea near Atlantic salmon farms and, after three months, pollutants bound to different polymers were analysed quantitatively by gas chromatography and mass spectrometry. Affinity of pollutants to four different polymers was evaluated to have a better understanding of the impact of different types of microplastics in the environment.

The specific objectives of the papers and supplementary file presented in this thesis are:

1. To establish optimized IFC protocols to study phagocytic activity of immune cells from both vertebrates and invertebrates as well as to study the response of cells to small microplastics (**Paper I**).

2. To determine tissue-specific responses of Atlantic salmon immune cells isolated from intestine, blood and head kidney tissues after 1, 24 and 48 h exposure to three concentrations of PE and/or PS microplastics, and compare such responses with those of blue mussel immune cells (**Paper II** and **Supplementary file**).

3. To determine whether patterns of pollutants bound to microplastics placed in the sea near Atlantic salmon farms reflect the composition of pollutants in aquafeeds. For this purpose, four different microplastic polymers (HDPE, PP, PET and PVC) were placed for three months near two Atlantic salmon farms, in a polluted urban harbour and an unpolluted reference station. In addition, a positive control was generated by incubating microplastic polymers with fish feed. Pollutants sorbed to microplastics were then analysed and patterns of pollutants compared (**Paper III**).

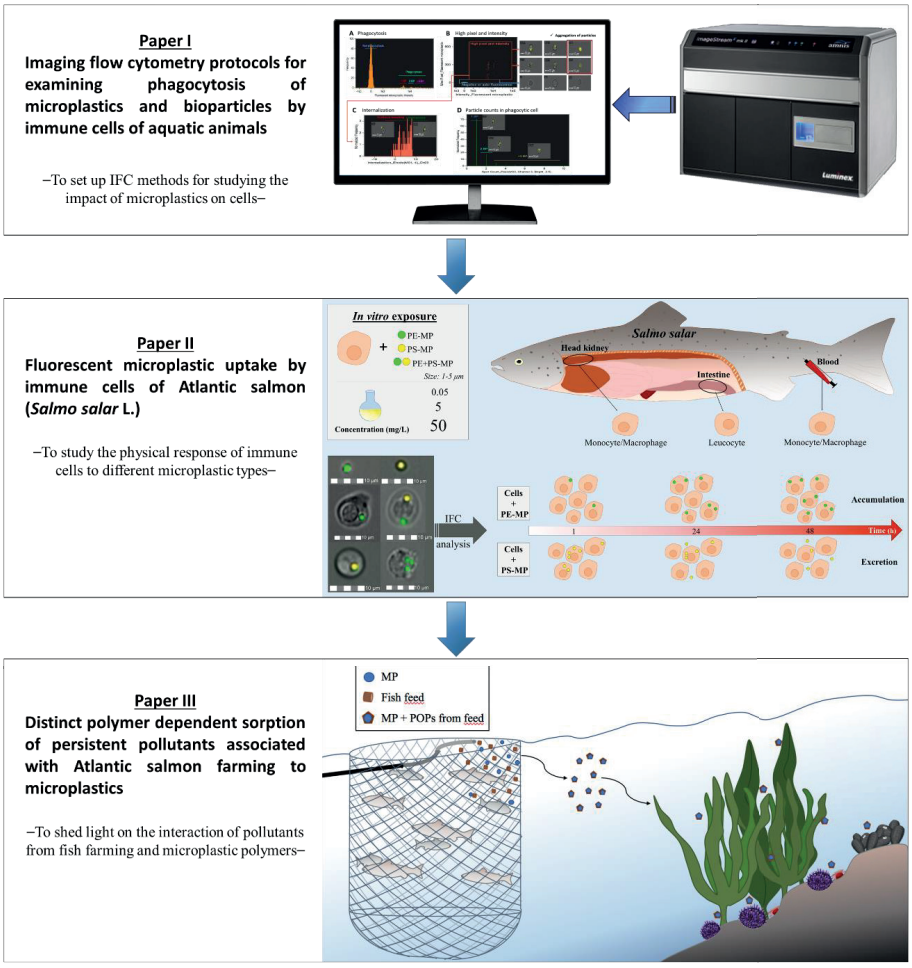

Figure 9: Schematic overview of the studies carried out in this dissertation. The names of the papers and their main aims are shown on the left, and on the right are shown the graphical illustrations of the studies.

3. General discussion

The ubiquitous presence of microplastics in the environment has raised public concern in recent years. Their impact on wildlife and their occurrence in our food and drinking water have led to an urgent need to promote a global treaty aimed at reducing and tackling the roots of microplastics (Group of Chief Scientific Advisors, 2019). However, although research on microplastics has increased exponentially, there are many unknowns concerning the toxicity and real impact of these particles. Atlantic salmon farming, one of the biggest commercial activities in Norway, is known to have an impact on the environment due to large emissions of organic materials and nutrients. However, until very recently, the role of aquaculture facilities as a source of microplastics in the marine environment was unknown. The large amount of plastic materials used in fish farming inevitably releases particles into the water (Gomiero et al., 2020b). Yet, little is known about the connection between microplastics and the environmental impact of salmon farming. The aim of this thesis was, therefore, to shed light on the potential role of microplastics in spreading contaminants associated to Atlantic salmon farming to the surrounding environment of the fish farms, as well as to understand the bioaccumulation and toxicity of microplastics in wild and farmed edible species such as salmon and mussels.

3.1. Setting up new methods for studying microplastics

The microplastic research field is relatively new and, consequently, methods for its study are constantly being developed. In this thesis, a novel protocol for studying the impact of microplastics at the cellular level was established by using imaging flow cytometry (IFC) (**Paper I**).

Flow cytometry has long been used to identify single cells and study cell populations by analysing their size and internal complexity. This tool is also widely used in immune studies, for instance, in the analysis of phagocytic activity of immune cells, one of the first immune responses of an organism against pathogens. In turn, flow cytometry enables us to measure cell damage by analysing parameters such as cell mortality. What

makes flow cytometry a powerful tool is especially the fact that a large number of cells can be processed in a single run. IFC combines traditional flow cytometry with fluorescence microscopy (McKinnon, 2018). Thus, IFC can process many cells at the same time than it provides with images of each cell and particle analysed, allowing accurate and reliable data collection while reducing false positives/negatives. The use of flow cytometry as a tool to quantify the accumulation of microplastics in cells was, however, never used before the start of this project. Flow cytometry has previously been used for studying the immune activity of immune cells, such as the phagocytic activity, from organisms exposed both *in vitro* and *in vivo* to microplastics (Espinosa et al., 2017, Espinosa et al., 2018). However, as far as we know, the use of flow cytometry as a tool to detect microplastics was not published until 2020. That year, acoustic focusing flow cytometry (AFFC) was employed by a few studies to measure the concentration of microplastics in water samples (Bringer et al., 2020, Kaile et al., 2020, Le Bihanic et al., 2020). This method enabled the quantification of non-stained microplastics of known sizes, and the quantification of unknown and broader size range of microplastics after staining them with Nile Red. AFFC is a promising tool for analysing microplastic concentrations in environmental water samples, and to check the water microplastic concentration in exposure experiments. However, this method cannot be used to detect microplastics in cells or tissues, due to potential false positives. In **Paper I**, an IFC protocol was established to study the cell's response to microplastics using fluorescent plastic particles. The novelty of this method lies not only on the quantitative assessment of microplastic accumulation in cells, but also on the possibility of studying the simultaneous impact of two different polymer types labelled with different fluorochromes (**Paper II** and **Supplementary file**). By using IFC and exposing cells to two microplastic polymer types labelled with different fluorochromes, we were able to show for the first time that single cells can ingest different microplastic types simultaneously (Figure 10). Moreover, the agglomeration rate of the plastic particles and their sphericity can be measured by IFC, as shown in the supplementary file of **Paper II**. The method described in **Paper I** has the potential to considerably increase our understanding on how microplastics accumulates in a broad range of species. The method can be used to

study a wide range of cells, both individually and simultaneously, from animals, plants and protists. Moreover, the versatility of this method lies on its ability to assess the impact of different microplastic types. Such findings could shed light on differential accumulation and effects of distinct polymer types, sizes and shapes. Nonetheless, this method is limited to exposure experiments using fluorescent labelled microplastics and cannot be applied to analyse accumulation of microplastics in environmental samples.

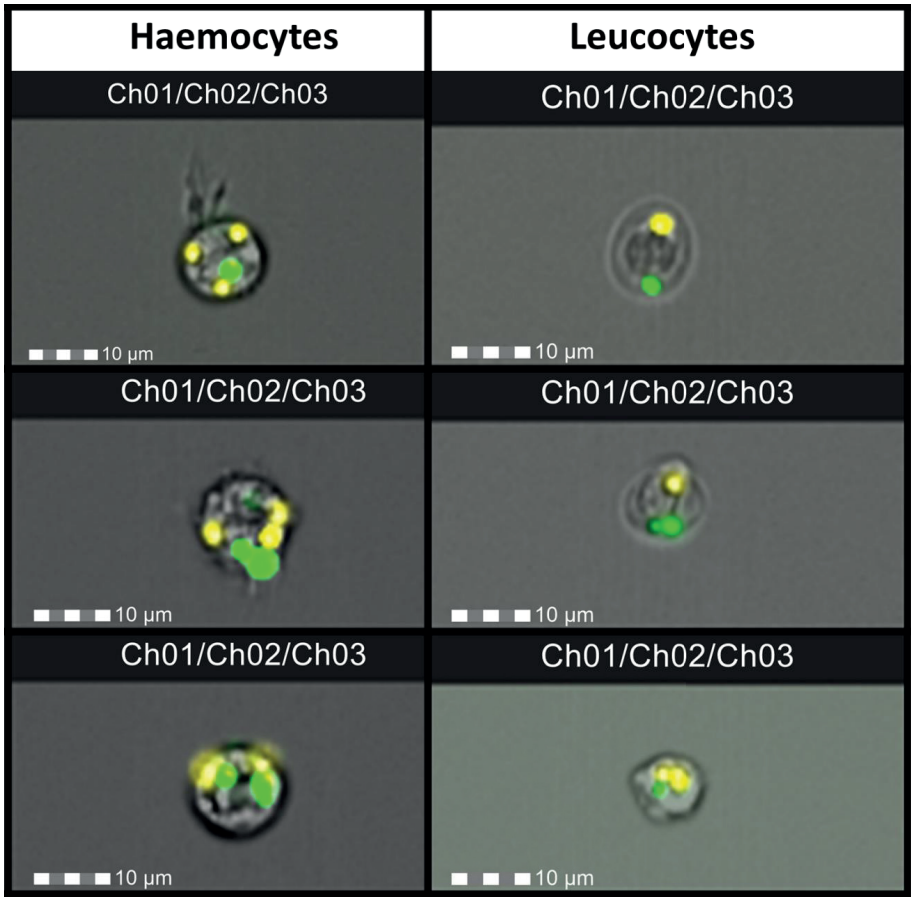

Figure 10: Haemocytes (left) and leucocytes (right) with internalized polyethylene (green) and polystyrene (yellow) microplastics. Pictures obtained from imaging flow cytometry (Image Stream®X Mk II Imaging Flow Cytometer, Luminex Corporation, Austin, TX, USA). Images detected by channels 1, 2 and 3 were merged for each object number to visualize the cell, and the green and yellow fluorescence, respectively. Scale = 10 µm.

3.2. Microplastic uptake by salmon and mussel immune cells

Cells from several organisms, especially immune cells with phagocytic activity, have been reported to ingest small microplastics. For instance, microplastics were detected in haemocytes of Mediterranean mussels (*Mytilus galloprovincialis*) exposed *in vivo* to LDPE by polarized light microscopy (Pittura et al., 2018). In human cells, confocal microscopy-based studies revealed that neutrophils and macrophages can ingest PS nanoparticles (460 nm), which were mainly located in the cytoplasm, while lymphocyte-like cells did not have particles in them after 4 days of exposure (Hwang et al., 2020). Uptake of microplastics has not only been observed in immune cells. Human intestinal epithelial cells Caco-2, for instance, were reported to internalize PE microplastics sized in the range of 1-4 µm and 10-20 µm after 24 h exposure (Stock et al., 2021).

In this PhD project, we studied the uptake of small microplastics by immune cells from Atlantic salmon and blue mussel (**Paper II** and **Supplementary file**). Our aim was to understand whether cells respond differently to different microplastic types as well as to evaluate the accumulation of the particles over time. In addition, we were interested in comparing responses of two very different marine species of economic and ecological importance. Hence, we selected a filter-feeder and sessile invertebrate, the blue mussel, and an active-feeding pelagic vertebrate, the Atlantic salmon. Our results showed that different microplastic types induced diverse cell responses in both species, and such responses were species-dependent.

The main differences observed in the response of salmon and mussel cells to microplastics are the percentages of phagocytic cells. Salmon leucocytes showed a lower ability to phagocytose microplastics in comparison to mussel haemocytes, with a maximum phagocytic ability of 5.5 ± 2.0 % in salmon blood cells exposed for 48 h to high concentrations of PE compared to a maximum phagocytic ability of 56.0 ± 3.3 % observed in mussel haemocytes exposed for 1 h to high concentrations of PS. However, mussel haemocytes and head kidney leucocytes from salmon showed similar phagocytic ability rates towards *Escherichia coli* particles (**Paper I**), suggesting that differences in the phagocytic ability are driven by the microplastics, rather than by the inherent

phagocytic ability of the cells. The different opsonizing mechanisms of vertebrates and invertebrates could be the reason for the observed differences. In vertebrates, receptors of the leucocytes perform self-nonself and microorganism recognition through non-opsonic and opsonic phagocytosis, mediated, for instance, by lectins and antibodies. However, invertebrates lacks adaptive immunity, and mainly relies on carbohydrate-binding proteins, such as lectins, as opsonizing mechanism for self-nonself and microorganism recognition (Canesi et al., 2002). Considering that plastic polymer structures are mainly made of carbon and hydrogen, carbohydrate-binding proteins might potentially bind to microplastics and be recognized in greater proportion by haemocytes. This could, in turn, explain differences in the phagocytosis rates of haemocytes to PS and PE, since each microplastic polymer type have distinct structure and lectins might bind differently to each of them.

The ability of blue mussel haemocytes to phagocytose microplastics (**Supplementary file)** was in accordance with phagocytic rates observed by Sendra et al. (2020b). In their study, Mediterranean mussel granulocytes exposed *in vitro* to 10 mg/L of 1 µm PS microplastics for 3 h showed a phagocytic ability of 59.3 ± 5.3 %. Nevertheless, Sendra et al. (2020b) observed relatively higher amounts of cells with internalised microplastic compared to our results, which could be due to diverse reasons. In our study, haemocytes were exposed to PS microplastics of 2.1 µm, particles of larger size than the one used by Sendra et al. (2020b). As observed in their study, cells exposed to smaller plastic particles (100 nm and 50 nm) at the same concentration (10 mg/L) have higher phagocytic abilities (60.6 ± 3.5 and 89.7 ± 1.9 %, respectively). Moreover, granulocytes are the haemocytes with higher phagocytic activity in *Mytilus* spp., followed by hyalinocytes and blast-like cells, the latter showing no phagocytic activity (Yang et al., 2015). Exclusion of hyalinocytes and blast-like cells in their study probably led to an increase in the percentage of phagocytic cells in comparison to our study, where the whole haemocyte fraction was used. The methods employed were also different in the two studies. Sendra et al. (2020b) quantified haemocytes with internalized microplastics by confocal microscopy and image processing, while our study used IFC, which enabled assessment of many cells per sample.

Comparison of the salmon and mussel results revealed an interesting finding: the differential response of leucocytes and haemocytes to different microplastic polymer types. Leucocytes exhibited higher uptake of PE microplastics, while haemocytes showed, overall, higher uptake of PS microplastics. Moreover, the accumulation of microplastics in leucocytes over time was polymer dependent. The number of cells containing PE increased over time, while the number of cells containing PS decreased, suggesting an accumulation of PE in the cells and exocytosis of PS particles. However, the accumulation or excretion of microplastics in haemocytes did not reveal a polymer dependent behaviour, but rather indicated a dose-dependent response. When haemocytes were exposed to high concentrations of PE, PS and the mixture of both polymers, the percentage of cells with microplastic decreased over time. However, when the cells were exposed to medium and low microplastic concentrations, the percentage increased over time (Figure S3). Phagocytosis of particles by leucocytes can be influenced by particle shape (Champion and Mitragotri, 2006). As shown in **Paper II**, PE microplastics used in the experiments had lower sphericity than PS microplastics, which might explain the higher uptake and accumulation of PE in leucocytes. On the other hand, haemocytes' functions are broader than the ones carried out by macrophages or other leucocytes, and comprise tasks such as digestion, shell repair, respiration, osmoregulation, transport and excretion (Sendra et al., 2020a). This could explain the high uptake rate of both PE and PS microplastics observed in the haemocytes. Considering the high percentage of cells affected by microplastics and the acute stress cause by it, haemocytes might excrete such particles to maintain their functionality. However, at lower concentrations, cells might try to digest or evoke responses to fight against such particles, inducing rather a chronic than acute stress and resulting in the accumulation of such particles over time.

This study compared the impact of mass-based concentrations of two similar-sized microplastic polymers, PE and PS, on phagocytic activity of immune cells. These two microplastic polymers had different densities and therefore the concentration of particles in the media differed among polymer treatments. This could have therefore led to a misinterpretation of the results when comparing cell responses to different

polymer types. PE microspheres used in this study had a density of 1.3 g/cc ($1.3x10^{-12}$ g/µm^3), while PS microspheres had a density of 1.05 g/cc ($1.05x10^{-12}$ g/µm^3). The size range of PE particles was 1 to 5 µm and the size of PS particles was 2.1 µm. Assuming that the average diameter of PE microplastics was 3 µm, the volume of these spherical particles was calculated as 7.95 µm^3, after which the mass of each particle was calculated to be $1.034x10^{-8}$ mg. From such calculations, the number of PE particles/L in 50, 5 and 0.05 mg/L was estimated to be $4.8x10^9$, $4.8x10^8$ and $4.8x10^6$, respectively. Using the same equations, volume and mass of PS microspheres were calculated to be, based on size and density, 2.73 µm^3 and $2.86x10^{-9}$ mg, respectively. Thus, $1.75x10^{10}$, $1.75x10^9$ and $1.75x10^7$ PS particles/L were present in 50, 5 and 0.05 mg/L of PS. Considering the above calculations, the number of particles/L was therefore higher for PS treatments than for PE treatments. Mussel haemocytes had higher uptake ability towards PS than PE, which could have been driven by the increased number of particles/L. However, salmon leucocytes phagocytosed more PE than PS, which emphasize the importance of considering particle shape and type when assessing the impact of microplastics in such specialised cells.

The use of similar methodology and experimental design applied in different species (**Paper II** and **Supplementary file**) sheds light on the differential impact of microplastics on species. Our results suggest that microplastics have a higher impact on mussels than on salmon, independent of the polymer type. The accumulation of microplastics in haemocytes exposed to low concentrations, equivalent to environmental concentrations of bigger-sized microplastics, suggest that the prolonged exposure of mussels to microplastics might impair their immune system, which could eventually have an effect at the population level. Moreover, mussels are in a relatively low trophic level and could be a potential source of microplastics to higher trophic animals, including humans (Zhang et al., 2019). Similarly, salmons exposed for a long time to microplastics such as PE, as might happen in the case of farmed salmon, could accumulate considerable amounts of microplastics and become a potential source of microplastics to human consumers. Microplastics might translocate from the digestive tract to other tissues and organs through uptake in cells, as suggested in **Paper II**. Intestinal leucocytes

of salmon showed the ability to phagocytose microplastics, and a previous study reported that intestinal epithelial cells can phagocytose such particles (Stock et al., 2021). Accordingly, microplastics were detected in the fillets of from both farmed and wild Atlantic salmon, and the major polymers detected were PS, PE, PP and PVC (Gomiero et al., 2020a). Nevertheless, studies have failed to observe a significant biomagnification of microplastics through the food web (Akhbarizadeh et al., 2019, Wang et al., 2021b).

3.3. Impact of microplastic translocation and accumulation

Uptake and translocation of small microplastics by immune cells might have important repercussion in the immune system. Impairment of immune activity in cells affected by microplastics has been observed in Mediterranean mussel. Haemocyte apoptosis significantly increased after 3 and 24 h exposure to 1 and 10 mg/L of 1 µm PS microplastics, and percentage of toxic radicals (ROS, H_2O_2, O_2 and NOS) in such cells as well as the phagocytic activity of granulocytes was affected by the microplastic exposure (Sendra et al., 2020a). Similarly, European seabass head-kidney leucocytes exposed to PVC and PE microplastics had decreased phagocytic capacity and increased respiratory burst activity, and Gilthead seabream head-kidney leucocytes exposed to PVC microplastics had increased respiratory burst activity (Espinosa et al., 2018). Elevated amounts of particles accumulated in immune cells, as observed in blue mussel (**Supplementary file**) and Atlantic salmon (**Paper II**), can thus disrupt the immune activity of such cells by reducing their ability to phagocytose pathogens and by increasing the energy consumption of the cells. Therefore, when relatively high percentages of immune cells are affected by microplastics, as observed in blue mussels and in Atlantic salmon exposed to 50 mg/L of microplastics (**Supplementary file** and **Paper II**), the overall immunity of the organism will probably be compromised.

Results of this thesis show that small microplastics can be taken up by leucocytes isolated from the Atlantic salmon intestine (**Paper II**). This suggests that microplastics translocated from the digestive tract can be taken up and accumulated in cells and be transported to internal tissues. Microplastics might then remain for long time in such

tissues, which increases the risk of trophic transfer and could potentially cause chronic stress in organisms. Nevertheless, an additional risk might be associated with translocation and accumulation of microplastics in organisms. Microplastics in the environment can sorb pollutants present in the water, and those could potentially be transferred to organisms after translocation of microplastics into animals. Currently, evidences of transfer of pollutants bound to microplastics into organisms are weak and the matter is still controversial (Koelmans et al., 2022). It has recently been demonstrated that the contribution of microplastics in accumulation of pollutants is insignificant compared to other uptake routes such as food ingestion. However, the contribution of microplastics in the transfer and accumulation of highly hydrophobic pollutants, such as PCBs, might be higher than other sources (Lee et al., 2019). In **Paper III**, we observed that microplastics sorbed high levels of PCBs and dioxins compared to other pollutants. Dioxins and PCBs are considered super-hydrophobic compounds because of their high octanol–water partition coefficient (log K_{ow}), normally above 6 and up to 12 (Wenning and Martello, 2008, Ahmad et al., 2019). Thus, the role of microplastics as vector of such pollutants to organisms could be significant. In addition, it has been reported that longer residence time of microplastics in organisms result in higher transfer of pollutants (Lee et al., 2019). Therefore, microplastics translocated and accumulated in internal tissues could be a source of pollutants to the organisms, especially in non-polluted environments.

Few studies have shown the impact of polluted microplastics collected from the environment on organisms. Japanese medaka larvae exposed to polluted microplastics collected from marine waters near Hawaii, which mainly bound PAHs, had increased DNA damage as well as decreased mobility and swimming speed as compared to larvae exposed to virgin microplastics (Pannetier et al., 2020). Similarly, Japanese medaka exposed to PE microplastics collected from San Diego Bay, USA, showed changed expression of endocrine-related genes (Rochman et al., 2014). Therefore, it is important to study interactions between pollutants and microplastics in the marine environment to critically assess the real impact of such particles on organisms in the environment.

3.4. Microplastics in aquaculture: potential vectors of pollutants in waters above the Arctic Circle

Aquaculture, and especially intensive farming, contributes to plastic discharge into the environment (Lusher et al., 2017a). Fish farming and fisheries has been estimated to generate 15 500 to 30 000 tonnes of plastic waste per year only in Norway, of which approximately 25% of the waste is recycled (Sundt et al., 2014, Syversen et al., 2020a). It has recently been reported that fish farming can also actively release microplastics into marine waters (Gomiero et al., 2020b, Lusher and Pettersen, 2021). Microplastics in the environment tend to bind pollutants due to the hydrophobic nature of such chemicals (Guo and Wang, 2019), and fish farming activities can release organic pollutants into the water, which mainly originate from fish feed (Sele et al., 2019). In this thesis project, we aimed to evaluate interactions between pollutants associated with fish farming and microplastics to better understand the environmental impact of marine fish farming and microplastics. Results of Paper III showed thatmicroplastics placed for 3 months at 10 m depth close to Atlantic salmon net pens sorbed pollutants associated with fish feed, suggesting that microplastics could potentially act as vectors of POPs associated with Atlantic salmon farming.

Microplastics found near fish farms can originate from local or remote sources. Equipment used in Atlantic salmon farms is mainly made of plastic and wear and tear of such materials results in emission of microplastics to the marine environment. Fish feeders used in open water farms, for example, have been estimated to generate 0.1 to 100 tonnes of microplastics due to abrasion (Gomiero et al., 2020b, Lusher and Pettersen, 2021). Such feeding pipes are commonly made of HDPE and, consequently, PE microplastics were the only polymer type detected in sludge collected from October 2020 to February 2021 from the bottom of net pens from a salmon farm located in Vestland County, Norway (unpublished data). Our results in **Paper III** showed that HDPE microplastics sorbed significantly less dioxins and PCBs than PET and PVC microplastics, while no significant differences were observed in the sorption of these POPs to PP and PET, PVC and HDPE. In terms of environmental footprint, these results could be

interpreted as positive for oceanic Atlantic salmon farming, since this activity mainly releases PE microplastics to the environment and such polymer binds relatively low amounts of POPs. However, Gomiero et al. (2020b) also detected PP, PS, PVC, PET and other microplastic polymers in marine sediments, suspended matter, and seawater collected from the surrounding areas of fish farms. Although the concentrations of those polymers near the salmon farms were comparable to those of the reference station of their study, results presented in **Paper III** showed that POPs from fish feed can bind to such microplastic polymers when present near the farms, potentially increasing the environmental risk of this activity. In addition, feeding pipes have been documented to release microplastic particles smaller than 15 µm in size (Gomiero et al., 2020b). These small microplastics can be phagocytosed by and accumulated in immune cells and tissues of marine organisms such as salmon and mussels, as observed in **Paper II** and **Supplementary file** and by Li et al. (2016) and Gomiero et al. (2020a).. Thus, small-sized microplastics associated with Atlantic salmon farming can have an impact in the environment as potential vectors of pollutants as they can accumulate in cells and tissues of marine organisms, thereby affecting their immune system.

Interestingly, the polymer type-specific sorption pattern observed in our study contradicts the observations reported in other studies. In San Diego Bay, California, microplastics placed for 3 months in the water showed that PCBs sorbed more strongly to HDPE than to PET and PVC (Rochman et al., 2013a). Sorption of organic compounds to plastic are affected by several physico-chemical and biological parameters, such as water temperature, salinity, pH or biofilm formation (Ziccardi et al., 2016, Rummel et al., 2017). Our study was carried out in winter season in waters above the Arctic Circle. In Bodø, average air temperature in December-January tend to be below or around 0 °C, and seawater temperature is around 6 °C, as observed in Leirvik, Morsdalsfjorden, in winter 2018-2019. Previous studies carried out in Skjerstadfjorden also reported that sub-surface waters (above 50 m depth) in the fjord in December had temperature of approximately 4 °C and down to 2 °C, and salinities of 29-30 ppm (Busch et al., 2014). Therefore, salinity and temperature of seawater during our study was lower than studies carried out at lower latitudes (NOAA, 2009). Patterns of pollutants sorbed to

microplastic polymers can vary considerably depending on the environmental conditions. To our knowledge there are no previous studies that have reported sorption patterns of POPs in microplastics in waters above the Arctic Circle or in Arctic waters. Hence, comparisons with previous studies are complicated and probably inaccurate. Nevertheless, incubation time of microplastics in sea water, deployment depth of the cages with microplastics and oceanographic differences among studied area are also important factors that might affect how pollutant interact with microplastics, resulting in differences with other reports. More studies are needed to better understand the interaction of POPs and microplastics in polar, temperate and tropical regions, and especially in Arctic waters now that the Arctic basin is considered a potential sink for microplastics (Lusher et al., 2015, Peeken et al., 2018, La Daana et al., 2020).

Paper III is, to our knowledge, the first study to report data on the interaction of microplastics and POPs from aquaculture. The results obtained in this study uncover the potential role of microplastics to further increase the environmental risk of human activities such as fish farming. Future in-depth studies are needed to fully understand the impact of microplastics in relation to aquaculture and other anthropogenic activities, but our results suggest that microplastics should be considered in future risk assessment of such activities.

4. Conclusions

The present thesis brings forward a novel method to the microplastic research field, enabling the study of the impact of such particles on a broad range of organisms. By using IFC, accumulation of microplastics in cells isolated from diverse tissues and organisms could be quantified, and the data were employed to compare the impact of microplastics on diverse species.

Blue mussel immune cells, i.e. haemocytes, were more strongly affected by exposure to microplastics than Atlantic salmon immune cells, i.e. leucocytes. Haemocytes were mainly affected by the exposure concentration rather than by polymer type. Low microplastic concentrations lead to accumulation of both PE and PS in haemocytes, while high microplastic concentrations caused cells to excrete such particles as a mechanism of defence. This suggests that environmental relevant concentrations of microplastics might induce chronic stress in blue mussel immune cells. Contrary to the observations on haemocytes, leucocytes were affected by both concentration and polymer type. PE microparticles accumulated in the cells while PS particles were excreted over time, but the number of cells affected increased directly with microplastic concentration. Thus, salmon might be less affected by environmental relevant concentrations of microplastics compared to mussels. These results show that marine species as diverse as mussel and salmon, an invertebrate and a vertebrate animal, respond differently to identical microplastic exposure. Hence, a better understanding of the impact of microplastics on different species is needed for a more accurate assessment of the risks of microplastics in the environment.

Furthermore, this thesis provides for the first time insight into the sorption pattern of pollutants to microplastics in waters above the Arctic circle, and sheds light on the potential of microplastics in increasing the environmental impact of fish farming. Microplastics placed near Atlantic salmon farms were found to sorb pollutants associated to fish feed, suggesting that such particles could potentially act as vectors of such pollutants to adjacent waters. In addition, PET and PVC microplastics showed significantly higher sorption of dioxins and PCBs than HDPE, and dioxins and PCBs

showed higher binding capacity to microplastics than other persistent organic pollutants (POPs). These findings bring new insight into the interaction of POPs and microplastic polymers and show that affinity of POPs to polymers is variable depending on study area.

5. Future perspectives

The results of this thesis unravel some of the unknowns around the impact of microplastics on the marine environment and provide new tools for future studies on microplastic. This work leads, in turn, to new ideas for future research, including:

1. Impact of different microplastic types (e.g. different shapes, sizes, polymers) on different cell types by IFC: from unicellular organisms such as microalgae, to cells from multicellular organisms such as human. (from **Paper I, II** and **Supplementary file**)
2. *In vivo* accumulation of microplastics in cells by IFC. (from **Paper I, II** and **Supplementary file**)
3. Interaction of POPs and microplastics in diverse conditions (e.g. temperatures, salinities, pH) and environments, focusing especially on dioxins and Arctic waters. (from **Paper III**)
4. Toxicity of microplastics marinated with pollutants from fish farming origin on marine wildlife and farmed organisms. (from **Paper III**)
5. Transfer of pollutants associated to fish farming from microplastics to non or low-polluted environments, and what impact such contaminated microplastics have in the environment. (from **Paper III**)

In terms of ecotoxicology, future microplastic research should focus more on the impact of particle shape of microplastics found in nature. The impact of microfibers on organisms should therefore be investigated more in depth. In addition, it is important to study the impact of microplastics along with other environmental stressors, such as climate change or ocean acidification, and to assess whether the combination of stressors might have synergistic or antagonistic effects on organisms to get a better understanding of the realistic impact of microplastic pollution in the environment.

6. References

Abarghouei, S., Hedayati, A., Raeisi, M., Hadavand, B.S., Rezaei, H. & Abed-Elmdoust, A. (2021). Size-dependent effects of microplastic on uptake, immune system, related gene expression and histopathology of goldfish (*Carassius auratus*). *Chemosphere*: 129977.

Abbas, A.K., Lichtman, A.H. & Pillai, S. (2014). *Cellular and molecular immunology.* Elsevier Sauders, Philadelphia, US.

Abihssira-García, I.S., Park, Y., Kiron, V. & Olsvik, P.A. (2020). Fluorescent microplastic uptake by immune cells of Atlantic salmon (*Salmo salar* L.). *Front Environ Sci,* 8: 233. DOI https://doi.org/10.3389/fenvs.2020.560206

Ahmad, I., Weng, J., Stromberg, A.J., Hilt, J.Z. & Dziubla, T.D. (2019). Fluorescence based detection of polychlorinated biphenyls (PCBs) in water using hydrophobic interactions. *Analyst,* 144: 677-684. DOI 10.1039/c8an00867a

Akhbarizadeh, R., Moore, F. & Keshavarzi, B. (2019). Investigating microplastics bioaccumulation and biomagnification in seafood from the Persian Gulf: a threat to human health? *Food Addit Contam - Chem Ana. Control Exp. Risk Assess,* 36: 1696-1708.

Alabi, O.A., Ologbonjaye, K.I., Awosolu, O. & Alalade, O.E. (2019). Public and environmental health effects of plastic wastes disposal: a review. *J Toxicol Risk Assess,* 5: 1-13.

Ammendolia, J., Saturno, J., Brooks, A.L., Jacobs, S. & Jambeck, J.R. (2021). An emerging source of plastic pollution: environmental presence of plastic personal protective equipment (PPE) debris related to COVID-19 in a metropolitan city. *Environ Pollut,* 269: 116160.

Amornphimoltham, P., Yuen, P.S., Star, R.A. & Leelahavanichkul, A. (2019). Gut leakage of fungal-derived inflammatory mediators: part of a gut-liver-kidney axis in bacterial sepsis. *Dig Dis Sci,* 64: 2416-2428.

Andrady, A.L. (2017). The plastic in microplastics: A review. *Mar Pollut Bull,* 119: 12-22.

Aoki, T., Takano, T., Santos, M.D., Kondo, H. & Hirono, I. (2008). Molecular innate immunity in teleost fish: review and future perspectives. *Fisheries for Global Welfare and Environment, Memorial Book of the 5th World Fisheries Congress.* Terrapub, Tokyo, Japan.

Arthur, C., Baker, J.E. & Bamford, H.A. (2009). Proceedings of the International Research Workshop on the Occurrence, Effects, and Fate of Microplastic Marine Debris, September 9-11, 2008, University of Washington, Tacoma, WA, USA.

Avio, C.G., Gorbi, S., Milan, M., Benedetti, M., Fattorini, D., D'errico, G., Pauletto, M., Bargelloni, L. & Regoli, F. (2015). Pollutants bioavailability and toxicological risk from microplastics to marine mussels. *Environ Pollut,* 198: 211-222.

Bachère, E., Rosa, R.D., Schmitt, P., Poirier, A.C., Merou, N., Charrière, G.M. & Destoumieux-Garzón, D. (2015). The new insights into the oyster antimicrobial defense: Cellular, molecular and genetic view. *Fish Shellfish Immunol,* 46: 50-64.

Baker, I. (2018). Bakelite. *Fifty Materials That Make the World.* Springer, Cham, Switzerland.

Bamai, Y.A., Shibata, E., Saito, I., Araki, A., Kanazawa, A., Morimoto, K., Nakayama, K., Tanaka, M., Takigawa, T. & Yoshimura, T. (2014). Exposure to house dust phthalates in relation to asthma and allergies in both children and adults. *Sci Total Environ,* 485: 153-163.

Barentswatch (2021). Fish Health. https://www.barentswatch.no/fiskehelse/

Barrows, A., Cathey, S.E. & Petersen, C.W. (2018). Marine environment microfiber contamination: Global patterns and the diversity of microparticle origins. *Environ Pollut,* 237: 275-284.

Berntssen, M.H., Julshamn, K. & Lundebye, A.-K. (2010). Chemical contaminants in aquafeeds and Atlantic salmon (*Salmo salar*) following the use of traditional-versus alternative feed ingredients. *Chemosphere,* 78: 637-646.

Beyer, J., Green, N.W., Brooks, S., Allan, I.J., Ruus, A., Gomes, T., Bråte, I.L.N. & Schøyen, M. (2017). Blue mussels (*Mytilus edulis* spp.) as sentinel organisms in coastal pollution monitoring: a review. *Mar Environ Res,* 130: 338-365.

Bikker, J., Lawson, J., Wilson, S. & Rochman, C. (2020). Microplastics and other anthropogenic particles in the surface waters of the Chesapeake Bay. *Mar Pollut Bull,* 156: 111257.

Biron, M. (2018). *Thermoplastics and thermoplastic composites*. William Andrew, Oxford, UK.

Boudry, G., Yang, P.-C. & Perdue, M.H. (2004). Small Intestine, Anatomy. In: Johnson, L.R. (Ed.) *Encyclopedia of Gastroenterology*. Elsevier, New York, US, pp 404-409. https://doi.org/10.1016/B0-12-386860-2/00648-1

Boyer, R.F. (1981). Anecdotal history of styrene and polystyrene. *J Macromol Sci A,* 15**:** 1411-1434.

Bråte, I.L.N., Eidsvoll, D.P., Steindal, C.C. & Thomas, K.V. (2016). Plastic ingestion by Atlantic cod (*Gadus morhua*) from the Norwegian coast. *Mar Pollut Bull,* 112**:** 105-110. DOI https://doi.org/10.1016/j.marpolbul.2016.08.034

Bråte, I.L.N., Hurley, R., Iversen, K., Beyer, J., Thomas, K.V., Steindal, C.C., Green, N.W., Olsen, M. & Lusher, A. (2018). *Mytilus* spp. as sentinels for monitoring microplastic pollution in Norwegian coastal waters: A qualitative and quantitative study. *Environ Pollut,* 243**:** 383-393.

Bråte, I.L.N., Huwer, B., Thomas, K.V., Eidsvoll, D.P., Halsband, C., Almroth, B.C. & Lusher, A. (2017). *Micro- and macro-plastics in marine species from Nordic waters*.Nordic Council of Ministers, Copenhagen, Denmark.

Bringer, A., Thomas, H., Prunier, G., Dubillot, E., Bossut, N., Churlaud, C., Clérandeau, C., Le Bihanic, F. & Cachot, J. (2020). High density polyethylene (HDPE) microplastics impair development and swimming activity of Pacific oyster D-larvae, *Crassostrea gigas*, depending on particle size. *Environ Pollut,* 260**:** 113978.

Browne, M.A., Dissanayake, A., Galloway, T.S., Lowe, D.M. & Thompson, R.C. (2008). Ingested microscopic plastic translocates to the circulatory system of the mussel, *Mytilus edulis* (L.). *Environ Sci Technol,* 42**:** 5026-5031.

Burgos-Aceves, M.A. & Faggio, C. (2017). An approach to the study of the immunity functions of bivalve haemocytes: physiology and molecular aspects. *Fish Shellfish Immunol,* 67**:** 513-517.

Busch, K.E.T., Iversen, K.R., Nashoug, B.F., Kiele, M.R., Gitmark, J., Remen, V., Velvin, R. & Krogstad, M. (2014). Miljøundersøkelse i Skjerstadfjorden (Report No. 1006). *SALT Lofoten AS, Svolvær, Norway*. https://docplayer.me/18417897-Rapport-miljoundersokelse-i-skjerstadfjorden-salt-rapport-nr-1006.html

Canesi, L., Gallo, G., Gavioli, M. & Pruzzo, C. (2002). Bacteria–hemocyte interactions and phagocytosis in marine bivalves. *Microsc Res Tech,* 57**:** 469-476.

Cappello, T., De Marco, G., Conti, G.O., Giannetto, A., Ferrante, M., Mauceri, A. & Maisano, M. (2021). Time-dependent metabolic disorders induced by short-term exposure to polystyrene microplastics in the Mediterranean mussel *Mytilus galloprovincialis*. *Ecotoxicol Environ Saf,* 209**:** 111780.

Carpenter, E. & Smith Jr, K. (1972). Plastics on the Sargasso sea surface. *Science,* 175**:** 1240-1241.

Carpenter, E.J., Anderson, S.J., Harvey, G.R., Miklas, H.P. & Peck, B.B. (1972). Polystyrene spherules in coastal waters. *Science,* 178**:** 749-750.

Carraher Jr, C.E. (2003). *Seymour/Carraher's polymer chemistry*. CRC press, New York, US.

Champion, J.A. & Mitragotri, S. (2006). Role of target geometry in phagocytosis. *PNAS,* 103**:** 4930-4934.

Cole, M., Liddle, C., Consolandi, G., Drago, C., Hird, C., Lindeque, P.K. & Galloway, T.S. (2020). Microplastics, microfibres and nanoplastics cause variable sub-lethal responses in mussels (*Mytilus* spp.). *Mar Pollut Bull,* 160**:** 111552.

Cole, M., Lindeque, P., Halsband, C. & Galloway, T.S. (2011). Microplastics as contaminants in the marine environment: a review. *Mar Pollut Bull,* 62**:** 2588-2597.

Collicutt, B., Juanes, F. & Dudas, S.E. (2019). Microplastics in juvenile Chinook salmon and their nearshore environments on the east coast of Vancouver Island. *Environ Pollut,* 244**:** 135-142.

Colton, J.B., Knapp, F.D. & Burns, B.R. (1974). Plastic particles in surface waters of the northwestern Atlantic. *Science,* 185**:** 491-497.

Costa, M. (2008). Estudio de la respuesta inmune y expresión génica del mejillón mediterráneo *Mytilus galloprovincialis*. Doctoral thesis, University of Vigo, Spain.

Covernton, G.A., Pearce, C.M., Gurney-Smith, H.J., Chastain, S.G., Ross, P.S., Dower, J.F. & Dudas, S.E. (2019). Size and shape matter: A preliminary analysis of microplastic sampling technique in seawater studies with implications for ecological risk assessment. *Sci Total Environ,* 667**:** 124-132.

Cunningham, E.M., Ehlers, S.M., Dick, J.T., Sigwart, J.D., Linse, K., Dick, J.J. & Kiriakoulakis, K. (2020). High abundances of microplastic pollution in deep-sea sediments: evidence from Antarctica and the Southern Ocean. *Environ Sci Technol,* 54**:** 13661-13671.

Danso, D., Schmeisser, C., Chow, J., Zimmermann, W., Wei, R., Leggewie, C., Li, X., Hazen, T. & Streit, W.R. (2018). New insights into the function and global distribution of polyethylene terephthalate (PET)-

degrading bacteria and enzymes in marine and terrestrial metagenomes. *Appl Environ Microbiol,* 84.

De-La-Torre, G.E. & Aragaw, T.A. (2021). What we need to know about PPE associated with the COVID-19 pandemic in the marine environment. *Mar Pollut Bull,* 163**:** 111879.

Desforges, J.-P.W., Galbraith, M., Dangerfield, N. & Ross, P.S. (2014). Widespread distribution of microplastics in subsurface seawater in the NE Pacific Ocean. *Mar Pollut Bull,* 79**:** 94-99.

Ding, J., Zhang, S., Razanajatovo, R.M., Zou, H. & Zhu, W. (2018). Accumulation, tissue distribution, and biochemical effects of polystyrene microplastics in the freshwater fish red tilapia (*Oreochromis niloticus*). *Environ Pollut,* 238**:** 1-9. DOI https://doi.org/10.1016/j.envpol.2018.03.001

Directorate of Fisheries (2020). Atlantic salmon and rainbow trout, Grown out: Sale 1994-2019. In: Directorate of Fisheries. https://www.fiskeridir.no/English/Aquaculture/Statistics/Atlantic-salmon-and-rainbow-trout

Dodiuk, H. & Goodman, S. H. (2013). *Handbook of thermoset plastics*. William Andrew, San Diego, CA, US.

Duncan, E.M., Botterell, Z.L., Broderick, A.C., Galloway, T.S., Lindeque, P.K., Nuno, A. & Godley, B.J. (2017). A global review of marine turtle entanglement in anthropogenic debris: a baseline for further action. *Endangered Species Res,* 34**:** 431-448.

Ec. (2011). *Commission Recommendation of 18 October 2011 on the definition of nanomaterial (2011/696/EU)*. Retrieved from https://ec.europa.eu/research/industrial_technologies/pdf/policy/commission-recommendation-on-the-definition-of-nanomater-18102011_en.pdf

Ec. (2020). *Commission regulation (EC) No 1881/2006 of 19 December 2006 setting maximum levels for certain contaminants in foodstuffs (Text with EEA relevance)*. Comission, E., 02006R1881 — EN — 14102020 — 027001. Retrieved from https://eur-lex.europa.eu/legal-content/EN/TXT/?uri=CELEX%3A02006R1881-20201014

Enders, K., Lenz, R., Stedmon, C.A. & Nielsen, T.G. (2015). Abundance, size and polymer composition of marine microplastics ≥10μm in the Atlantic Ocean and their modelled vertical distribution. *Mar Pollut Bull,* 100**:** 70-81. DOI https://doi.org/10.1016/j.marpolbul.2015.09.027

Endo, S. & Koelmans, A.A. (2016). Sorption of hydrophobic organic compounds to plastics in the marine environment: equilibrium. In: Takada, H. & Karapanagioti, H.K. (Eds.) *Hazardous chemicals associated with plastics in the marine environment.* Springer, Switzerland.

Espinosa, C., Beltrán, J.M.G., Esteban, M.A. & Cuesta, A. (2018). In vitro effects of virgin microplastics on fish head-kidney leucocyte activities. *Environ Pollut,* 235**:** 30-38.

Espinosa, C., Cuesta, A. & Esteban, M.Á. (2017). Effects of dietary polyvinylchloride microparticles on general health, immune status and expression of several genes related to stress in gilthead seabream (*Sparus aurata* L.). *Fish Shellfish Immunol,* 68**:** 251-259.

Espinosa, C., Esteban, M.Á. & Cuesta, A. (2019). Dietary administration of PVC and PE microplastics produces histological damage, oxidative stress and immunoregulation in European sea bass (*Dicentrarchus labrax* L.). *Fish Shellfish Immunol,* 95**:** 574-583.

Evangeliou, N., Grythe, H., Klimont, Z., Heyes, C., Eckhardt, S., Lopez-Aparicio, S. & Stohl, A. (2020). Atmospheric transport is a major pathway of microplastics to remote regions. *Nat commun,* 11**:** 1-11.

Fao (2020). The State of World Fisheries and Aquaculture 2020. Sustainability in action. In: *The State of World Fisheries and Aquaculture (SOFIA).* FAO, Rome, Italy.

Farrell, P. & Nelson, K. (2013). Trophic level transfer of microplastic: *Mytilus edulis* (L.) to *Carcinus maenas* (L.). *Environ Pollut,* 177**:** 1-3.

Fisner, M., Majer, A., Taniguchi, S., Bícego, M., Turra, A. & Gorman, D. (2017). Colour spectrum and resin-type determine the concentration and composition of Polycyclic Aromatic Hydrocarbons (PAHs) in plastic pellets. *Mar Pollut Bull,* 122**:** 323-330.

Free, C.M., Jensen, O.P., Mason, S.A., Eriksen, M., Williamson, N.J. & Boldgiv, B. (2014). High-levels of microplastic pollution in a large, remote, mountain lake. *Mar Pollut Bull,* 85**:** 156-163.

Fuglem, B., Jirillo, E., Bjerkås, I., Kiyono, H., Nochi, T., Yuki, Y., Raida, M., Fischer, U. & Koppang, E.O. (2010). Antigen-sampling cells in the salmonid intestinal epithelium. *Dev Comp Immunol,* 34**:** 768-774. DOI https://doi.org/10.1016/j.dci.2010.02.007

Gaitán-Espitia, J.D., Quintero-Galvis, J.F., Mesas, A. & D'elía, G. (2016). Mitogenomics of southern hemisphere blue mussels (Bivalvia: Pteriomorphia): Insights into the evolutionary characteristics of the *Mytilus edulis* complex. *Sci rep,* 6**:** 1-10.

Galgani, F., Fleet, D., Franeker, J., Katsanevakis, S., Maes, T., Mouat, J., Oosterbaan, L., Poitou, I., Hanke, G. & Thompson, R. (2010). *Marine strategy framework directive: Task group 10 report, Marine litter (Report No. EUR 24340 EN - 2010)*. Office for Official Publications of the European Communities, Luxembourg. *https://ec.europa.eu/environment/marine/pdf/9-Task-Group-10.pdf* .

Gallart-Jornet, L., Barat, J., Rustad, T., Erikson, U., Escriche, I. & Fito, P. (2007). A comparative study of brine salting of Atlantic cod (*Gadus morhua*) and Atlantic salmon (*Salmo salar*). *J Food Eng,* 79**:** 261-270.

Galloway, T.S. (2015). Micro-and nano-plastics and human health. *Marine anthropogenic litter*. Springer, Cham, Switzerland.

Goldberg, E.D. (1975). The mussel watch: a first step in global marine monitoring. Mar Pollut Bull, 6: 111-114. http://dx.doi.org/10.1016/0025-326X(75)90271-4

Gomiero, A., Haave, M., Bjorøy, Ø., Herzke, D., Kögel, T., Nikiforov, V. & Øysæd, K.B. (2020a). *Quantification of microplastic in fillet and organs of farmed and wild salmonids-a comparison of methods for detection and quantification* (Report No. 8/2020). NORCE Environment. https://norceresearch.brage.unit.no/norceresearch-xmlui/bitstream/handle/11250/2687619/NORCE%20Report%208_2020%20SalmoDetect%20project.pdf?sequence=1&isAllowed=y

Gomiero, A., Haave, M., Kögel, T., Bjorøy, Ø., Gjessing, M., Lea, T.B., Horve, E., Martins, C. & Olafsen, T. (2020b). *TRACKing of PLASTtic emissions from aquaculture industry* (Report No. 4/2020). NORCE Environment. https://norceresearch.brage.unit.no/norceresearch-xmlui/bitstream/handle/11250/2649891/Rapport%20NORCE%20milj%C3%B8%204-2020.pdf?sequence=4

Gomiero, A., Øysæd, K.B., Agustsson, T., Van Hoytema, N., Van Thiel, T. & Grati, F. (2019). First record of characterization, concentration and distribution of microplastics in coastal sediments of an urban fjord in south west Norway using a thermal degradation method. *Chemosphere,* 227**:** 705-714.

Gray, A.D. & Weinstein, J.E. (2017). Size-and shape-dependent effects of microplastic particles on adult daggerblade grass shrimp (*Palaemonetes pugio*). *Environ Toxicol Chem,* 36**:** 3074-3080.

Green, N.W., Schøyen, M., Hjermann, D.Ø., Øxnevad, S., Ruus, A., Grung, M., Beylich, B., Lund, E., Tveiten, L. & Jenssen, M.T. (2020). *Contaminants in coastal waters of Norway 2019* (Report No. 7555/2020). NIVA Reports. https://www.miljodirektoratet.no/globalassets/publikasjoner/m1766/m1766.pdf

Gregory, M.R. (1978). Accumulation and distribution of virgin plastic granules on New Zealand beaches. *N Z J Mar Freshwat Res,* 12**:** 399-414.

Gregory, M.R. (1999). Plastics and South Pacific Island shores: environmental implications. *Ocean Coast Manag,* 42**:** 603-615.

Group of Chief Scientific Advisors (2019). *Environmental and Health Risks of Microplastic Pollution* (Report No. 4). EuropeanUnion, Luxembourg.

Grumezescu, V. & Grumezescu, A. (2019). *Materials for biomedical engineering: thermoset and thermoplastic polymers*: Elsevier Science.

Guo, X. & Wang, J. (2019). The chemical behaviors of microplastics in marine environment: A review. *Mar Pollut Bull,* 142**:** 1-14.

Hamed, M., Soliman, H.a.M., Osman, A.G.M. & Sayed, A.E.-D.H. (2019). Assessment the effect of exposure to microplastics in Nile Tilapia (*Oreochromis niloticus*) early juvenile: I. blood biomarkers. *Chemosphere,* 228**:** 345-350. DOI https://doi.org/10.1016/j.chemosphere.2019.04.153

Hamm, T. & Lenz, M. (2021). Negative impacts of realistic doses of spherical and irregular microplastics emerged late during a 42 weeks-long exposure experiment with blue mussels. *Sci Total Environ,* 778**:** 146088. DOI https://doi.org/10.1016/j.scitotenv.2021.146088

Harris, P.T. (2020). The fate of microplastic in marine sedimentary environments: A review and synthesis. *Mar Pollut Bull,* 158**:** 111398. DOI https://doi.org/10.1016/j.marpolbul.2020.111398

Hartmann, N.B., HüFfer, T., Thompson, R.C., HassellöV, M., Verschoor, A., Daugaard, A.E., Rist, S., Karlsson, T., Brennholt, N. & Cole, M. (2019). Are we speaking the same language? Recommendations for a definition and categorization framework for plastic debris. *Environ Sci Technol*. DOI https://doi.org/10.1021/acs.est.8b05297

Heinrich, P., Hanslik, L., Kämmer, N. & Braunbeck, T. (2020). The tox is in the detail: technical fundamentals for designing, performing, and interpreting experiments on toxicity of microplastics and associated substances. *Environ Sci Pollut Res,* 27**:** 22292-22318.

Hirai, H., Takada, H., Ogata, Y., Yamashita, R., Mizukawa, K., Saha, M., Kwan, C., Moore, C., Gray, H. & Laursen, D. (2011). Organic micropollutants in marine plastics debris from the open ocean and remote and urban beaches. *Mar Pollut Bull,* 62**:** 1683-1692. DOI https://doi.org/10.1016/j.marpolbul.2011.06.004

Hognes, E.S. & Skaar, C. (2017). *Avfallshåndtering fra sjøbasert havbruk* (report No. OC2017 A-218). SINTEF Ocean, Trondheim, Norway. https://sintef.brage.unit.no/sintef-xmlui/handle/11250/2477326

Huntington, A., Corcoran, P.L., Jantunen, L., Thaysen, C., Bernstein, S., Stern, G.A. & Rochman, C.M. (2020). A first assessment of microplastics and other anthropogenic particles in Hudson Bay and the surrounding eastern Canadian Arctic waters of Nunavut. *Facets,* 5**:** 432-454.

Hwang, J., Choi, D., Han, S., Choi, J. & Hong, J. (2019). An assessment of the toxicity of polypropylene microplastics in human derived cells. *Sci Total Environ,* 684**:** 657-669.

Hwang, J., Choi, D., Han, S., Jung, S.Y., Choi, J. & Hong, J. (2020). Potential toxicity of polystyrene microplastic particles. *Sci rep,* 10**:** 1-12.

Iftimie, S., López-Azcona, A.F., Vallverdú, I., Hernández-Flix, S., De Febrer, G., Parra, S., Hernández-Aguilera, A., Riu, F., Joven, J., Andreychuk, N., Baiges-Gaya, G., Ballester, F., Benavent, M., Burdeos, J., Català, A., Castañé, È., Castañé, H., Colom, J., Feliu, M., Gabaldó, X., Garrido, D., Garrido, P., Gil, J., Guelbenzu, P., Lozano, C., Marimon, F., Pardo, P., Pujol, I., Rabassa, A., Revuelta, L., Ríos, M., Rius-Gordillo, N., Rodríguez-Tomàs, E., Rojewski, W., Roquer-Fanlo, E., Sabaté, N., Teixidó, A., Vasco, C., Camps, J. & Castro, A. (2021). First and second waves of coronavirus disease-19: A comparative study in hospitalized patients in Reus, Spain. *PLOS ONE,* 16**:** e0248029. DOI 10.1371/journal.pone.0248029

Jeong, C.-B., Won, E.-J., Kang, H.-M., Lee, M.-C., Hwang, D.-S., Hwang, U.-K., Zhou, B., Souissi, S., Lee, S.-J. & Lee, J.-S. (2016). Microplastic size-dependent toxicity, oxidative stress induction, and p-JNK and p-p38 activation in the monogonont rotifer (*Brachionus koreanus*). *Environ Sci Technol,* 50**:** 8849-8857.

Jepsen, E.M. & De Bruyn, P.N. (2019). Pinniped entanglement in oceanic plastic pollution: A global review. *Mar Pollut Bull,* 145**:** 295-305.

Jiang, Y., Yang, F., Zhao, Y. & Wang, J. (2020). Greenland Sea Gyre increases microplastic pollution in the surface waters of the Nordic Seas. *Sci Total Environ,* 712**:** 136484.

Jones, K.C. & De Voogt, P. (1999). Persistent organic pollutants (POPs): state of the science. *Environ Pollut,* 100**:** 209-221. DOI https://doi.org/10.1016/S0269-7491(99)00098-6

Jovanović, B. (2017). Ingestion of microplastics by fish and its potential consequences from a physical perspective. *Integr Environ Assess Manag,* 13**:** 510-515.

Kaile, N., Lindivat, M., Elio, J., Thuestad, G., Crowley, Q.G. & Hoell, I.A. (2020). Preliminary results from detection of microplastics in liquid samples using flow cytometry. *Front Mar Sci,* 7**:** 856.

Kang, J.-H., Kwon, O.Y., Lee, K.-W., Song, Y.K. & Shim, W.J. (2015). Marine neustonic microplastics around the southeastern coast of Korea. *Mar Pollut Bull,* 96**:** 304-312.

Kato, G., Miyazawa, H., Nakayama, Y., Ikari, Y., Kondo, H., Yamaguchi, T., Sano, M. & Fischer, U. (2018). A Novel Antigen-Sampling Cell in the Teleost Gill Epithelium With the Potential for Direct Antigen Presentation in Mucosal Tissue. *Front Immunol,* 9**:** 2116-2116. DOI 10.3389/fimmu.2018.02116

Khonakdar, H., Jafari, S. & Hässler, R. (2007). Glass-transition-temperature depression in chemically crosslinked low-density polyethylene and high-density polyethylene and their blends with ethylene vinyl acetate copolymer. *J Appl Polym Sci,* 104**:** 1654-1660.

Kinjo, A., Mizukawa, K., Takada, H. & Inoue, K. (2019). Size-dependent elimination of ingested microplastics in the Mediterranean mussel *Mytilus galloprovincialis*. *Mar Pollut Bull,* 149**:** 110512.

Koelmans, A.A. (2015). Modeling the role of microplastics in bioaccumulation of organic chemicals to marine aquatic organisms. A critical review. In: Melanie Bergmann, Lars Gutow & Klages, M. (Eds.) *Marine Anthropogenic Litter.* Springer, Cham Switzerland, pp 309-324.

Koelmans, A.A., Diepens, N.J. & Mohamed Nor, N.H. (2022). Weight of evidence for the microplastic vector effect in the context of chemical risk assessment. *Microplastic in the Environment: Pattern and Process.* Springer, Cham, Switzerland.

Krogdahl, Å., Gajardo, K., Kortner, T.M., Penn, M., Gu, M., Berge, G.M. & Bakke, A.M. (2015). Soya Saponins Induce Enteritis in Atlantic Salmon (*Salmo salar* L.). *J Agric Food Chem,* 63**:** 3887-3902. DOI 10.1021/jf506242t

Krüger, L., Casado-Coy, N., Valle, C., Ramos, M., Sanchez-Jerez, P., Gago, J., Carretero, O., Beltran-Sanahuja, A. & Sanz-Lazaro, C. (2020). Plastic debris accumulation in the seabed derived from coastal fish farming. *Environ Pollut,* 257**:** 113336.

La Daana, K.K., Gardfeldt, K., Krumpen, T., Thompson, R.C. & O'connor, I. (2020). Microplastics in sea ice and seawater beneath ice floes from the Arctic Ocean. *Sci rep,* 10**:** 1-11.

Le Bihanic, F., Clérandeau, C., Cormier, B., Crebassa, J.-C., Keiter, S.H., Beiras, R., Morin, B., Bégout, M.-L., Cousin, X. & Cachot, J. (2020). Organic contaminants sorbed to microplastics affect marine medaka fish early life stages development. *Mar Pollut Bull,* 154**:** 111059.

Le Foll, F., Rioult, D., Boussa, S., Pasquier, J., Dagher, Z. & Leboulenger, F. (2010). Characterisation of *Mytilus edulis* hemocyte subpopulations by single cell time-lapse motility imaging. *Fish Shellfish Immunol,* 28**:** 372-386.

Lee, H., Lee, H.-J. & Kwon, J.-H. (2019). Estimating microplastic-bound intake of hydrophobic organic chemicals by fish using measured desorption rates to artificial gut fluid. *Sci Total Environ,* 651**:** 162-170. DOI https://doi.org/10.1016/j.scitotenv.2018.09.068

Li, J., Lusher, A.L., Rotchell, J.M., Deudero, S., Turra, A., Bråte, I.L.N., Sun, C., Hossain, M.S., Li, Q. & Kolandhasamy, P. (2019). Using mussel as a global bioindicator of coastal microplastic pollution. *Environ Pollut,* 244**:** 522-533.

Li, J., Qu, X., Su, L., Zhang, W., Yang, D., Kolandhasamy, P., Li, D. & Shi, H. (2016). Microplastics in mussels along the coastal waters of China. *Environ Pollut,* 214**:** 177-184.

Li, L.-L., Amara, R., Souissi, S., Dehaut, A., Duflos, G. & Monchy, S. (2020). Impacts of microplastics exposure on mussel (*Mytilus edulis*) gut microbiota. *Sci Total Environ,* 745**:** 141018. DOI https://doi.org/10.1016/j.scitotenv.2020.141018

Lindeque, P.K., Cole, M., Coppock, R.L., Lewis, C.N., Miller, R.Z., Watts, A.J., Wilson-Mcneal, A., Wright, S.L. & Galloway, T.S. (2020). Are we underestimating microplastic abundance in the marine environment? A comparison of microplastic capture with nets of different mesh-size. *Environ Pollut,* 265**:** 114721.

Loker, E.S. (2012). Macroevolutionary immunology: a role for immunity in the diversification of animal life. *Front Immunol,* 3**:** 25.

Lundebye, A.-K., Lock, E.-J., Rasinger, J.D., Nøstbakken, O.J., Hannisdal, R., Karlsbakk, E., Wennevik, V., Madhun, A.S., Madsen, L. & Graff, I.E. (2017). Lower levels of persistent organic pollutants, metals and the marine omega 3-fatty acid DHA in farmed compared to wild Atlantic salmon (*Salmo salar*). *Environ Res,* 155**:** 49-59. DOI https://doi.org/10.1016/j.envres.2017.01.026

Lusher, A. (2015). Microplastics in the marine environment: distribution, interactions and effects. *Marine Anthropogenic Litter.* Springer, Cham, Switzerland, pp 245-307.

Lusher, A., Hollman, P. & Mendoza-Hill, J. (2017a). *Microplastics in fisheries and aquaculture: status of knowledge on their occurrence and implications for aquatic organisms and food safety* (Report No 615). FAO Fisheries and Aquaculture Technical Paper, Rome, Italy.

Lusher, A. & Pettersen, R. (2021). *Sea-based sources of microplastics to the Norwegian marine environment* (Report No. 7568-2021). NIVA. file:///C:/Users/06023852/Downloads/M1911%20(1).pdf

Lusher, A., Welden, N., Sobral, P. & Cole, M. (2017b). Sampling, isolating and identifying microplastics ingested by fish and invertebrates. *Anal methods,* 9**:** 1346-1360.

Lusher, A.L., Tirelli, V., O'connor, I. & Officer, R. (2015). Microplastics in Arctic polar waters: the first reported values of particles in surface and sub-surface samples. *Sci rep,* 5**:** 1-9.

Ma, H., Pu, S., Liu, S., Bai, Y., Mandal, S. & Xing, B. (2020). Microplastics in aquatic environments: Toxicity to trigger ecological consequences. *Environ Pollut,* 261**:** 114089.

Mankidy, R., Wiseman, S., Ma, H. & Giesy, J.P. (2013). Biological impact of phthalates. *Toxicol Lett,* 217**:** 50-58.

Mazurais, D., Ernande, B., Quazuguel, P., Severe, A., Huelvan, C., Madec, L., Mouchel, O., Soudant, P., Robbens, J. & Huvet, A. (2015). Evaluation of the impact of polyethylene microbeads ingestion in European sea bass (*Dicentrarchus labrax*) larvae. *Mar Environ Res,* 112**:** 78-85.

Mckinnon, K.M. (2018). Flow cytometry: an overview. *Curr protoc immunol,* 120**:** 5.1. 1-5.1. 11.

Medzhitov, R. (2001). Toll-like receptors and innate immunity. *Nat Rev Immunol,* 1**:** 135-145.

Meijer, L.J.J., Emmerik, T.V., Ent, R.V.D., Schmidt, C. & Lebreton, L. (2021). More than 1000 rivers account for 80% of global riverine plastic emissions into the ocean. *Sci Adv,* 7**:** eaaz5803. DOI doi:10.1126/sciadv.aaz5803

Mendoza, L.M.R., Karapanagioti, H. & Álvarez, N.R. (2018). Micro (nanoplastics) in the marine environment: current knowledge and gaps. *Current Opinion in Environmental Science & Health,* 1**:** 47-51.

Miller, M.E., Kroon, F.J. & Motti, C.A. (2017). Recovering microplastics from marine samples: A review of current practices. *Mar Pollut Bull,* 123**:** 6-18.

Moore, C.J., Moore, S.L., Leecaster, M.K. & Weisberg, S.B. (2001). A comparison of plastic and plankton in the North Pacific central gyre. *Mar Pollut Bull,* 42**:** 1297-1300.

Mulder, K. & Knot, M. (2001). PVC plastic: a history of systems development and entrenchment. *Technol Soc,* 23**:** 265-286.

Müller, Y.K., Wernicke, T., Pittroff, M., Witzig, C.S., Storck, F.R., Klinger, J. & Zumbülte, N. (2020). Microplastic analysis—are we measuring the same? Results on the first global comparative study for microplastic analysis in a water sample. *Anal Bioanal Chem,* 412**:** 555-560.

Nagasawa, T., Nakayasu, C., Rieger, A.M., Barreda, D.R., Somamoto, T. & Nakao, M. (2014). Phagocytosis by thrombocytes is a conserved innate immune mechanism in lower vertebrates. *Front Immunol,* 5**:** 445.

Natta, G. & Corradini, P. (1960). Structure and properties of isotactic polypropylene. *Il Nuovo Cimento (1955-1965),* 15**:** 40-51.

Naveira, C., Rodrigues, N., Santos, F.S., Santos, L.N. & Neves, R.A. (2021). Acute toxicity of Bisphenol A (BPA) to tropical marine and estuarine species from different trophic groups. *Environ Pollut,* 268**:** 115911.

Neves, D., Sobral, P., Ferreira, J.L. & Pereira, T. (2015). Ingestion of microplastics by commercial fish off the Portuguese coast. *Mar Pollut Bull,* 101**:** 119-126.

Noaa (2009). World Ocean Atlas 2009 Figures. National Oceanic and Atmospheric Administarion. https://www.nodc.noaa.gov/OC5/WOA09F/pr_woa09f.html

Ogata, Y., Takada, H., Mizukawa, K., Hirai, H., Iwasa, S., Endo, S., Mato, Y., Saha, M., Okuda, K. & Nakashima, A. (2009). International Pellet Watch: Global monitoring of persistent organic pollutants (POPs) in coastal waters. 1. Initial phase data on PCBs, DDTs, and HCHs. *Mar Pollut Bull,* 58**:** 1437-1446. DOI https://doi.org/10.1016/j.marpolbul.2009.06.014

Pannetier, P., Morin, B., Le Bihanic, F., Dubreil, L., Clérandeau, C., Chouvellon, F., Van Arkel, K., Danion, M. & Cachot, J. (2020). Environmental samples of microplastics induce significant toxic effects in fish larvae. *Environ Int,* 134**:** 105047.

Park, Y. (2021). Transcriptomic and cellular studies on the intestine of Atlantic salmon. Discovering intestinal macrophages using omic tools. Doctoral thesis, Faculty of Biosciences and Aquaculture, Nord University, Bodø, Norway.

Peacock, A. (2000). *Handbook of polyethylene: structures: properties, and applications*. CRC press, Basel, Switzerland.

Pedà, C., Caccamo, L., Fossi, M.C., Gai, F., Andaloro, F., Genovese, L., Perdichizzi, A., Romeo, T. & Maricchiolo, G. (2016). Intestinal alterations in European sea bass *Dicentrarchus labrax* (Linnaeus, 1758) exposed to microplastics: preliminary results. *Environ Pollut,* 212**:** 251-256.

Peeken, I., Primpke, S., Beyer, B., Gütermann, J., Katlein, C., Krumpen, T., Bergmann, M., Hehemann, L. & Gerdts, G. (2018). Arctic sea ice is an important temporal sink and means of transport for microplastic. *Nat commun,* 9**:** 1-12.

Peng, X., Chen, M., Chen, S., Dasgupta, S., Xu, H., Ta, K., Du, M., Li, J., Guo, Z. & Bai, S. (2018). Microplastics contaminate the deepest part of the world's ocean. *Geocheml Perspect Lett,* 9**:** 1-5.

Pittura, L., Avio, C.G., Giuliani, M.E., D'errico, G., Keiter, S.H., Cormier, B., Gorbi, S. & Regoli, F. (2018). Microplastics as vehicles of environmental PAHs to marine organisms: combined chemical and physical hazards to the Mediterranean mussels, *Mytilus galloprovincialis*. *Front Mar Sci,* 5**:** 103.

Plasticseurope (2021). European Plastics Industry Market Data. *Plastics - the Facts.*

Primpke, S., Cross, R.K., Mintenig, S.M., Simon, M., Vianello, A., Gerdts, G. & Vollertsen, J. (2020). Toward the systematic identification of microplastics in the environment: evaluation of a new independent software tool (siMPle) for spectroscopic analysis. *Appl Spectrosc,* 74**:** 1127-1138.

Prokić, M.D., Radovanović, T.B., Gavrić, J.P. & Faggio, C. (2019). Ecotoxicological effects of microplastics: Examination of biomarkers, current state and future perspectives. *TrAC,* 111**:** 37-46. DOI https://doi.org/10.1016/j.trac.2018.12.001

Revel, M., Lagarde, F., Perrein-Ettajani, H., Bruneau, M., Akcha, F., Sussarellu, R., Rouxel, J., Costil, K., Decottignies, P., Cognie, B., Châtel, A. & Mouneyrac, C. (2019). Tissue-Specific Biomarker Responses in the Blue Mussel Mytilus spp. Exposed to a Mixture of Microplastics at Environmentally Relevant Concentrations. *Front Environ Sci,* 7. DOI 10.3389/fenvs.2019.00033

Rist, S., Almroth, B.C., Hartmann, N.B. & Karlsson, T.M. (2018). A critical perspective on early communications concerning human health aspects of microplastics. *Sci Total Environ,* 626**:** 720-726.

Rochman, C.M., Hoh, E., Hentschel, B.T. & Kaye, S. (2013a). Long-term field measurement of sorption of organic contaminants to five types of plastic pellets: implications for plastic marine debris. *Environ Sci Technol,* 47**:** 1646-1654. DOI https://doi.org/10.1021/es303700s

Rochman, C.M., Hoh, E., Kurobe, T. & Teh, S.J. (2013b). Ingested plastic transfers hazardous chemicals to fish and induces hepatic stress. *Sci Rep,* 3**:** 1-7.

Rochman, C.M., Kurobe, T., Flores, I. & Teh, S.J. (2014). Early warning signs of endocrine disruption in adult fish from the ingestion of polyethylene with and without sorbed chemical pollutants from the marine environment. *Sci Total Environ,* 493**:** 656-661. DOI https://doi.org/10.1016/j.scitotenv.2014.06.051

Rummel, C.D., Jahnke, A., Gorokhova, E., KüHnel, D. & Schmitt-Jansen, M. (2017). Impacts of biofilm formation on the fate and potential effects of microplastic in the aquatic environment. *Environl Sci Technol Lett,* 4**:** 258-267. DOI https://doi.org/10.1021/acs.estlett.7b00164

Ryan, P.G. (2018). Entanglement of birds in plastics and other synthetic materials. *Mar Pollut Bull,* 135**:** 159-164.

Scheirs, J. & Priddy, D. (2003). *Modern styrenic polymers: polystyrenes and styrenic copolymers*. John Wiley & Sons, Chichester, UK.

Schymanski, D., Goldbeck, C., Humpf, H.-U. & Fürst, P. (2018). Analysis of microplastics in water by micro-Raman spectroscopy: release of plastic particles from different packaging into mineral water. *Water Res,* 129**:** 154-162.

Sele, V., Sanden, M., Berntssen, M., Storesund, J.E., Lie, K.K., Espe, M., Lundebye, A.-K., Hemre, G.I., Waagbø, R. & Ørnsrud, R. (2019). *Program for overvåking av fiskefôr-Årsrapport for prøver innsamlet i 2018*. Institute of Marine Research. https://www.mattilsynet.no/dyr_og_dyrehold/for/rapport_overvaakingsprogram_fiskefor_2018.35512/binary/Rapport:%20Overv%C3%A5kingsprogram%20fiskef%C3%B4r%202018

Sendra, M., Carrasco-Braganza, M.I., Yeste, P.M., Vila, M. & Blasco, J. (2020a). Immunotoxicity of polystyrene nanoplastics in different hemocyte subpopulations of *Mytilus galloprovincialis*. *Sci rep,* 10**:** 1-14.

Sendra, M., Saco, A., Yeste, M.P., Romero, A., Novoa, B. & Figueras, A. (2020b). Nanoplastics: From tissue accumulation to cell translocation into Mytilus galloprovincialis hemocytes. resilience of immune cells exposed to nanoplastics and nanoplastics plus Vibrio splendidus combination. *J Hazard Mater,* 388**:** 121788.

Simon, A., Arbiol, C., Nielsen, E.E., Couteau, J., Sussarellu, R., Burgeot, T., Bernard, I., Coolen, J.W., Lamy, J.B. & Robert, S. (2020). Replicated anthropogenic hybridisations reveal parallel patterns of admixture in marine mussels. *Evol Appl,* 13**:** 575-599.

Sjödin, A., Patterson Jr, D.G. & Bergman, Å. (2003). A review on human exposure to brominated flame retardants—particularly polybrominated diphenyl ethers. *Environ Int,* 29**:** 829-839.

Sorensen, R.M. & Jovanović, B. (2021). From nanoplastic to microplastic: A bibliometric analysis on the presence of plastic particles in the environment. *Mar Pollut Bull,* 163**:** 111926.

Stock, V., Laurisch, C., Franke, J., Dönmez, M.H., Voss, L., Böhmert, L., Braeuning, A. & Sieg, H. (2021). Uptake and cellular effects of PE, PP, PET and PVC microplastic particles. *Toxicol In Vitro,* 70**:** 105021.

Stone, V., Miller, M.R., Clift, M.J., Elder, A., Mills, N.L., Møller, P., Schins, R.P., Vogel, U., Kreyling, W.G. & Alstrup Jensen, K. (2017). Nanomaterials versus ambient ultrafine particles: an opportunity to exchange toxicology knowledge. *Environ Health Perspect,* 125**:** 106002.

Suaria, G., Avio, C.G., Mineo, A., Lattin, G.L., Magaldi, M.G., Belmonte, G., Moore, C.J., Regoli, F. & Aliani, S. (2016). The Mediterranean Plastic Soup: synthetic polymers in Mediterranean surface waters. *Sci Rep,* 6**:** 37551. DOI 10.1038/srep37551

Sundt, P., Schulze, P.-E. & Syversen, F. (2014). *Sources of microplastic-pollution to the marine environment* (Report No. M-321/2015). Norwegian Environment Agency. https://www.miljodirektoratet.no/globalassets/publikasjoner/M321/M321.pdf

Syversen, F., Sundt, P., Kirkevaag, K. & Briedis, R. (2020a). *Materialstrømmen til plast i Norge – hva vet vi?* (Report No. 2). Handelens Miljøfond, Oslo, Norway. https://s3.eu-west-1.amazonaws.com/handelensmiljofond/PDF/Materialstr%C3%B8mmen-til-plast-i-Norge-Hva-vet-vi.pdf

Syversen, T., Vollstad, J., Lilleng, G. & Hanssen, B.J. (2020b). *Slitasje på fiskeredskap. Kvantifisering av slitasje fra ulike redskapstyper* (Report No. 2020:01296). Directorate of Fisheries. file:///C:/Users/06023852/Downloads/Slitasje%20fra%20fiskeredskap-sluttrapport%20(5).pdf

Taranger, G.L., Karlsen, Ø., Bannister, R.J., Glover, K.A., Husa, V., Karlsbakk, E., Kvamme, B.O., Boxaspen, K.K., Bjørn, P.A. & Finstad, B. (2015). Risk assessment of the environmental impact of Norwegian Atlantic salmon farming. *ICES J Mar Sci,* 72**:** 997-1021. DOI https://doi.org/10.1093/icesjms/fsu132

Ter Halle, A., Jeanneau, L., Martignac, M., Jardé, E., Pedrono, B., Brach, L. & Gigault, J. (2017). Nanoplastic in the North Atlantic subtropical gyre. *Environ Sci Technol,* 51**:** 13689-13697.

Thompson, R.C. (2015). Microplastics in the marine environment: sources, consequences and solutions. In: *Marine anthropogenic litter.* Springer, Cham, Switezerland, pp 185-200.

Thompson, R.C., Olsen, Y., Mitchell, R.P., Davis, A., Rowland, S.J., John, A.W., Mcgonigle, D. & Russell, A.E. (2004). Lost at sea: where is all the plastic? *Science,* 304**:** 838.

Thushari, G. & Senevirathna, J. (2020). Plastic pollution in the marine environment. *Heliyon,* 6**:** e04709.

Tomazic-Jezic, V.J., Merritt, K. & Umbreit, T.H. (2001). Significance of the type and the size of biomaterial particles on phagocytosis and tissue distribution. *J Biomed Mater Res A,* 55**:** 523-529.

Tort, L., Balasch, J. & Mackenzie, S. (2003). Fish immune system. A crossroads between innate and adaptive responses. *Inmunología,* 22**:** 277-286.

Van Der Gon, H.D., Van Het Bolscher, M., Visschedijk, A. & Zandveld, P. (2007). Emissions of persistent organic pollutants and eight candidate POPs from UNECE–Europe in 2000, 2010 and 2020 and the emission reduction resulting from the implementation of the UNECE POP protocol. *Atmos Environ,* 41**:** 9245-9261.

Van Der Vegt, A. (2006). *From polymers to plastics*. VSSD Delft, Netherlands.

Vangelsten, B.V., Bay-Larsen, I.A., Nogueira, L.A., Pedersen, V. & Johannessen, E.R. (2019). *Delrapport HAVPLAST: Marint avfall fra havbruksnæringen* (Report No. 10/2019). Nordland Research Institute, Norway. https://nforsk.brage.unit.no/nforsk-xmlui/handle/11250/2727368

Von Moos, N., Burkhardt-Holm, P. & KöHler, A. (2012). Uptake and effects of microplastics on cells and tissue of the blue mussel *Mytilus edulis* L. after an experimental exposure. *Environ Sci Technol,* 46**:** 11327-11335.

Wabnitz, C. & Nichols, W.J. (2010). Plastic pollution: An ocean emergency. *Mar Turtle Newsl***:** 1.

Waller, C.L., Griffiths, H.J., Waluda, C.M., Thorpe, S.E., Loaiza, I., Moreno, B., Pacherres, C.O. & Hughes, K.A. (2017). Microplastics in the Antarctic marine system: an emerging area of research. *Sci Total Environ,* 598**:** 220-227.

Wang, C., Zhao, J. & Xing, B. (2020a). Environmental Source, Fate, and Toxicity of Microplastics. *J Hazard Mater***:** 124357. DOI https://doi.org/10.1016/j.jhazmat.2020.124357

Wang, S., Hu, M., Zheng, J., Huang, W., Shang, Y., Fang, J.K.-H., Shi, H. & Wang, Y. (2021a). Ingestion of nano/micro plastic particles by the mussel *Mytilus coruscus* is size dependent. *Chemosphere,* 263**:** 127957.

Wang, T., Hu, M., Xu, G., Shi, H., Leung, J.Y. & Wang, Y. (2021b). Microplastic accumulation via trophic transfer: Can a predatory crab counter the adverse effects of microplastics by body defence? *Sci Total Environ,* 754**:** 142099.

Wang, W., Ge, J. & Yu, X. (2020b). Bioavailability and toxicity of microplastics to fish species: A review. *Ecotoxicol Environ Saf,* 189**:** 109913.

Webb, S., Gaw, S., Marsden, I. & Mcrae, N. (2020). Biomarker responses in New Zealand green-lipped mussels *Perna canaliculus* exposed to microplastics and triclosan. *Ecotoxicol Environ Saf,* 201**:** 110871.

Wenning, R.J. & Martello, L.B. (2008). Dioxin. In: Jørgensen, S.E. & Fath, B.D. (Eds.) *Encyclopedia of Ecology.* Elsevier Science. https://doi.org/10.1016/B978-008045405-4.00385-2

Whinfield, J.R. & Dickson, J.T. (1941). Improvements relating to the manufacture of highly polymeric substances (UK Patent No. GB578079A).

Whyte, S.K. (2007). The innate immune response of finfish–a review of current knowledge. *Fish Shellfish Immunol,* 23**:** 1127-1151.

Woods, M.N., Stack, M.E., Fields, D.M., Shaw, S.D. & Matrai, P.A. (2018). Microplastic fiber uptake, ingestion, and egestion rates in the blue mussel (Mytilus edulis). *Mar Pollut Bull,* 137**:** 638-645. DOI https://doi.org/10.1016/j.marpolbul.2018.10.061

Wootton, E., Dyrynda, E. & Ratcliffe, N. (2003). Bivalve immunity: comparisons between the marine mussel (*Mytilus edulis*), the edible cockle (*Cerastoderma edule*) and the razor-shell (*Ensis siliqua*). *Fish Shellfish Immunol,* 15**:** 195-210.

Worm, B., Lotze, H.K., Jubinville, I., Wilcox, C. & Jambeck, J. (2017). Plastic as a Persistent Marine Pollutant. *Annual Review of Environment and Resources,* 42**:** 1-26. DOI 10.1146/annurev-environ-102016-060700

Wright, S.L., Thompson, R.C. & Galloway, T.S. (2013). The physical impacts of microplastics on marine organisms: A review. *Environ Pollut,* 178**:** 483-492. DOI https://doi.org/10.1016/j.envpol.2013.02.031

Xu, G., Cheng, H., Jones, R., Feng, Y., Gong, K., Li, K., Fang, X., Tahir, M.A., Valev, V.K. & Zhang, L. (2020). Surface-Enhanced Raman Spectroscopy Facilitates the Detection of Microplastics< 1 μm in the Environment. *Environ Sci Technol*.

Yang, H.-S., Hong, H.-K., Donaghy, L., Noh, C.-H., Park, H.-S., Kim, D.-S. & Choi, K.-S. (2015). Morphology and Immune-related activities of hemocytes of the mussel Mytilus coruscus (Gould, 1861) from East Sea of Korea. *OSJ,* 50**:** 77-85.

Zapata, A., Diez, B., Cejalvo, T., Gutierrez-De Frias, C. & Cortes, A. (2006). Ontogeny of the immune system of fish. *Fish Shellfish Immunol,* 20**:** 126-136.

Zhang, F., Wang, X., Xu, J., Zhu, L., Peng, G., Xu, P. & Li, D. (2019). Food-web transfer of microplastics between wild caught fish and crustaceans in East China Sea. *Mar Pollut Bull,* 146**:** 173-182.

Ziccardi, L.M., Edgington, A., Hentz, K., Kulacki, K.J. & Driscoll, S.K. (2016). Microplastics as vectors for bioaccumulation of hydrophobic organic chemicals in the marine environment: A state-of-the-science review. *Environ Toxicol Chem,* 35**:** 1667-1676. DOI https://doi.org/10.1002/etc.3461

Zitouni, N., Bousserrhine, N., Belbekhouche, S., Missawi, O., Alphonse, V., Boughatass, I. & Banni, M. (2020). First report on the presence of small microplastics (≤ 3 μm) in tissue of the commercial fish *Serranus scriba* (Linnaeus. 1758) from Tunisian coasts and associated cellular alterations. *Environ Pollut***:** 114576.

Zitouni, N., Bousserrhine, N., Missawi, O., Boughattas, I., Chèvre, N., Santos, R., Belbekhouche, S., Alphonse, V., Tisserand, F. & Balmassiere, L. (2021). Uptake, tissue distribution and toxicological effects of environmental microplastics in early juvenile fish *Dicentrarchus labrax*. *J Hazard Mater,* 403**:** 124055.

Paper I

frontiers in Immunology

METHODS
published: 18 February 2020
doi: 10.3389/fimmu.2020.00203

Imaging Flow Cytometry Protocols for Examining Phagocytosis of Microplastics and Bioparticles by Immune Cells of Aquatic Animals

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Imaging flow cytometry (IFC) is a powerful tool which combines flow cytometry with digital microscopy to generate quantitative high-throughput imaging data. Despite various advantages of IFC over standard flow cytometry, widespread adoption of this technology for studies in aquatic sciences is limited, probably due to the relatively high equipment cost, complexity of image analysis-based data interpretation and lack of core facilities with trained personnel. Here, we describe the application of IFC to examine phagocytosis of particles including microplastics by cells from aquatic animals. For this purpose, we studied (1) live/dead cell assays and identification of cell types, (2) phagocytosis of degradable and non-degradable particles by Atlantic salmon head kidney cells and (3) the effect of incubation temperature on phagocytosis of degradable particles in three aquatic animals–Atlantic salmon, Nile tilapia, and blue mussel. The usefulness of the developed method was assessed by evaluating the effect of incubation temperature on phagocytosis. Our studies demonstrate that IFC provides significant benefits over standard flow cytometry in phagocytosis measurement by allowing integration of morphometric parameters, especially while identifying cell populations and distinguishing between different types of fluorescent particles and detecting their localization.

Keywords: ImageStream®^{X}, IFC, Atlantic salmon, Nile tilapia, blue mussel, phagocytosis

OPEN ACCESS

Edited by:
Kim Dawn Thompson, Moredun Research Institute, United Kingdom

Reviewed by:
Sebastian Reyes-Cerpa, Universidad Mayor, Chile
Caroline Fossum, Swedish University of Agricultural Sciences, Sweden

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Specialty section:
This article was submitted to Comparative Immunology, a section of the journal Frontiers in Immunology

Received: *20 October 2019*
Accepted: *27 January 2020*
Published: *18 February 2020*

Citation:
Park Y, Abihssira-García IS, Thalmann S, Wiegertjes GF, Barreda DR, Olsvik PA and Kiron V (2020) Imaging Flow Cytometry Protocols for Examining Phagocytosis of Microplastics and Bioparticles by Immune Cells of Aquatic Animals. Front. Immunol. 11:203. doi: 10.3389/fimmu.2020.00203

INTRODUCTION

Flow cytometry (FC) is widely employed for studying mammalian cells in particular and detecting biomarkers in clinical studies. FC systems quantify cell data within seconds and can provide information on cell phenotypes and functions. However, conventional FC is not designed to measure morphological and spatial information of single cells, and the technology is not able to efficiently detect dim and small particles (<300 nm) (1) as well as to distinguish aggregates of these small particles. Furthermore, although conventional FC can measure intra- and extracellular marker expressions, it does not provide information on marker localization. Another obstacle connected to conventional FC is auto-fluorescence. While the system cannot always precisely distinguish between false-positive and false-negative events (2), fine-tuning of instrument settings and protocol optimization can minimize the problem (3). Nevertheless, cell phenotype identification and functional analyses using conventional FC cannot be entirely objective as the equipment lacks image-capturing features. To overcome inaccuracies in acquiring cell data,

quantitative studies preferably rely on both conventional FC and fluorescent cell imaging.

Imaging flow cytometry (IFC), also called multispectral imaging flow cytometry, is a powerful tool that enables us to collect information from single cells, including those from fluorescent images. Major advantages of IFC are (1) high fluorescence sensitivity, (2) high image resolution capability, (3) high speed processing, (4) ability to analyse changes in cell or nuclear morphology, (5) rare cell detection ability, and (6) capacity to understand cell-cell interaction (2). Certain disciplines of biology, namely hematology (4), immunology (5), cell biology (6), and microbiology (7) have already benefited from IFC. However, application of IFC is still in its infancy when it comes to studies in aquatic sciences.

Researchers have reported IFC-based analyses of fish cells, using nucleus staining to understand cell morphology and employing fluorescent particles to determine phagocytic activity in goldfish (8, 9). Different particles such as fluorescent latex beads (10), zymosan-APC (8), and nanoparticles (11) have been used to analyse phagocytosis using IFC. These methods can be further optimized, depending on the characteristics of the particles, e.g., latex beads that are not degraded vis-à-vis pHrodo™ BioParticles® that emit fluorescent light upon acidification following ingestion by the target cells (10). Researchers have also improved the protocol for measuring particles' intensity in IFC (12). Overall, these studies provide the first information on the use of IFC to identify different cells and understand cell functions such as phagocytosis and the localization of markers of interest in cells from aquatic animals.

Though previous studies on aquatic animals have reported phagocytosis, here we present (1) basic, but optimized protocols for live/dead cell assay and identification of cell types (2), an improved protocol for examining phagocytosis of non-degradable (microplastic) and degradable (bioparticles) particles by immune cell types of fish, and (3) an optimized phagocytosis assay using cells harvested from three very different aquatic animals: cold water-adapted carnivorous marine fish (Atlantic salmon, *Salmo salar*), warm water-adapted omnivorous, freshwater fish (Nile tilapia, *Oreochromis niloticus*) and a cold water-adapted detritivorous/planktivorous marine mollusc (blue mussel, *Mytilus edulis*). Effect of incubation temperature was studied to verify the sensitivity and usefulness of the optimized phagocytosis assay.

METHODS

Ethics Statement

The studies were approved (Atlantic salmon: FOTS ID 10050, Nile tilapia: FOTS ID 1042) by the National Animal Research Authority in Norway (Mattilsynet). The fish rearing and handling procedures were according to the approved protocols of FDU.

Animals

Atlantic salmon (*S. salar*) in the weight range 700–900 g were used in this experiment. They were purchased from a commercial producer (Sundsfjord Smolt, Nygårdsjøen, Norway) and maintained at the Research Station of Nord University, Bodø, Norway. Fish were fed a commercial feed (Ewos AS, Bergen, Norway) and reared in a flow-through sea water system (temperature: 7–8°C, dissolved oxygen saturation: 87–92%, 24–h light cycle).

Nile tilapia (*O. niloticus*, 400–600 g) were bred and reared at the Research Station of Nord University in a freshwater recirculating aquaculture system (temperature: 28°C, pH: 7.6, dissolved oxygen saturation: 80% in outlet and 115% in inlet, 11 h dark/13 h light cycle). The fish were fed commercial feeds (Skretting, Stavanger, Norway) during the rearing period.

Adult blue mussels (*M. edulis*) were collected from a beach along the Saltenfjorden, Bodø, Norway (67°12′01″ N 14°37′56″ E) and transported to the Research Station, Nord University. Prior to isolation of hemocytes, they were kept for 2 days in running seawater at 7–8°C.

Cell Isolation

Cells from salmon and tilapia head kidney (HK) were grown in Leibovitz's L-15 Medium (L-15; Sigma-Aldrich, Oslo, Norway), supplemented with 100 μg/mL gentamicin sulfate (Sigma), 2 mM L-glutamine (Sigma) and 15 mM HEPES (Sigma). Osmolality of medium was adjusted by adding a solution consisting of 5% (v/v) 0.41 M NaCl, 0.33 M $NaHCO_3$, and 0.66 (w/v) D-glucose. Cell culture media were adjusted to 380 mOsm for salmon and 320 mOsm for tilapia. To culture the mussel hemocytes, filtered (through a 0.2 μm mesh) sea water was used as the medium.

Head kidney from salmon ($n = 6$) were sampled after the fish were killed with an overdose of MS-222 (Tricaine methane sulphonate; Argent Chemical Laboratories, Redmond, USA; 80 mg/L). Thereafter, the HK cells were isolated as described previously (13) with minor modifications. Briefly, HK was dissected out, and the tissues were transferred to 15 mL centrifuge tubes to make a total volume of 4 mL in ice-cold L-15+ (L-15 medium with 50 U/mL penicillin, 50 μg/mL streptomycin, 2% fetal bovine serum (FBS) and 10 U/mL heparin). The tissue was placed on a sterile 100 μM cell strainer (Falcon) and the cells were disrupted with the help of a syringe plunger. The harvested cells were washed twice in ice-cold L-15+. The cell suspension from salmon HK was then layered on 40/60% Percoll (Sigma) to separate HK leukocytes for magnetic-activated cell sorting (MACS) or layered on 34/51% Percoll to separate monocytes/macrophages for subsequent phagocytosis assays. After centrifugation (500 × g, 30 min, 4°C), the cells at the interface between the two Percoll gradients were collected and washed twice with ice-cold L-15-FBS free (L-15 medium with 50 U/mL penicillin, 50 μg/mL streptomycin) by centrifugation (500 × g, 5 min, 4°C). Cells were then kept in L-15+. HK phagocytic cells that were separated based on 34/51% Percoll gradient, were allowed to adhere on a petri dish for 3 days at 12°C. After removing the supernatant containing non-adherent cells, the petri dish with the adherent cells was placed on ice for 10 min, and the cells were collected by washing three times with 1.5 mL ice-cold PBS supplemented with 5 mM EDTA. Next, these collected cells were centrifuged (500 × g, 5 min, 4°C) and used for further analyses.

Head kidney from tilapia ($n = 6$) were collected after killing the fish with an overdose of clove oil (Sigma Aldrich, St. Louis,

MO, USA), and cells were harvested as described previously (14, 15), with minor modifications. Briefly, the HK tissues were transferred to 15 mL centrifuge tubes to make a total volume of 4 mL in ice-cold L-15+. The cells were harvested from the HK and washed twice as described for salmon. The cell suspension was layered on 34/51% Percoll to separate phagocytic cells, and then after centrifugation, cells at the interface were collected and washed twice in L-15-FBS free. The cells in the suspension were allowed to adhere on a petri dish containing L-15+ for 3 days at 25°C. After removing the supernatant containing non-adherent cells, the petri dish with the adherent cells was placed on ice for 10 min, and the cells were collected by washing three times with 1.5 mL ice-cold PBS supplemented with 5 mM EDTA. Next, the collected cells were centrifuged (500 × g, 5 min, 4°C) and used for further analyses.

In the fish experiments, the cells were counted using a portable cell counter (Scepter™ 2.0 cell counter, EMD Millipore, Darmstadt, Germany).

Hemocytes from adult mussels ($n = 6$) were isolated as described previously (16) with minor modifications. Briefly, hemolymph was drawn from the posterior adductor muscle using a 2 mL syringe equipped with a 23G-needle. The hemocytes from each mussel were counted using a Neubauer chamber, and 0.2×10^6 cells per sample were collected and re-suspended in 1 mL of filtered sea water to avoid formation of clumps.

Magnetic-Activated Cell Sorting (MACS) of Salmon IgM$^+$ Cells

The isolated salmon HK leukocytes (2×10^6 cells) were incubated with mouse anti-trout/salmon IgM (6.06 μg/mL; Aquatic Diagnostics Ltd, Sterling, UK) for 60 min at 4°C. After two washes with L-15+, the cells were incubated for 15 min at 4°C in a cocktail with a total volume of 100 μL, which contained L-15+, 1 μL of goat anti-mouse IgG-FITC (0.75 mg/mL; Thermo Fisher Scientific, Oslo, Norway) and 40 μL of goat anti-mouse IgG microbeads as per the instructions of the manufacturer (Miltenyi Biotec, Bergisch Gladbach, Germany). First, MACS LD columns (Miltenyi Biotec) that were placed in a magnetic separator of the multistand were washed using L-15+. The cell suspension was then transferred into the LD column. Following cell sorting, the positive cells were harvested and re-suspended in L-15+.

Live/Dead Cell Assay

In the studies on cells from salmon and tilapia, aliquots containing 1×10^6 cells in 50 μL PBS were transferred to 1.5 mL microcentrifuge tubes. Then 1 μL of propidium iodide (PI; 1 mg/mL, Sigma) was added to each sample to detect the dead cells in the cell suspension. In the case of mussel, aliquots containing 0.2×10^6 cells in 50 μL filtered sea water were transferred to 1.5 mL microcentrifuge tubes, and then 1 μL of DRAQ5™ (25 mM, Thermo Fisher Scientific) was added to each sample to detect the dead cells in the cell suspension. The tubes were gently mixed before the samples were run through the ImageStream$^{®X}$ Mk II Imaging Flow Cytometer (Luminex Corporation, Austin, TX, USA). Cell analyses were performed on 10,000 cells acquired at a rate of 300 objects/second at low speed and a magnification of 40×. Dead cells were estimated as the percent of cells positive for either PI or DRAQ5™ (red fluorescent cells). After excluding the dead cells, viable cells were analyzed to generate brightfield (BF) area (size) vs. side scatter (SSC) intensity (complexity) dot plots. Instrument settings were kept identical throughout the study.

Phagocytosis Assay

In the first phagocytosis experiment, phagocytic cells from salmon HK were employed to study the uptake of two types of particles; non-degradable fluorescent polystyrene microplastic beads (2.1 μm; Magsphere Inc., California, USA) and degradable fluorescent bio-particles (>0.2 μm; pHrodo™ Red *Escherichia coli* Bioparticles, Thermo Fisher Scientific). In the second experiment, we used degradable fluorescent bio-particles only; to determine phagocytic ability and capacity of the cells from salmon, tilapia and mussel at two different incubation temperatures. Phagocytic ability was measured as the percent of phagocytic cells among the total macrophage-like cells or hemocytes. On the other hand, phagocytic capacity was measured as the mean number of particles per phagocytic cell. Phagocytic index (PI) or phagocytic activity was determined employing the equation (17, 18):

Phagocytic index (PI) = [% phagocytic cells containing at least one particle] × [mean particle count per phagocytic cell].

Briefly, fluorescent bio-particles were added at a cell:particle ratio of 1:5 per sample, both in the case of HK macrophages (0.5×10^6 cells) and hemocytes (0.2×10^6 cells). Cells suspensions and bio-particles were mixed and incubated for 2 h at different temperatures. Following incubation, cell suspensions were washed twice with 500 μL L-15+ by centrifugation (500 × g, 5 min, 4°C). The supernatant was discarded, and the resulting cell pellets were re-suspended in 50 μL PBS. The cell samples were run in an imaging flow cytometer (Luminex), equipped with a 10 mW 488 nm argon-ion laser, to detect the bio-particle fluorescence (577/35 nm bandpass; Channel 3). Thereafter, the images were analyzed using IDEAS 6.1.822.0 software (Luminex).

Data and Statistical Analyses

Statistical analysis was performed in RStudio version 1.1.463. Normality of the data was tested by Shapiro-Wilk Test, and the assumption of equal variance was checked by Bartlett's Test. Comparisons between the two groups were performed using unpaired Student's *t*-test. Statistically significant differences ($p < 0.05$) are reported for the phagocytosis data.

RESULTS

Live/Dead Cells and Leukocyte Populations From Salmon Head Kidney

To determine single cell area and to identify cell populations, we employed a basic gating strategy using the Brightfield Gradient Root Mean Square (RMS) feature of the imaging flow cytometer (see **Figure 1**). This strategy helped us to select the cells in best focus, i.e., this allowed us to obtain high quality images with RMS values >50 (**Figure 1A**). Next, we separated single cells from others (debris, doublets and aggregates; **Figure 1B**). Dead cells were excluded based on

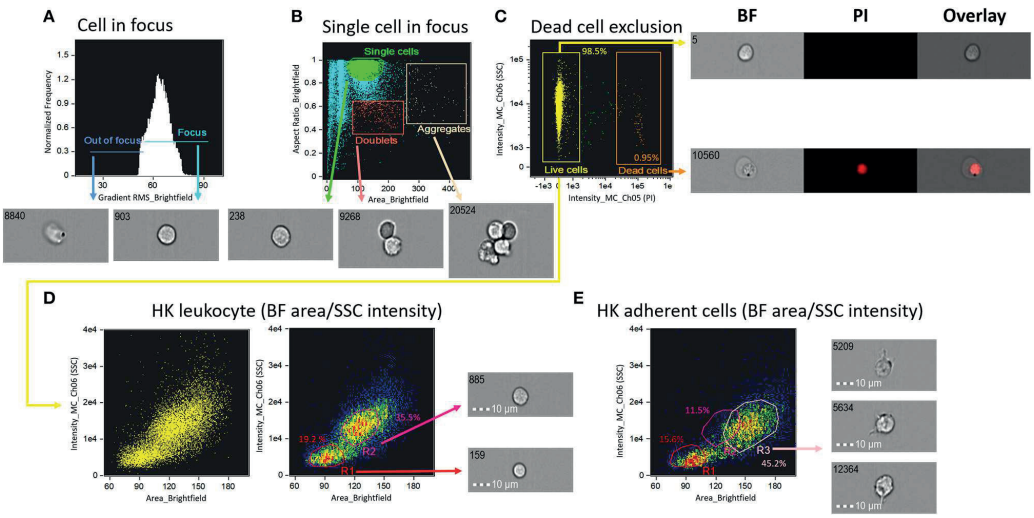

FIGURE 1 | Schematic of the gating strategy to determine areas and populations of head kidney cells from Atlantic salmon. **(A)** Focused cells were gated using IDEAS® gradient RMS feature which helps to select high-quality images with RMS values above 50. **(B)** Representative image showing the gated cells; single cells, doublets and aggregates are shown in a dot plot. Aspect ratio is a feature that evaluates an elliptical shape fitted around the detected object. The ratio is calculated as the minor axis divided by the major axis; a value close to 1 indicates that the cell has a circular shape, while a value in the range 0.4–0.8 signals the presence of doublets or aggregates if the individual cells are of oval morphology. **(C)** Orange dots are the dead cells that were excluded using propidium iodide, to separate the live cells (yellow dots). **(D)** HK leukocyte population is shown in a brightfield (BF) area (cell size) vs. side scatter (SSC) intensity (cell internal complexity) plot. **(E)** HK adherent cell (macrophage-like cell) population is shown in a BF area vs. SSC intensity plot. All cell images were captured with 40 × objective. Scale bar = 10 μm. BF, brightfield; PI, propidium iodide; R, region; R1, lymphocyte-like cells; R2, monocytes/macrophages-like cells; R3, macrophage-like cells.

positivity for PI (**Figure 1C**). The percentage of live cells were 98.5%. The brightfield (BF) area and side scatter (SSC) intensity of the live, single cells were assessed. We prepared a BF area vs. SSC intensity dot plot to show the salmon HK leukocyte populations (**Figure 1D**). Cells with smaller size (low BF area) and low SSC intensity were possibly lymphocyte-like cells (19.2%; R1 in **Figure 1D**) while those with larger size (BF area) and higher SSC intensity compared to lymphocyte-like cells were considered as monocytes/macrophages (35.5%; R2 in **Figure 1D**). **Figure 1E** shows salmon HK adherent cell populations in a BF area vs. SSC intensity dot plot; here, R3 is probably HK macrophage-like cells (45.2%). We conclude that using IFC, dead cells can be excluded, and different single cell populations can be better detected than in conventional FC.

Salmon Head Kidney IgM$^+$ Lymphocyte Identification

Salmon head kidney IgM$^+$ lymphocytes separated using MACS were used to ascertain their localization in a BF area vs. SSC intensity dot plot (**Figure 2**). For this purpose, cells were extracellularly stained with IgM-FITC, which enabled us to identify areas of negatively- and positively-stained B lymphocyte populations. Before MACS (**Figure 2A**), all cells were located in the IgM$^-$ area (right panel **Figure 2A**). After staining with IgM-FITC and performing MACS (**Figure 2B**), most cells were located in the IgM$^+$ area (89.8%; right panel **Figure 2B**). These data confirmed that the IgM$^+$ cells matched the location of the lymphocyte-like cells (R1 population in **Figure 1D**). Thus, we confirmed the localization of salmon IgM$^+$ cells using IFC.

Examining Phagocytosis Using Non-degradable Fluorescent Microplastic Beads

To determine the phagocytosis of microplastics by salmon HK cells, first, we plotted histograms of fluorescence intensity of non-degradable fluorescent polystyrene microplastic beads in live cells (**Figure 3A**). Because all the polystyrene beads were of similar size, we assumed that fluorescence intensity is proportional to the number of beads taken up by each phagocytic cell. Using IFC, we could exclude auto-fluorescence and could gate images with more pixels and higher intensity (phagocytic images with pixel value > 30 were considered to be of high quality) (**Figure 3B**). Caution was taken to exclude aggregates; addition of many microplastic beads can cause bead aggregation, leading to false identification of aggregates as phagocytic cells, especially in conventional FC. Next, we gated phagocytic cells that engulfed microplastic beads using an internalization score (**Figure 3C**). This score is the ratio of the particle intensity inside a cell to the intensity of the whole cell, and it is calculated after masking (which selects pixels

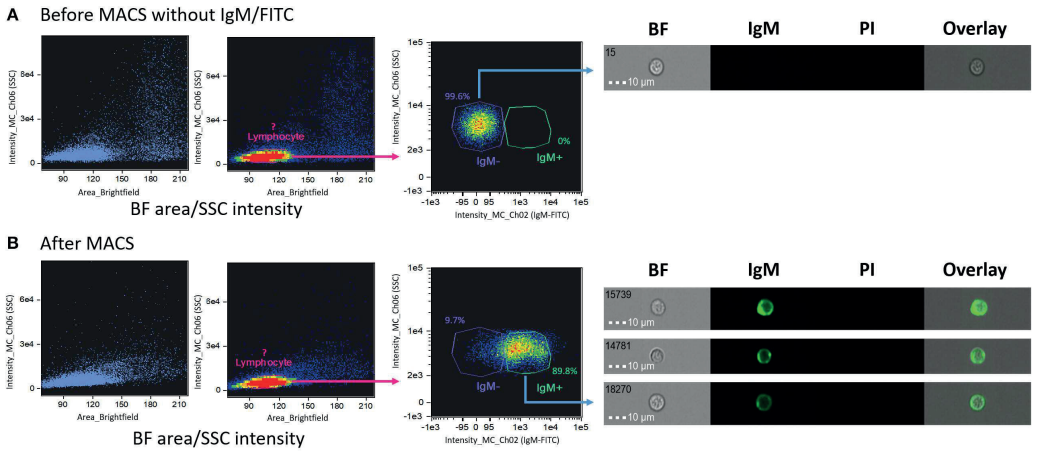

FIGURE 2 | Identification of salmon IgM$^+$ lymphocyte after magnetic-activated cell sorting (MACS). Dot plots of **(A)** HK leukocytes before and **(B)** after MACS separation. The left and middle plots in **(A,B)** show brightfield (BF) area vs. SSC intensity and the right plot in **(A,B)** shows the density of the target cells. Cells without IgM-FITC are shown in the IgM$^-$ gate (**A**, right panel) while IgM$^+$ cells after MACS are shown in the IgM$^+$ gate (**B**, right panel). All cell images were captured with 40 × objective. Scale bar = 10 μm. BF, brightfield; IgM, immunoglobulin M; PI, propidium iodide.

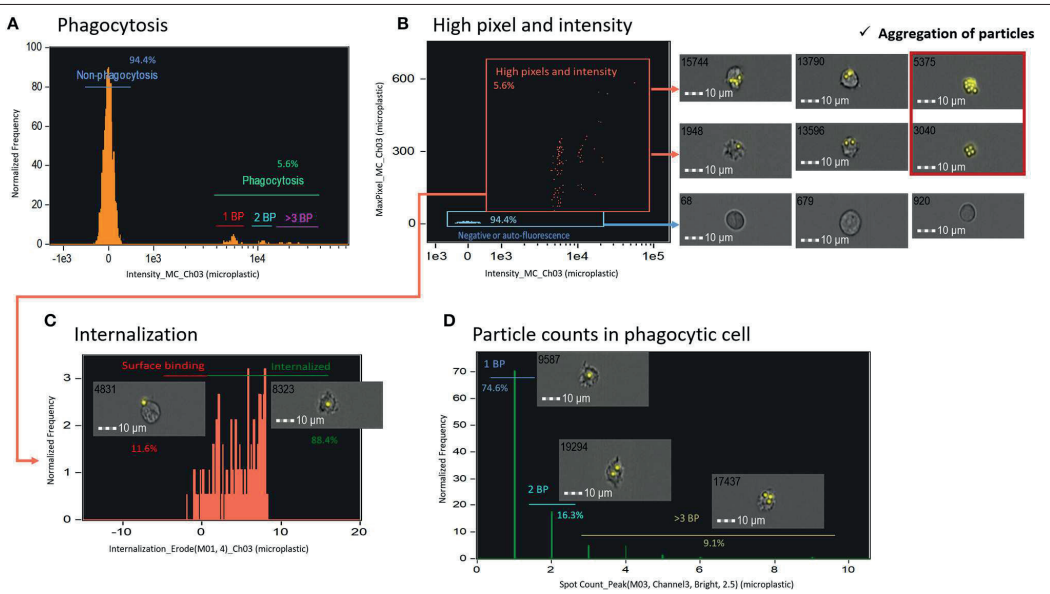

FIGURE 3 | Measurement of phagocytosis using non-degradable fluorescent particles (microplastics). **(A)** A histogram of fluorescent microplastic intensity in live cells. **(B)** To exclude auto-fluorescence and find the best images, images with more pixels and high intensity were gated. Errors may occur in conventional FC due to aggregation of microplastics. **(C)** Internalized cells were gated using internalization score. **(D)** Cells with internalized particles were plotted in a histogram of spot count, which shows the number of particles. All cell images were captured with 40 × objective. Scale bar = 10 μm. BP, bio-particle.

within an image based on their intensity and localization) with the following mask function [Erode (M01, 4)_Ch03]. The ratio indicated the proximity of microplastic to the center of the cell; cells with a score of > 0.3 were considered to have internalized particles and those with a score of < 0.3 were considered to have surface-bound particles (11). Finally, only cells with internalized particles were presented in a histogram of spot count feature, which is an ideal approach to quantify the masked spots in the cell (**Figure 3D**). Overall, IFC can be applied for detecting non-degradable microplastic beads inside the phagocytic cells and quantifying the number of beads. In addition, salmon HK phagocytic cells could recognize microplastics as foreign bodies although we observed only few phagocytosed particles.

Examining Phagocytosis Using Degradable Fluorescent Bio-particles

To determine phagocytosis of degradable bio-particles by salmon HK cells compared with non-degradable microplastics, first, we plotted histograms of fluorescence intensity of degradable bio-particles (**Figure 4A**). In comparison to the histogram of the non-degradable microplastic beads described above (**Figure 3**), it was more difficult to distinguish the number of bio-particles in this histogram. To exclude auto-fluorescence and obtain high-quality images, we adopted a gating strategy based on high pixel (pixel value > 30) and intensity of images (**Figure 4B**). We created two gates, one to include particles with high pixel and high intensity and the other one with negative or auto-fluorescence (histogram in **Figure 4B**). From the histogram, it is clear that overlapping particle intensity (orange) and auto-fluorescence (blue) curves can cause detection errors. Cells that had engulfed the bio-particles were gated using the internalization score as described in the previous section (**Figure 4C**). Finally, only cells with internalized particles are presented in a histogram of particle intensity to understand the number of particles in the phagocytic cells (**Figure 4D**). We found that to quantify the number of degradable particles, particle intensity-based protocol is a better strategy compared to the method employing spot count feature.

Optimizing IFC-Based Method for Phagocytosis Assay

To verify the validity of our IFC-based method, we used degradable fluorescent bio-particles from *E. coli* to assess the effect of incubation temperature on the phagocytic activity and capacity of phagocytic cells from three aquatic animals. The phagocytic ability of HK phagocytic cells from salmon (**Figure 5A**) and tilapia (**Figure 6A**) incubated at 12 and 25°C, respectively, was significantly higher compared to cells incubated at 4°C, but temperature did not significantly affect the phagocytic ability of hemocytes from blue mussel (**Figure 7A**). In contrast, the phagocytic capacity of none of the aquatic species tested

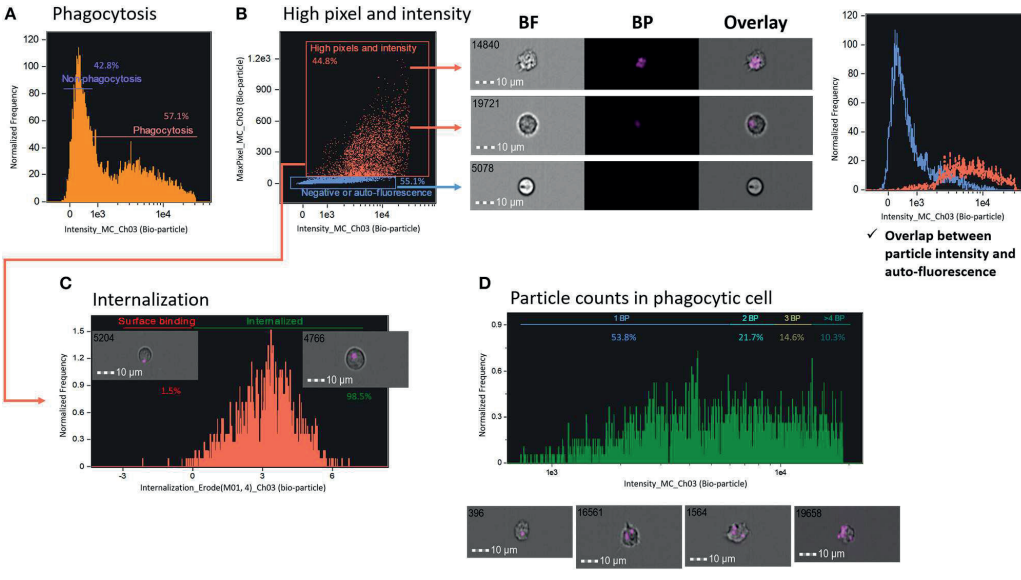

FIGURE 4 | Measurement of phagocytosis using degradable fluorescent particles (pHrodo™). **(A)** Live cells are plotted in a histogram of fluorescent bio-particle intensity. **(B)** To exclude auto-fluorescence and find the best image, images with more pixels and high intensity were gated. The histogram (right panel) points to the detection error caused by overlapping curves of particle intensity (orange) and auto-fluorescence (blue) in conventional flow cytometry. **(C)** Internalized cells were gated using internalization score. **(D)** Cells with internalized particles were plotted in a histogram of bio-particle intensity, which shows the number of particles. All cell images were captured with 40 × objective. Scale bar = 10 µm. BF, brightfield; BP, bio-particle.

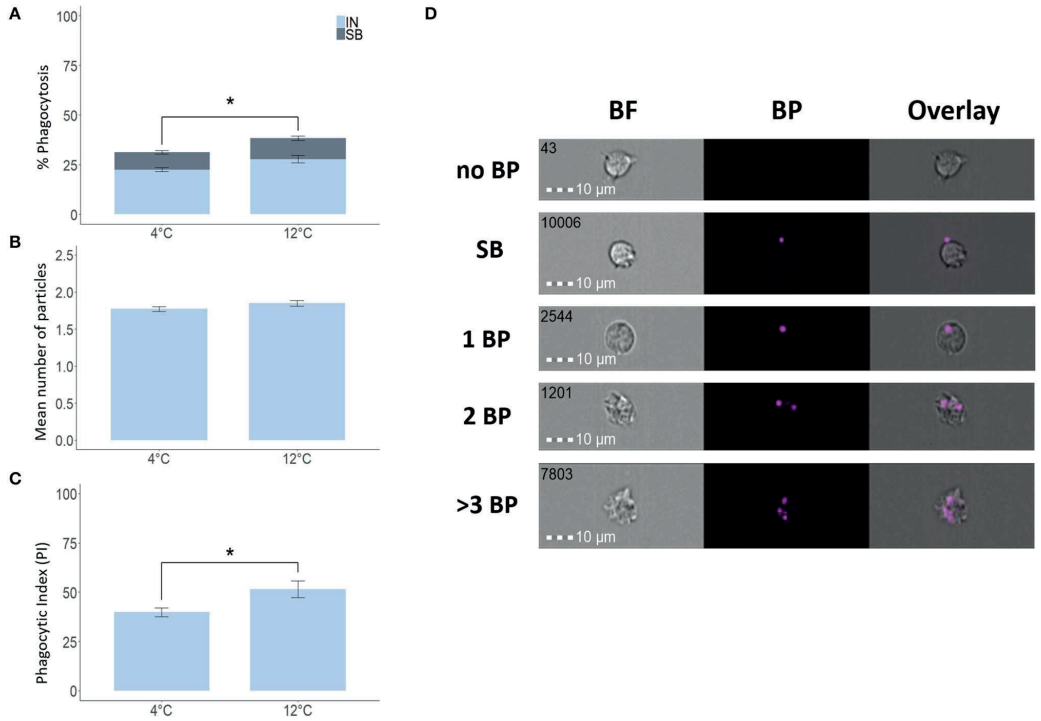

FIGURE 5 | Phagocytosis of Atlantic salmon head kidney macrophages incubated at different temperatures. Percent of phagocytic cells **(A)**, mean number of particles ingested per phagocytic cell **(B)** and phagocytic index, PI **(C)**. Representative cell images that show cells with no BP, SB, and 1BP, 2BP, and > 3BP **(D)**. Statistically significant differences ($p < 0.05$) are indicated using asterisks. Bar plots show the mean ± SD. Sample size = 6. All cell images were captured with 40 × objective. Scale bar = 10 µm. IN, internalization; SB, surface-binding particles; 1 BP, 2 BP, and > 3 BP, 1–3 internalized bio-particles; BF, brightfield.

was significantly affected by temperature (**Figures 5B,D**, **6B,D**, **7B,D**). The phagocytic index of only the salmon cells incubated at 12°C was significantly higher compared to cells incubated at 4°C (**Figure 5C**). This temperature effect could not be detected for the phagocytic index of tilapia HK cells (**Figure 6C**) and blue mussel hemocytes (**Figure 7C**) although the cells were incubated at higher values, i.e., 12 and 25°C, respectively. The optimized method for phagocytosis assay was well-applied to phagocytic cells from three aquatic animals. The results showed that unlike that of phagocytic cells from fishes, phagocytosis of the cells from mussel was not significantly affected by incubation temperature.

DISCUSSION

The major advantage of imaging flow cytometry (IFC) over conventional flow cytometry (FC) is its ability to distinguish between false-positive and false-negative events by considering additional features of the captured cellular images (2). The two systems share the basic principle (19). Although IFC has been widely adopted to study mammalian cell types, it is not yet commonly employed to investigate other organisms, including aquatic animals. There is a paucity of appropriate tools such as cell-specific markers, which hampers the wider adoption of new technologies like IFC. Furthermore, the associated protocols require thorough refining before IFC can be used to study cell types from aquatic animals. For example, as the weak and small fluorescence cannot be detected by the system, we employ masking and features within IDEAS software to accurately select the area of interest during image analysis (20). Our study describes procedures to accurately identify cellular phenotypes and quantify phagocytosis by cells from three very different aquatic animals.

In the present IFC study, we could successfully exclude dead cells and cell aggregates and could identify single leukocytes from Atlantic salmon HK based on bright field (BF) area and SSC intensity. We observed two distinct populations: cells located in the low BF area and low SSC intensity, and cells located in the high BF area and high SSC intensity. Our IFC results are in agreement with conventional FC data on HK leukocytes from salmon (21). A study on goldfish primary kidney macrophages

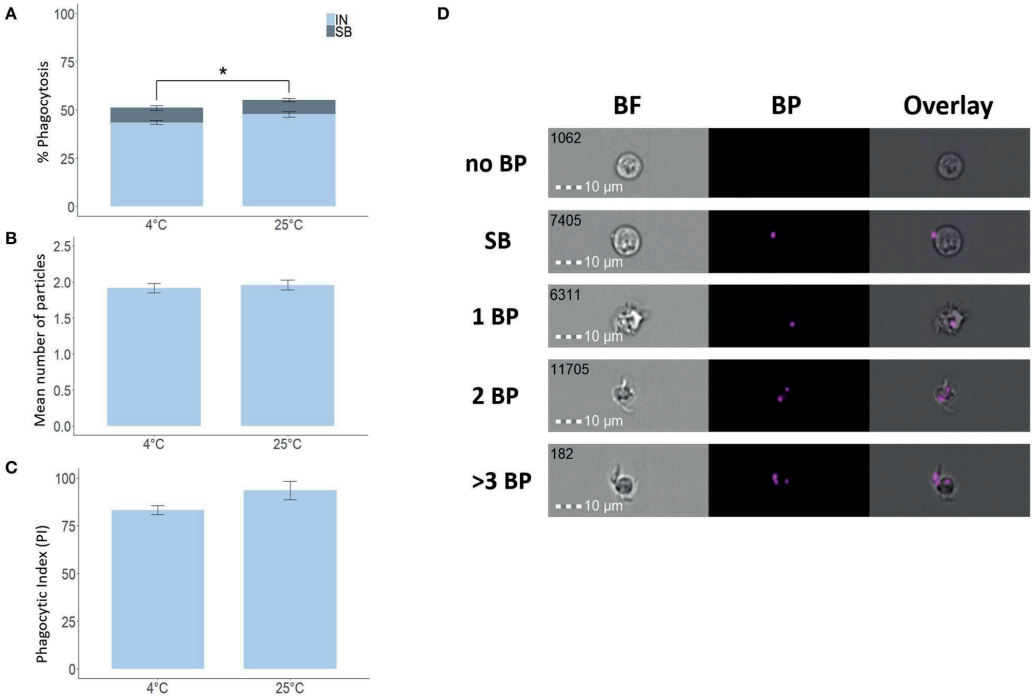

FIGURE 6 | Phagocytosis of Nile tilapia head kidney macrophages incubated at different temperatures. Percent of phagocytic cells **(A)**, mean number of particles ingested per phagocytic cell **(B)** and phagocytic index, PI **(C)**. Representative cell images that show cells with no BP, SB, and 1BP, 2BP, and > 3BP **(D)**. Statistically significant differences ($p < 0.05$) are indicated using asterisks. Bar plots show the mean ± SD. Sample size = 6. All cell images were captured with 40 × objective. Scale bar = 10 µm. IN, internalization; SB, surface-binding particles; 1 BP, 2 BP, and > 3 BP, 1–3 internalized bio-particles; BF, brightfield.

also compared IFC results with those from conventional FC data; both systems were used to identify cell sub-populations. Similar dot plots were generated for both flow cytometry systems, indicating that the replacement of forward scatter (FSC) which measures cell size in conventional FC by BF area in IFC (22) is a reliable approach, independent of fish species.

Interestingly, adherent cells from Atlantic salmon HK (R3, macrophage-like cells) were located in a higher BF area than R2 cells from the same organ (**Figures 1D,E**). The proportion of macrophage-like cells was approximately 45.2%. The macrophage-like cells in the R3 region displayed a similar morphology to that of the adherent TO cells, a cell line originating from salmon HK leukocytes (23). Furthermore, in another study that employed conventional FC, salmon macrophage-like cells were presented in an FSC vs. SSC plot (24). Similar to our gating, the author gated three regions in the plot and assumed that the two higher FSC regions contained macrophage-like cells which was ∼56% out of the total number of cells.

After optimizing the method to distinguish between lymphocytes from monocytes/macrophages, magnetic cell sorting (MACS) was performed to sort target lymphocytes using an IgM-specific antibody. The purity of IgM^+ cells after MACS was 89.8% which is similar to 92% in a salmon study (25). MACS enabled us to ascertain the area of lymphocyte-like cells as defined/interpreted from the BF area vs. SSC intensity plots. The sorted salmon IgM^+ cells were located in the low BF area and low SSC intensity gate, confirming a close area match to that of the lymphocyte-like cells. Similarly, a previous study on trout HK confirmed lymphocyte localization (low FSC and low SSC) using conventional FC, based on $CD4^+$T cell markers (26). In addition, employing conventional FC, percentage of IgM^+ and IgT^+ B cells in salmon HK cells were determined by gating the same area (25). The gate areas in **Figures 1**, **2** confirm the presence of lymphocytes.

After confirming the identity of the B lymphocytes in the low BF area vs. low SSC intensity gate, we explored the phagocytosis of the adherent monocytes/macrophages HK fraction. Phagocytosis is an important initial immune response with final entry of antigens into the phagosomes/lysosomes that stimulates the production of reactive oxygen species (27). Phagocytic activity is influenced by many factors such as cell

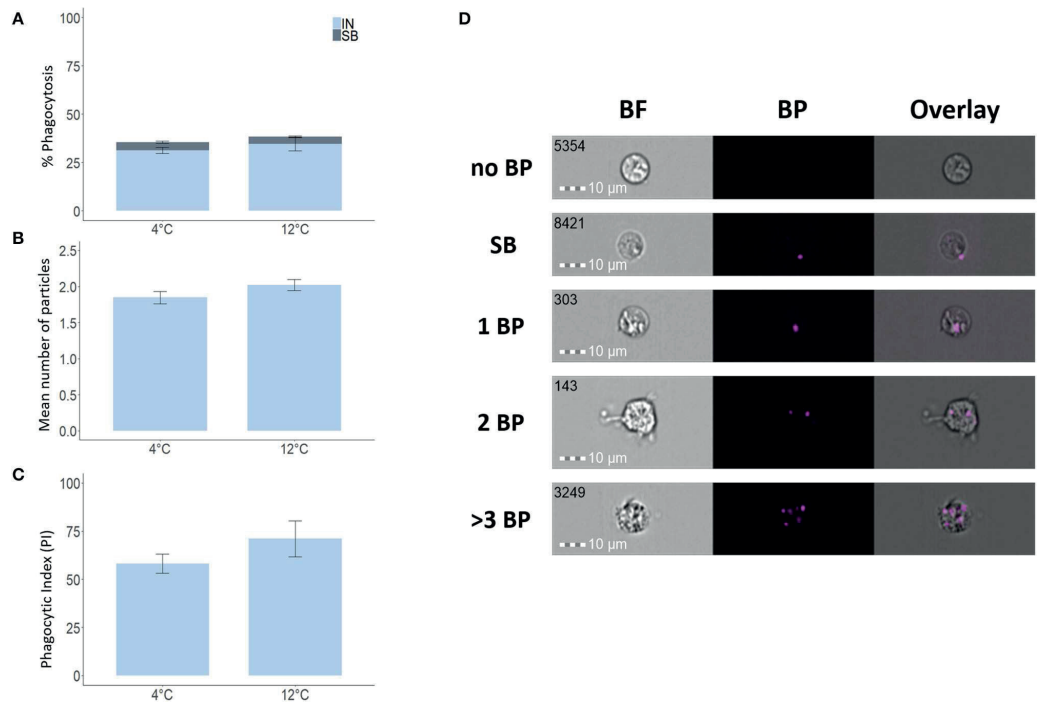

FIGURE 7 | Phagocytosis of blue mussel hemocytes incubated at different temperatures. Percent of phagocytic cells **(A)**, mean of number of particles ingested per phagocytic cell **(B),** and phagocytic index, PI **(C)**. Representative cell images that show cells with no BP, SB, and 1BP, 2BP, and > 3BP **(D)**. Statistically significant differences ($p < 0.05$) are indicated using asterisks. Bar plots show the mean ± SD. Sample size = 6. All cell images were captured with 40 × objective. Scale bar = 10 µm. IN, internalization; SB, surface-binding particles; 1 BP, 2 BP, and > 3 BP, 1–3 internalized bio-particles; BF, brightfield.

maturity, cytokine response, antigen presenting cell activation status (28) and the characteristics of phagocytosed antigens or particles (29). We explored the phagocytic activity of salmon HK cells using IFC, which allowed for not only quantification of the number of cells with internalized particles but also the localization of particles inside the cells. The IFC methods for assessing phagocytosis are complex, and researchers are yet to standardize them for different particle types. In the present study, we tested two different types of particles, non-degradable and degradable particles. This is the first IFC study that reports the use of microplastic as non-degradable particles and bio-particles from *E. coli* as degradable particles. Considering the growing debate on microplastic pollution of the marine ecosystem, studying phagocytosis of microplastics by immune cells from aquatic animals can be of particular interest from an environmental perspective. In our studies, with our sensitive IFC methodology, we could clearly detect microplastic particles engulfed by salmon macrophages, although only few particles were detected inside these cells. We, therefore, assume that these cell types can phagocytose microplastics as (foreign) particles. It should be pointed out that the salmon HK phagocytic cells were not able to uptake more microplastics; the reason could be that the cells can efficiently recognize microbe-derived particles (bio particle from *E. coli*) due to their natural antigenicity and phagocytose it more easily than an "unknown particle" such as the microplastic. Furthermore, the microplastic beads are not coated with any compound recognizable by the phagocytic cells, and they are larger compared to the bioparticles.

Compared to microplastic particles, the bio-particles are known to emit fluorescence within cells. However, this occurs only upon acidification, i.e., they emit fluorescence of a particular wavelength, depending on the pH level that the particle encounters. Hence, we suggest the use of fluorescent intensity feature rather than spot count feature to accurately assess the counts of degradable particles in phagocytes. Although a different feature was used to count the number of particles per phagocytic cell, a publication (12) has reported an IFC method for counting internalized fluorescent-labeled bacteria. The author succeeded in distinguishing between cells with high bright detail similarity score and those with low bright detail similarity score; the former one had internalized particles while other cells had external particles. Although the method of Smirnov et al. (12) gives

information on the overall degree of phagocytosis in phagocytic cells, it cannot accurately count the internalized particles. Thus, the bright detail similarity score and fluorescent intensity feature are effective in detecting and counting (as in this report) the mean number of internalized particles per phagocytic cell.

Although we did not perform a direct comparison between IFC and conventional FC, from our results we understand that false events such as auto-fluorescence and aggregated particles can be misinterpreted in the case of conventional FC. Pixel and intensity features were adjusted carefully in the present study to exclude the false-positive events. Caution should be exercised when gating phagocytic cells using these features because in IFC, cell size is measured based on pixels, and the sensitivity of the measurement is dependent on the cell size (19). Thus, in order to include the region of interest for analysis, the mask that identifies the intracellular compartment has to be adjusted for different types of cells and particles.

After standardizing the protocols for monocytes/macrophage phagocytosis, we optimized the methods for measuring phagocytosis, using degradable bio-particles, by cells from three very different aquatic animals—two fishes, Atlantic salmon and Nile tilapia and a mollusc, blue mussel—to evaluate the effect of incubation temperature on their phagocytic abilities and capacities. Our results indicated that phagocytosis of cells from the fishes can be affected by the incubation temperature. Although not directly comparable, phagocytosis of human leukocytes was reduced at higher and lower temperature compared to the normal host temperature range (30). Interestingly, the phagocytosis by hemocytes from blue mussel, a eurythermal species that can tolerate a broad temperature range from −1 to 20°C (31, 32), was not affected by incubation temperature.

In summary, IFC was used to study phagocytosis in fish and mussel cells. We were able to identify cell populations and determine the phagocytosis of different kinds of particles by quantifying the number of internalized particles and detecting the localization of particles in the phagocytes. This study provides important information about how IFC can be used in the field of fish immunology and ecotoxicology. Furthermore, the procedures described in this report may have wider application in aquatic sciences, to unravel the effects of microplastic-ingestion by living organisms in the oceans.

DATA AVAILABILITY STATEMENT

All datasets generated for this study are included in the article/supplementary material.

ETHICS STATEMENT

The animal study was reviewed and approved by National Animal Research Authority in Norway (Mattilsynet).

AUTHOR CONTRIBUTIONS

YP and VK conceived and designed the study. YP and IA-G performed the experiments. YP analyzed the data and wrote the first draft of the manuscript while IA-G wrote a section of it. ST, GW and DB provided suggestions to improve the IFC protocols. YP, IA-G, ST, GW, DB, PO, and VK read, revised, and approved the manuscript for submission.

FUNDING

This study was supported by the INFISH project (272004) funded by Regional Forskningsfond Nord-Norge (RFF Nord-Norge). YP was financially supported by Korean Government Scholarship—National Institute for International Education, South Korea.

ACKNOWLEDGMENTS

The support of the staff at the Research Station, Nord University, Norway is acknowledged. YP is grateful to the members of the Cell Biology and Immunology Group, Wageningen University, the Netherlands for teaching him the principles of flow cytometry. Bisa Saraswathy is thanked for her support in data analysis, helpful discussion, and manuscript preparation.

REFERENCES

1. Görgens A, Bremer M, Ferrer-Tur R, Murke F, Tertel T, Horn PA, et al. Optimisation of imaging flow cytometry for the analysis of single extracellular vesicles by using fluorescence-tagged vesicles as biological reference material. *J Extracell Vesicles.* (2019) 8:1587567. doi: 10.1080/20013078.2019.1587567
2. Barteneva NS, Fasler-Kan E, Vorobjev IA. Imaging flow cytometry: coping with heterogeneity in biological systems. *J Histochem Cytochem.* (2012) 60:723–33. doi: 10.1369/0022155412453052
3. Hulspas R, O'gorman MR, Wood BL, Gratama JW, Sutherland DR. Considerations for the control of background fluorescence in clinical flow cytometry. *Cytom B Clin Cytom.* (2009) 76:355–64. doi: 10.1002/cyto.b.20485
4. Van Beers EJ, Samsel L, Mendelsohn L, Saiyed R, Fertrin KY, Brantner CA, et al. Imaging flow cytometry for automated detection of hypoxia-induced erythrocyte shape change in sickle cell disease. *Am J Hematol.* (2014) 89:598–603. doi: 10.1002/ajh.23699
5. Ofir-Birin Y, Abou Karam P, Rudik A, Giladi T, Porat Z, Regev-Rudzki N. Monitoring extracellular vesicle cargo active uptake by imaging flow cytometry. *Front Immunol.* (2018) 9:1011. doi: 10.3389/fimmu.2018.01011
6. Piancone F, Saresella M, Marventano I, La Rosa F, Santangelo MA, Caputo D, et al. Monosodium urate crystals activate the inflammasome in primary progressive multiple sclerosis. *Front Immunol.* (2018) 9:983. doi: 10.3389/fimmu.2018.00983
7. Jenner D, Ducker C, Clark G, Prior J, Rowland CA. Using multispectral imaging flow cytometry to assess an *in vitro* intracellular *Burkholderia thailandensis* infection model. *Cytom A.* (2016) 89:328–37. doi: 10.1002/cyto.a.22809
8. Rieger AM, Konowalchuk JD, Grayfer L, Katzenback BA, Havixbeck JJ, Kiemele MD, et al. Fish and mammalian phagocytes differentially regulate pro-inflammatory and homeostatic responses *in vivo*. *PLoS ONE.* (2012) 7:e47070. doi: 10.1371/journal.pone.0047070
9. Rességuier J, Delaune E, Coolen A-L, Levraud J-P, Boudinot P, Le Guellec D, et al. Specific and efficient uptake of surfactant-free poly (lactic acid)

nanovaccine vehicles by mucosal dendritic cells in adult zebrafish after bath immersion. *Front Immunol.* (2017) 8:190. doi: 10.3389/fimmu.2017.00190
10. Parra D, Rieger AM, Li J, Zhang YA, Randall LM, Hunter CA, et al. Pivotal advance: peritoneal cavity B-1 B cells have phagocytic and microbicidal capacities and present phagocytosed antigen to CD4+ T cells. *J Leukocyte Biol.* (2012) 91:525–36. doi: 10.1189/jlb.0711372
11. Phanse Y, Ramer-Tait AE, Friend SL, Carrillo-Conde B, Lueth P, Oster CJ, et al. Analyzing cellular internalization of nanoparticles and bacteria by multi-spectral imaging flow cytometry. *J Visual Exp.* (2012) 64:e3884. doi: 10.3791/3884
12. Smirnov A, Solga MD, Lannigan J, Criss AK. An improved method for differentiating cell-bound from internalized particles by imaging flow cytometry. *J Immunol Methods.* (2015) 423:60–9. doi: 10.1016/j.jim.2015.04.028
13. Paulsen SM, Engstad RE, Robertsen B. Enhanced lysozyme production in Atlantic salmon (*Salmo salar* L.) macrophages treated with yeast β-glucan and bacterial lipopolysaccharide. *Fish Shellfish Immunol.* (2001) 11:23–37. doi: 10.1006/fsim.2000.0291
14. Pirarat N, Pinpimai K, Endo M, Katagiri T, Ponpornpisit A, Chansue N, et al. Modulation of intestinal morphology and immunity in Nile tilapia (*Oreochromis niloticus*) by *Lactobacillus rhamnosus* GG. *Res Vet Sci.* (2011) 91:e92–7. doi: 10.1016/j.rvsc.2011.02.014
15. Wen C-M. Development and characterization of a cell line from tilapia head kidney with melanomacrophage characteristics. *Fish Shellfish Immunol.* (2016) 49:442–9. doi: 10.1016/j.fsi.2016.01.013
16. Antoun SW. *Mussel (Mytilus edulis) hemocytes for in vitro testing* (dissertation/master's thesis). University of Oslo, Oslo, Norway (2011).
17. Barbuddhe SB, Malik SVS, Gupta LK. Effect of *in vitro* monocyte activation by *Listeria Monocytogenes* antigens on phagocytosis and production of reactive oxygen and nitrogen radicals in bovines. *Vet Immunol Immunopathol.* (1998) 64:149–59. doi: 10.1016/S0165-2427(98)00129-9
18. Fuentes A-L, Millis L, Vapenik J, Sigola L. Lipopolysaccharide-mediated enhancement of zymosan phagocytosis by RAW 264.7 macrophages is independent of opsonins, laminarin, mannan, and complement receptor 3. *J Surg Res.* (2014) 189:304–12. doi: 10.1016/j.jss.2014.03.024
19. Basiji DA. *Principles of Amnis Imaging Flow Cytometry in Imaging Flow Cytometry*. Berlin: Springer (2016) 13–21. doi: 10.1007/978-1-4939-3302-0_2
20. Grimwade LF, Fuller KA, Erber WN. Applications of imaging flow cytometry in the diagnostic assessment of acute leukaemia. *Methods.* (2017) 112:39–45. doi: 10.1016/j.ymeth.2016.06.023
21. Kalgraff CA, Wergeland HI, Pettersen EF. Flow cytometry assays of respiratory burst in Atlantic salmon (*Salmo salar* L.) and in Atlantic cod (*Gadus morhua* L.) leucocytes. *Fish Shellfish Immunol.* (2011)31:381–8. doi: 10.1016/j.fsi.2011.05.028
22. Rieger AM, Hall BE, Barreda DR. Macrophage activation differentially modulates particle binding, phagocytosis and downstream antimicrobial mechanisms. *Dev Compar Immunol.* (2010) 34:1144–59. doi: 10.1016/j.dci.2010.06.006
23. Pettersen EF, Ingerslev H-C, Stavang V, Egenberg M, Wergeland HI. A highly phagocytic cell line TO from Atlantic salmon is CD83 positive and M-CSFR negative, indicating a dendritic-like cell type. *Fish Shellfish Immunol.* (2008) 25:809–19. doi: 10.1016/j.fsi.2008.08.014
24. Ulvestad JS. *Studies on the stimulation of Atlantic salmon macrophage-like cells with emphasis on respiratory burst* (dissertation/master's thesis). UiT The Arctic University of Norway, Tromsø, Norway (2017).
25. Jenberie S, Thim HL, Sunyer JO, Skjødt K, Jensen I, Jørgensen JB. Profiling Atlantic salmon B cell populations: CpG-mediated TLR-ligation enhances IgM secretion and modulates immune gene expression. *Sci Rep.* (2018) 8:3565. doi: 10.1038/s41598-018-21895-9
26. Maisey K, Montero R, Corripio-Miyar Y, Toro-Ascuy D, Valenzuela B, Reyes-Cerpa S, et al. Isolation and characterization of salmonid CD4+ T cells. *J Immunol.* (2016) 196:4150–63. doi: 10.4049/jimmunol.1500439
27. Gartlan KH, Krashias G, Wegmann F, Hillson WR, Scherer EM, Greenberg PD, et al. Sterile inflammation induced by Carbopol elicits robust adaptive immune responses in the absence of pathogen-associated molecular patterns. *Vaccine.* (2016) 34:2188–96. doi: 10.1016/j.vaccine.2016.03.025
28. Gordon S. Phagocytosis: an immunobiologic process. *Immunity.* (2016) 44:463–75. doi: 10.1016/j.immuni.2016.02.026
29. Underhill DM, Goodridge HS. Information processing during phagocytosis. *Nat Rev Immunol.* (2012) 12:492. doi: 10.1038/nri3244
30. Peterson P, Verhoef J, Quie P. Influence of temperature on opsonization and phagocytosis of staphylococci. *Infect Immun.* (1977) 15:175–9. doi: 10.1128/IAI.15.1.175-179.1977
31. Hiscock K, Tyler-Walters H. Assessing the sensitivity of seabed species and biotopes–the Marine Life Information Network (MarLIN). *Hydrobiologia.* (2006) 555:309–20. doi: 10.1007/s10750-005-1127-z
32. Thyrring J, Rysgaard S, Blicher ME, Sejr MK. Metabolic cold adaptation and aerobic performance of blue mussels (*Mytilus edulis*) along a temperature gradient into the high Arctic region. *Marine Biol.* (2015) 162:235–43. doi: 10.1007/s00227-014-2575-7

Conflict of Interest: ST is an employee of Luminex B.V., which is a subsidiary of Luminex Corporation. Luminex Corporation is the manufacturer of the ImageStream$^{®X}$ Mk II Imaging Flow Cytometer.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Paper II

frontiers in Environmental Science

ORIGINAL RESEARCH
published: 20 November 2020
doi: 10.3389/fenvs.2020.560206

Fluorescent Microplastic Uptake by Immune Cells of Atlantic Salmon (*Salmo salar* L.)

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The ubiquitous presence of microplastics and their marine ecotoxicity are major public concerns. Microplastics are ingested accidentally by the marine fauna or are taken up indirectly through the food chain. These particles can accumulate in cells and tissues and affect the normal biological functions of organisms, including their defense mechanisms. There is limited information available about the response of immune cells to microplastics; the degree of uptake by the cells, the response of different organs or the impact of environmental concentrations of microplastic are matters that remain unclear. Moreover, very little is known about the toxicity of different polymer types. This study aimed to shed light on the physical impact of small microplastics (1–5 μm) on cells from Atlantic salmon. Immune cells from intestine, blood, and head kidney were exposed to green fluorescent polyethylene microplastic (PE-MP), yellow fluorescent polystyrene microplastic (PS-MP) and both. High (50 mg/L), medium (5 mg/L), and low (0.05 mg/L) concentrations were tested for 1, 24, 48, and 72 h to study cell mortality and microplastic uptake. Quantitative data of microplastic uptake by fish immune cells were obtained for the first time by imaging flow cytometry. Salmon immune cells showed a relatively low ability to phagocytose microplastics. Less than 6% of the cells ingested the particles after 48 h of exposure to high concentrations. Cells also phagocytosed microplastics at low concentrations although at low rates (<0.1%). PE-MPs was phagocytosed by higher percentage of cells compared to PS-MPs and the former bioaccumulated in time while the latter decreased over time. However, each cell generally phagocytosed more PS-MPs particles than PE-MPs. Cells from different tissues showed different responses to the microplastic polymers. In conclusion, this study shows that immune cells of Atlantic salmon can phagocytose microplastics, and the impact is dependent on the microplastic type. PE-MPs, the most abundant polymer in the oceans and a widely used plastic in salmon aquaculture, was more easily taken up than PS-MPs. Furthermore, the study demonstrates how imaging flow cytometry can be applied in microplastics research.

Keywords: microplastic (MP), ecotoxicology, phagocytosis, imaging flow cytometry (IFC), Atlantic salmon (*Salmo salar* L.), polystyrene (PS), polyethylene (PE), immune cells

OPEN ACCESS

Edited by:
Cristina Panti,
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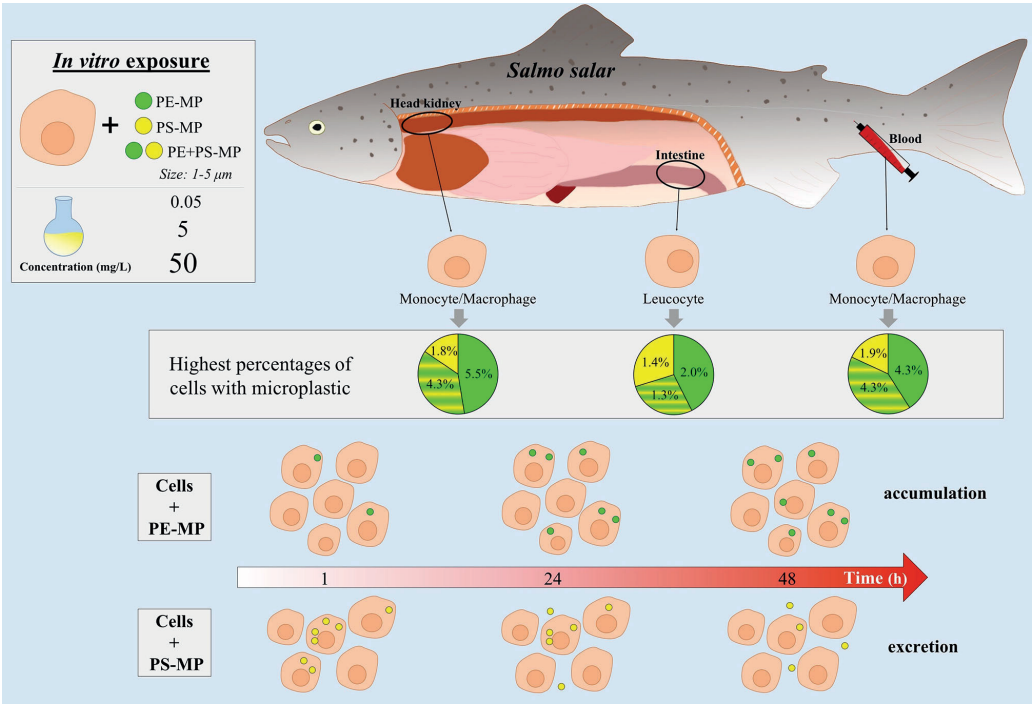
Specialty section:
This article was submitted to Toxicology, Pollution and the Environment, a section of the journal Frontiers in Environmental Science

Received: *08 May 2020*
Accepted: *27 October 2020*
Published: *20 November 2020*

Citation:
Abihssira-García IS, Park Y, Kiron V and Olsvik PA (2020) Fluorescent Microplastic Uptake by Immune Cells of Atlantic Salmon (Salmo salar L.). Front. Environ. Sci. 8:560206. doi: 10.3389/fenvs.2020.560206



GRAPHICAL ABSTRACT |

INTRODUCTION

The increasing presence of microplastics (MPs) in the marine environment is of global concern. MPs are defined as plastic particles smaller than 5 mm (Arthur et al., 2009), although it has been recently proposed to redefine the limits of MPs to 1 to <1,000 μm to fit the SI nomenclature and for a better consensus among scientists (Hartmann et al., 2019). MPs in the marine environment mainly come from the degradation and fragmentation of different plastic items (GESAMP, 2015), resulting in rough and irregular shaped particles (Choi et al., 2018). Plastic debris mainly consist of fossil fuel-derived synthetic polymers, which can be classified into thermoplastic and thermoset (UNEP, 2016). Thermoplastic polymers are the most abundant plastic types found in the marine environment; a study conducted in 2014 found that almost half of the MPs collected in the North Atlantic ocean was polyethylene (PE) and polypropylene (PP) (Enders et al., 2015). Similarly, a study carried out in 2015 found that PE accounted for 73% of the small MPs (20–999 μm) collected in the North Atlantic Subtropical Gyre, followed by PP, PVC, and PS (Ter Halle et al., 2017). Other synthetic polymers such as polyethylene terephthalate (PET) and polyamides (PA) are also widely detected in the marine environment (Suaria et al., 2016; Erni-Cassola et al., 2019).

MPs are ingested by a wide range of marine organisms (Lusher et al., 2017b; Alimba and Faggio, 2019). Particles can accumulate in the digestive tract of animals and affect, among other parameters, their behavior, growth and survival (Von Moos et al., 2012; Choi et al., 2018; Foley et al., 2018; Assas et al., 2020). For example, a 7-days exposure of zebrafish (*Danio rerio*) to 5 μm PS-MPs resulted in their accumulation in the gills, liver, and gut of the fish, leading to increased oxidative stress; underlining the potential toxic effect of the MP (Lu et al., 2016). A few studies have documented that fine MPs can adhere to tissues [for example fish skin] and translocate across living cells into the circulatory system, dispersing the particles to other organs (Wang et al., 2020) and impacting the health of the cells and the immune system of the organisms (Wright and Kelly, 2017). Small MPs (<3 μm) have been found in both the digestive tract and muscles of painted comber (*Serranus scriba*) fish collected in the coast of Tunisia (Zitouni et al., 2020). MPs can affect several immune parameters such as the phagocytic activity, respiratory burst and the expression of immune-related genes in fishes (Espinosa et al., 2017; Choi et al., 2018). Therefore, it is important to study

the impact of MPs on fish immune cells. An important aspect to consider when studying the toxicity of microplastics is the polymer type. The type of polymers ingested by an organism depends on its ecological niche. Pelagic fish tend to ingest low-density polymers since they normally float in seawater, while demersal fish preferably ingest polymers with higher densities than seawater as such particles sink and are mainly present in deep waters (Bråte et al., 2017). A few studies have reported that different MP polymers induce different toxicities in fish. A study on fathead minnow (*Pimephales promelas*) showed that both PS and PC nanoparticle aggregates (41 nm−1,710 μm) induced degranulation of primary granules of neutrophils, but only PC nanoparticles affected the oxidative burst of the cells (Greven et al., 2016). PVC-MP and PE-MP (40–150 μm) induced different responses in gilthead seabream (*Sparus aurata*) and European sea bass (*Dicentrarchus labrax*); while PVC-MPs decreased the phagocytic capacity of leucocytes, high concentrations (100 mg/ml) of PE-MPs significantly increased oxidative stress (Espinosa et al., 2018). In zebrafish, 10 days of exposure to 10 mg/L of PP-MP decreased the survival rate of the fish by 27%, while exposure to the same concentration of PA-MP, PE-MP, PVC-MP, and PS-MP (70 μm) did not affect survival (Lei et al., 2018). Recently, a study on Japanese medaka (*Oryzias latipes*) showed that environmental concentrations of a mixture of virgin PE-MP and PP-MP (∼400 μm in size) affected development and behavior of the larvae. After 30 days of exposure to the plastic mixture, fish had a higher head/body length ratio than the control group, and their swimming speed was also affected (Pannetier et al., 2020). These findings highlight the need to study polymer-specific impacts on fish immunity.

Even though publications related to MP accumulation in fish and invertebrates have increased considerably since 2010 (Lusher et al., 2017b), research into how MPs affect living organisms is still in its infancy, and many questions remain unanswered. For instance, the effects of various types of polymers, the degree of uptake of MP by immune cells, the response of different tissues and organs to MP, and the response of the organisms to environmentally relevant concentrations of MPs, have to be delineated through further research.

In this work, we studied the impact of two MPs, PE and PS, on Atlantic salmon (*Salmo salar* L.), the commercially most important farmed marine fish species (FAO, 2018). A recent study revealed that PE accounted for ∼40% of the MPs ingested by five fish species from the North and Baltic Sea (Rummel et al., 2016). Moreover, marine litter in Arctic, North Atlantic, North Sea, Skagerrak, and Baltic Sea is dominated by PS plastic (Strand et al., 2015). In addition, the weathering of plastic equipment used in aquaculture, such as cage floats or feed pipes that are mainly made of PE, is littering the marine environment (Lusher et al., 2017a). Hence, it is important to understand how Atlantic salmon, a farmed species that is produced mainly in open sea cages, is affected by PE and PS. We examined the physical impact of MPs in terms of uptake by cells. For this purpose, immune cells from three tissues, distal intestine (DI), blood, and head kidney (HK), were exposed to two types of polymers, PE-MP and PS-MP; either to each polymer singly or to a combination of the two, at three concentrations (50, 5, and 0.05 mg/L) for 1, 24, 48, and 72 h. The DI is the main entry point of MPs in the body, and blood cells can act as vehicles to carry MPs to other organs (Ding et al., 2018). The HK is one of the main organs where the hematopoiesis occurs and it is considered as a lymphoid organ, where immune cells are produced (Zapata et al., 2006; Whyte, 2007). After the exposure of leucocytes to the MPs, the mortality of the salmon immune cells as well as their phagocytic ability and capacity to ingest MP were determined by employing imaging flow cytometry. The present study sheds light on (1) the quantitative uptake of MPs by fish immune cells, (2) the response of immune cells exposed to two different polymers, (3) the effect of relatively low MP concentrations on immune cells, and (4) the organ-specific response of immune cells to MP exposure.

MATERIALS AND METHODS

Microplastics and Reagents for Cell Studies

PS (2.1 ± 0.12 μm) and PE (1–5 μm) microspheres, were acquired from Magsphere Inc. (California, USA) and Cospheric (California, USA), respectively. PS-MPs had a more uniform sphericity than PE-MPs. The agglomeration rate of PS particles was 2.04 ± 2.12%, and of PE particles, 3.22 ± 1.32% (**Supplementary File 1**). In order to distinguish the two polymers, we obtained PS-MPs and PE-MPs that emit yellow and green fluorescence, respectively. For the exposure studies with each polymer, stock solutions (2.5 g/L, 250 mg/L, and 2.5 mg/L) were prepared prior to the experiments.

Leibovitz's L-15 Medium (L-15) (Sigma, Oslo, Norway), employed for cell isolation, was adjusted to a pH of 7.3 and osmolarity of 380 mOsm by adding a solution consisting of 5% (v/v) 0.41 M NaCl, 0.33 M $NaHCO_3$, and 0.66 (w/v) D-glucose, and supplemented with 100 μg/mL gentamicin sulfate (Sigma), 2 mM L-glutamine (Sigma), 15 mM HEPES (Sigma), and 50 U/mL penicillin, 50 μg/mL streptomycin (Sigma). An enriched L-15 medium supplemented with 2% fetal bovine serum and 10 U/mL heparin (L-15+) was used to isolate and culture the cells from DI, blood and HK tissues. Percoll solutions (Sigma) adjusted to 380 mOsm by adding NaCl were also prepared for the cell isolation process. DTT (0.145 mg/mL dithiothreitol + 0.37 mg/mL EDTA in Ca^{2+} & Mg^{2+} free HBSS, Sigma), DNAse I (0.05 mg/mL; Sigma) and digestive (0.37 mg collagenase IV/mL washing medium, Thermo Fisher Scientific, Oslo, Norway) solutions were employed to extract cells from the DI.

Leucocyte Harvesting From Atlantic Salmon

Atlantic salmon smolts (60 g) were procured from Sundsfjord Smolt, Nygårdsjøen, Norway and transferred to the Research Station of Nord University, Bodø, Norway. The fish were maintained in a seawater flow-through system (7–8°C) and fed on commercial feeds (Skretting AS, Stavanger, Norway) for several months. Fish in the size range 500–800 g were then employed for the study. The selected fish were starved for 24 h and euthanized with an overdose of MS-222 (Tricaine methane

sulphonate; Argent Chemical Laboratories, Redmond, USA; 160 mg/L) prior to cell extraction.

Immune cells from DI, blood and HK tissues were extracted as described by Attaya et al. (2018), Haugland et al. (2012), and Park et al. (2020), respectively, with some minor modifications. Briefly, 2 mL of blood from each individual was withdrawn with a heparinized syringe and mixed in 6 mL of L-15+. HK was dissected and placed in 4 mL of L-15+, and the cells were harvested by passing the tissue through a 40 μm cell strainer and washing twice with L-15+ by centrifugation (500 × g, 5 min, 4°C). The monocytes/macrophages from both the blood and the HK cell suspensions were then separated using Percoll 34/51% (by centrifugation; 500 × g, 30 min, 4°C) and then washed twice with L-15 by centrifugation (500 × g, 5 min, 4°C). The resulting cell layer was carefully collected and transferred to a petri dish for a 2-day incubation at 12°C to allow the macrophage-like cells to adhere. Thereafter, the supernatant was removed and the adherent cells on the petri dish were detached with ice-cold PBS supplemented with 5 mM EDTA. The collected cells were centrifuged (500 × g, 5 min, 4°C) and employed for the exposure experiment. As for the DI tissue, it was dissected out, washed with 1x PBS, and then incubated in 6 mL of DTT solution for 20 min at RT in order to remove as much mucus as possible. The tissue was washed with L-15+ supplemented with DNAse I to avoid cell clumping and remove excess DTT. The tissue fragment was then transferred into 6 mL of the digestive solution and placed on a shaking incubator (200 rpm) for 60 min at RT. The tissue and supernatant were then harvested by passing through a 100 μm cell strainer and the resulting cell suspension was washed twice with L-15+ by centrifugation (500 × g, 5 min, 4°C) and layered on a Percoll solution of 25/75%. After centrifugation (500 × g, 30 min, 4°C), the leucocyte-rich Percoll intermediate layer was collected, washed twice with L-15 by centrifugation (500 × g, 5 min, 4°C) and later used in the exposure experiment. For the exposure study, cells from 36 individuals were employed to prepare the 6 replicates of the experiment, each of which consisted of a random pool of cells from six fish.

Exposure Experiment

To understand the polymer-specific response of salmon immune cells, they were exposed to either PE-MP or PS-MP or a mixture of both (PE+PS-MP). The treatment employing a combination of PE-MP and PS-MP was selected to ascertain the synergetic or antagonistic effect of the two polymers. Three different concentrations of the polymer types were tested: high (50 mg/L), medium (5 mg/L), and low (0.05 mg/L) concentration. The low concentration was selected based on the highest concentration of MP reported for the North Pacific Gyre (California Current Ecosystem LTER and Goldstein, 2017). However, this concentration does not reflect the environmental concentration of the microplastic size used in this experiment since mesh size used for the sampling was 333 μm. The medium and high concentrations were higher than the concentrations found in the environment, but still lower than what is being used in several MP exposure experiments. A negative control (C), where cells were not exposed to MP, was also set up to ascertain the viability of the cells. DI (0.2×10^6 cells/100 μL), blood (0.5×10^6 cells/100 μL), and HK (0.5×10^6 cells/100 μL) cells were incubated in L-15+ along with the MPs at the different concentrations mentioned above, and the tubes with the cells were maintained at 12°C on a rotator (Rotator PTR-60, Grant-bio, Cambridge, IK) for 1, 24, 48, or 72 h. Six replicates per treatment were employed for the study.

After the exposure, the cells were analyzed by ImageStream$^{®X}$ Mk II Imaging Flow Cytometer (Luminex Corporation, Austin, TX, USA) as described by Park et al. (2020). Briefly, cells were washed by centrifugation (500 × g, 5 min, 4°C), resuspended in 50 μL of 1x PBS, and 1 μL of propidium iodide (PI, 1 mg/mL, Sigma) was added prior to loading the sample in the imaging flow cytometer to assess the mortality. A total of 30,000 images per sample were acquired with the 488 nm argon-ion laser set up at 0.15 mW to detect MP particles and dead cells. Cell mortality, phagocytic ability and phagocytic capacity were analyzed using IDEAS 6.1.822.0 software (Luminex) (Park et al., 2020). Cell mortality was determined as the percentage of cells with red fluorescence (stained by propidium iodide) among the total cells. The phagocytosis assay, consisting of phagocytic ability and phagocytic capacity, was used to study the uptake and bioaccumulation of MPs in the immune cells. The phagocytic ability was defined as the percentage of cells with MP particles inside (i.e., cells with green fluorescence, yellow fluorescence, or both). The phagocytic capacity was defined as the number of MP particles ingested by each phagocytic cell, which were detected by the function "spot count" of the IDEAS software. The phagocytic capacity was calculated and normalized using the following equation:

$$Phagocytic\ capacity = \frac{\sum_{i=1}^{7} \mathrm{i} \cdot \mathrm{Ci}}{\mathrm{n}} \cdot 100$$

Where *Ci* is the number of phagocytic cells with *i* phagocytosed particles and *n* the total number of phagocytic cells. Cells with surface binding particles were counted as phagocytic cells and included in the phagocytic ability and capacity data analysis. Surface binding is considered an early stage of the phagocytosis since it triggers the physical exploration of the particle by the extension of pseudopods by the cells, the intracellular signaling and eventually the internalization of the particle (Underhill and Goodridge, 2012).

Statistical Analysis

Statistical analyses were done with the software RStudio 1.1.463. Poisson regression models were fitted to the data on cell mortality, phagocytic ability and phagocytic capacity. This approach helped us to understand the relationship between the response variable and the predictor variables. Data from each tissue were analyzed separately and statistical differences were considered significant at $p < 0.05$.

For the cell mortality ($n = 6$), two factor regressions were carried out, considering *Time* (1, 24, 48, and 72 h) and *Treatment* (C, PE-0.05, PE-5, PE-50, PS-0.05, PS-5, PS-50, PE+PS-0.05, PE+PS-5, and PE+PS-50) as the predictor variables. The factors *Polymer type* and *Concentration* were grouped into *Treatment* to avoid incorrect comparisons. For both the phagocytic ability (*n*

= 6) and phagocytic capacity ($n = 6$), three factor regressions were carried out, considering *Time* (1, 24, and 48 h; 72 h was excluded), *Polymer type* (PE-MP, PS-MP, and PE+PS-MP) and *Concentration* (50, 5, and 0.05 mg/L) as the predictor variables. For the DI data, the *Concentration* 0.05 mg/L was excluded from both phagocytic ability and phagocytic capacity analyses due to the fewer number of samples with phagocytic cells.

For all three response variables and for each tissue, several Poisson regression models were fitted to detect the differences of interest. For this purpose, one specific level of each factor was fixed as first line in each model, with which the comparisons were made.

RESULTS

Cell Mortality

Cell mortality was assessed to examine the response of leucocytes to MPs, in terms of time, polymer type and MP concentration. MP type or concentration was not found to have a significant effect on cell mortality. On the other hand, time affected the cell mortality significantly, based on the statistical analyses described below (**Figure 1**; **Supplementary File 2**, **Supplementary Tables 1–3**).

The mortality of DI cells increased significantly at 24 and 48 h compared to 1 h ($p < 0.05$) in 90% of the cases, while only 40% increased at 72 h compared to 1 h ($p < 0.01$). On the other hand, mortality was higher at 72 h for blood and HK cells. In the case of blood, mortality at 72 h was significantly higher in almost 50% of the cases ($p < 0.05$), and 24 h was the timepoint with the lowest mortality. For the HK cells, the mortality increased significantly at 72 h compared to 1 and 24 h ($p < 0.05$) in 95% of the cases, and at 48 h the mortality was significantly higher than 1 h ($p < 0.05$) in 40% of the cases. Yet the mortality at this timepoint was still lower than at 72 h.

Considering the general trend in mortality, the 72 h timepoint was not considered for the subsequent studies.

Microplastic Uptake

Immune cells from all the three tissues (DI, blood, and HK) of Atlantic salmon phagocytosed PE-MPs and PS-MPs of 1–5 μm in size (**Figures 2–5**). Phagocytosis was documented at different concentrations including the lower MP concentration (0.05 mg/L) (**Figure 3**). Single cells from blood and HK, but not from DI, had the capacity to phagocytose both PE-MPs and PS-MPs simultaneously (**Figure 5**).

Phagocytic Ability to PE-MP and PS-MP

The ability of immune cells from all three tissues to phagocytose different MP polymer types is shown in **Figures 3**, **4**. The cells had higher phagocytic ability toward PE-MP than PS-MP, and in some cases the values for the former MP were six times higher than those observed for the latter polymer type. The phagocytic ability was found to increase with the MP concentration. In **Figure 3**, the phagocytic ability of the three concentrations at 48 h is shown, the timepoint with most distinct differential responses. Cells from all the three tissues presented higher MP uptake when the concentration increased independently of the polymer type; there were statistical differences for comparisons based on concentrations at all timepoints and polymer types ($p < 0.001$) (**Supplementary File 2**, **Supplementary Tables 4–6**).

The ability of DI immune cells to phagocytose MP varied with the polymer type (**Figures 4A,B**). The phagocytic ability of cells exposed to high and medium concentrations of PS-MP decreased significantly with time ($p < 0.05$; * and • in **Figures 4A,B**). In contrast, at high concentrations of PE-MPs and PE+PS-MPs the phagocytic ability increased at 24 and 48 h compared to 1 h ($p < 0.001$; * in **Figure 4B**). PE-MPs were more phagocytosed than PS-MPs by DI cells exposed to high and medium concentrations at both 24 and 48 h ($p < 0.001$; ***a*** and ***b*** in **Figures 4A,B**). Similarly, cells exposed to high and medium concentrations of PE+PS-MPs had higher phagocytic ability than those exposed to PS-MPs at 48 h ($p < 0.001$; ***a*** and ***b*** in **Figures 4A,B**).

The phagocytic ability of blood cells exposed to high and medium concentrations of PE-MPs and PE+PS-MPs was significantly higher than to PS-MPs at all three timepoints ($p < 0.001$; ***a***, ***b***, and ***c*** in **Figures 4C,D**). However, differences in the ability to phagocytose PE-MPs and PE+PS-MPs varied depending on the concentration. Cells exposed to the medium concentration of PE-MPs presented a higher phagocytic ability than the mixture at all times ($p < 0.05$; ***a*** and ***b*** in **Figure 4C**). Nevertheless, at 48 h, the phagocytic ability of cells exposed to high concentrations of PE-MPs and PE+PS-MPs were similar (***a*** at 48 h in **Figure 4D**). Overall, the ability of blood immune cells to phagocytose all the three polymer types increased at 24 and 48 h compared to 1 h ($p < 0.05$; * in **Figures 4C,D**). However, cells exposed to high and medium concentrations of PS-MPs had less phagocytic cells at 48 h compared to 24 h ($p < 0.001$).

The differences in the phagocytosis of different polymer types by the HK immune cells were distinct as shown in **Figures 4E,F**. The phagocytic ability of the cells exposed to high and medium concentrations of PE-MPs was significantly higher than those of PE+PS-MPs and PS-MPs at all times ($p < 0.01$; ***a***, ***b***, and ***c*** in **Figures 4E,F**). Moreover, low concentrations of PE-MPs induced a significantly higher phagocytic ability than PS-MPs ($p < 0.05$). Cells exposed to high and medium concentrations of PE+PS-MPs showed a higher phagocytic ability than those of cells exposed to PS-MPs ($p < 0.001$; ***b*** and ***c*** in **Figures 4E,F**). Different trends in time depending on the polymers were evident in HK cells (**Figures 4E,F**). The phagocytic ability to PE-MPs increased significantly with time after exposures to high and medium concentrations ($p < 0.01$; * and • in **Figures 4E,F**). By contrast, the phagocytic ability to medium and high concentrations of PS-MPs decreased with time ($p < 0.05$; * and • in **Figures 4E,F**). However, the phagocytic ability of HK cells exposed to high concentrations of PE+PS-MPs increased at 24 and 48 h ($p < 0.001$; * in **Figure 4F**) while that of the cells exposed to medium concentrations had lower phagocytic ability after 48 h of exposure ($p < 0.001$; *• in **Figure 4E**).

Overall, blood and HK cells had higher abilities to phagocytose MP (**Figure 3**). HK cells exposed to 50 mg/L of PE-MPs were found to have the highest phagocytic ability, observed at 48 h, with an average value of 5.5% (SD 2.0). The highest ability to

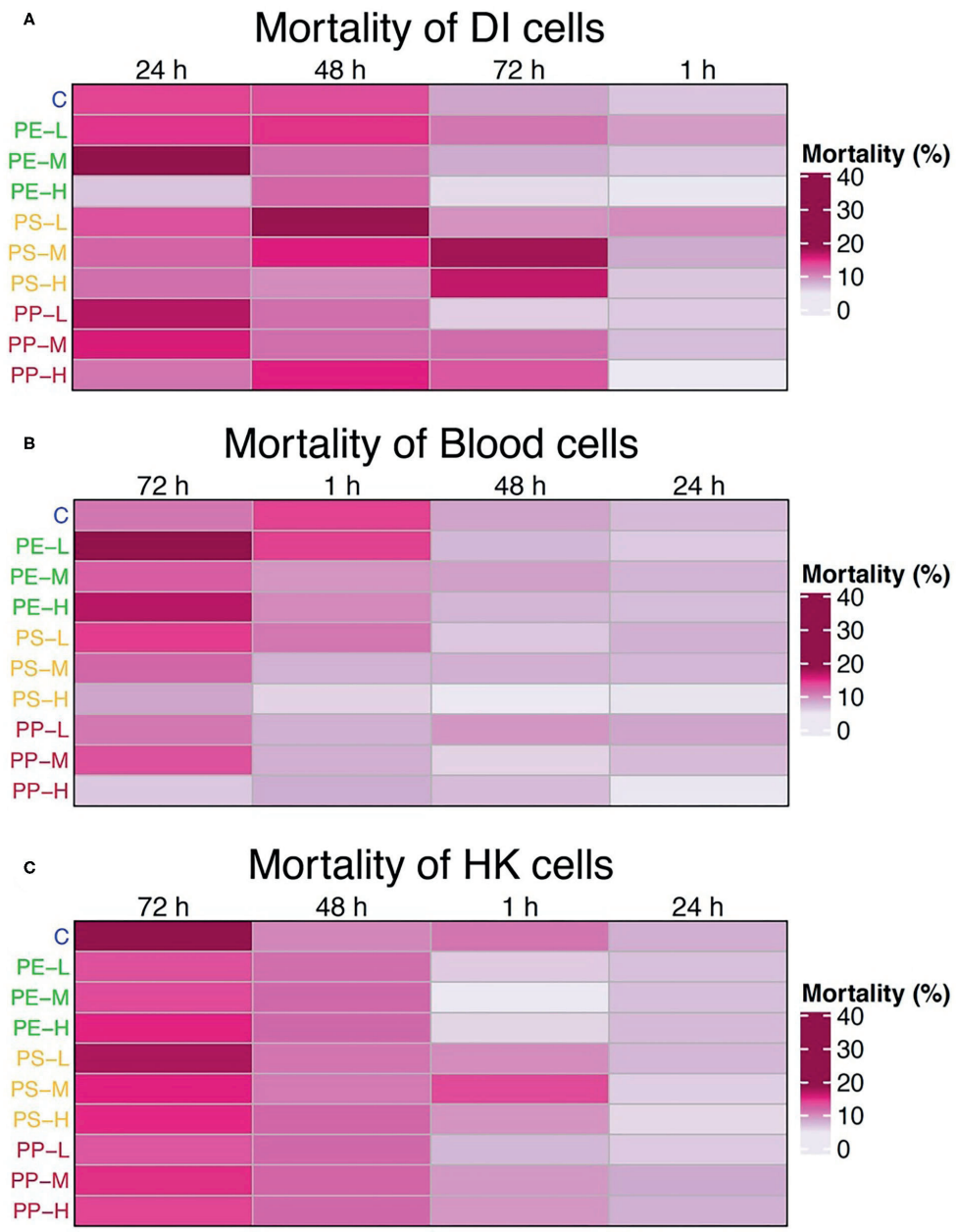


FIGURE 1 | Heatmaps showing the average mortality of **(A)** intestinal, **(B)** blood, and **(C)** HK cells at 1, 24, 48, and 72 h for each treatment group. Time is arranged based on the decreasing level of mortality, from left to right. Cell treatments on the left of the map are color coded based on the polymer types: blue = control, green = PE, yellow = PS, and red (PP) = PE + PS; and ordered by concentration (L = 0.05 mg/L, M = 5 mg/L, and H = 50 mg/L). The mortality is expressed as the percentage of dead cells (cells with red fluorescence) from the total number of cells.

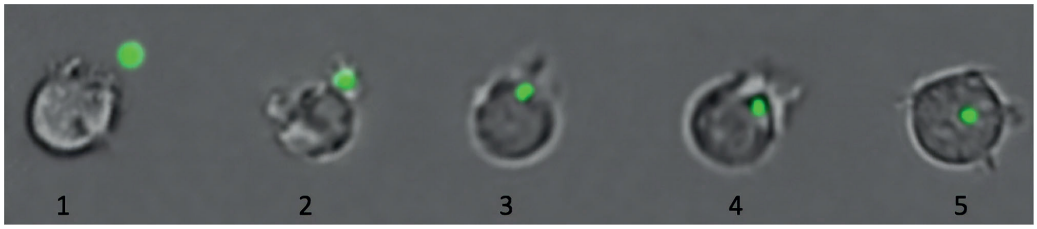


FIGURE 2 | Phagocytosis of microplastics (MPs) by immune cells of Atlantic salmon. As shown in this figure, cells ingest MP (green fluorescent particle) by performing the first steps of phagocytosis: detection and recognition of the MP (1), attachment of the cell to the MP by formation of pseudopods (2), and internalization of the MP (3–5) (Esteban et al., 2015). The last steps of the phagocytosis, involved in the digestion of the particle, are not present due to the lack of specific pathways to degrade plastic (Gewert et al., 2015). The collated images are from representative pictures of five different cells captured at different phagocytic stages. Cells are not to scale.

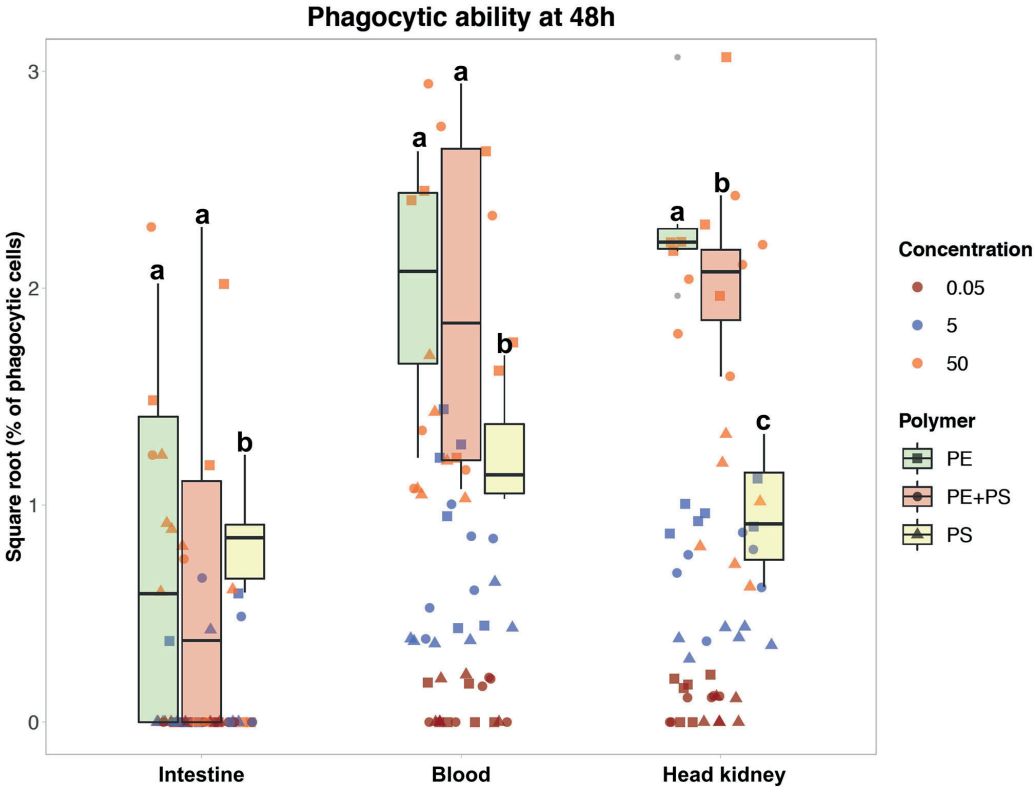


FIGURE 3 | Phagocytic ability of Atlantic salmon immune cells after 48 h of exposure to MP. The color codes for the three MP concentrations are: maroon (0.05 mg/L), blue (5 mg/L), and orange (50 mg/L). The boxplots represent the phagocytic ability of the cells exposed to the high MP concentration (50 mg/L) of PE (green), PE + PS (red), and PS (yellow) at 48 h. Different letters above the box plots represent statistical differences in the ability of the cells from the same tissue to phagocytose different polymers.

phagocytose PS-MPs was observed at 24 h in blood cells exposed to 50 mg/L (1.9%, SD 1.0), and the highest ability to phagocytose PE+PS-MPs was found at 48 h in blood cells exposed to the high concentration (4.3%, SD 3.3). DI cells had the lowest phagocytic ability among the three tissues; those exposed to 50 mg/L of PE-MP had the highest phagocytic ability (2.0%, SD 1.6), at 24 h.

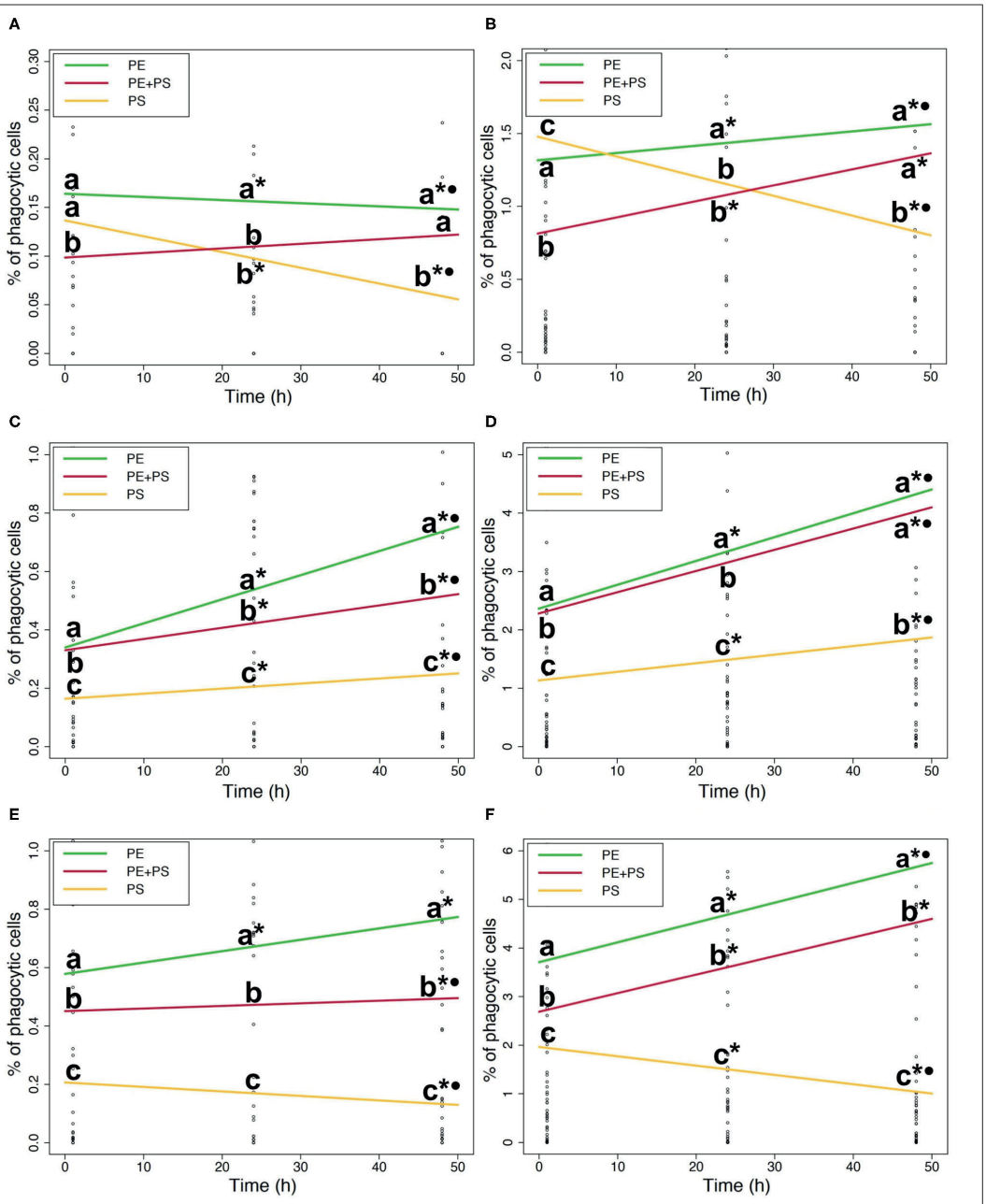


FIGURE 4 | (A). Phagocytic ability of DI cells to microplastic (5 mg/L). **(B)**. Phagocytic ability of DI cells to microplastic (50 mg/L). **(C)**. Phagocytic ability of blood cells to microplastic (5 mg/L). **(D)**. Phagocytic ability of blood cells to microplastic (50 mg/L). **(E)** Phagocytic ability of HK cells to microplastic (5 mg/L). **(F)** Phagocytic ability

(Continued)

FIGURE 4 | of HK cells to microplastic (50 mg/L). Phagocytic ability of DI **(A,B)**, blood **(C,D)**, and HK **(E,F)** cells exposed to the medium (left) and the high (right) MP concentrations. The lines show the general MP accumulation trends in time. The color codes for the MPs are: PE (green), PS (yellow), and PE + PS (red). Different letters above the lines represent statistical differences between polymer types at each time point. Asterisk and dot represent statistical differences between time points of a polymer type; *shows that there are differences compared to 1 h and •shows that there are differences compared to 24 h.

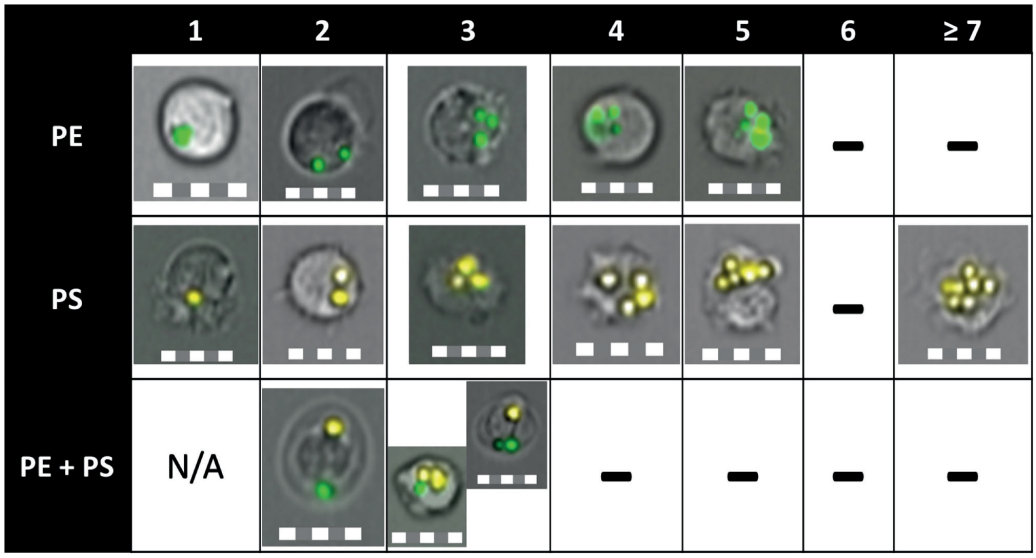


FIGURE 5 | Phagocytic capacity of Atlantic salmon immune cells to ingest PE-MP, PS-MP, or both. The column names indicate the number of particles inside a cell. "-" indicate the absence of cells that engulfed that number of particles. Cells were able to ingest up to five PE particles and up to eight PS particles, which translated to a higher phagocytic capacity of the cells toward PS-MP. HK and blood cells were able to phagocytose two different polymer types at the same time (shown on 3rd row, engulfed PE-MP and PS-MP), a fact proven for the first time in this study. Pictures were obtained by imaging flow cytometry from all tissues, timepoints, and MP concentrations. Scale = 10 μm.

Cells from all three tissues had a low degree of phagocytosis at 0.05 mg/L, the lower concentration of MP; few cells were able to phagocytose MP (0.08% was the highest phagocytic ability).

Phagocytic Capacity to PE-MP and PS-MP

The phagocytic capacity (i.e., the number of particles ingested by an immune cell) of Atlantic salmon to MP differed from tissue to tissue (**Supplementary File 2**, **Supplementary Tables 7–9**). DI immune cells had high phagocytic capacity to PE-MPs and PE+PS-MPs as compared to PS-MPs after 48 h of exposure to the highest MP concentration ($p < 0.05$). However, unlike the phagocytic ability, the phagocytic capacity of DI cells was not affected by the MP concentration.

Blood immune cells had higher phagocytic capacity to engulf PE-MPs at 24 h, while it was at 48 h for the other MPs ($p < 0.05$). However, statistical differences in the phagocytic capacity of blood cells to take up the different polymers were not evident, although PS-MPs were, in average, more ingested than the other MP types at both medium and high concentrations.

HK immune cells had the highest capacity to phagocytose MPs. While more than 7 PS-MP particles were observed inside these cells at 1 and 24 h of exposure, a maximum of five particles were seen in cells exposed to PE-MP for 24 h (**Figure 5**). Yet, the capacity of the cells to ingest the different MP polymer types did not vary significantly. The phagocytic capacity of HK cells increased with the MP concentration. Cells exposed to high concentrations of PE-MPs and PE+PS-MPs had higher phagocytic capacity than cells exposed to low concentrations at all timepoints ($p < 0.05$), as well as cells exposed to the medium concentration at 48 h ($p < 0.05$). However, HK cells exposed to 50 mg/L of PS-MPs only showed high phagocytic activity as compared to 0.05 and 5 mg/L at 1 and 24 h ($p < 0.01$).

DISCUSSION

The adverse effects of microplastics (MPs) on the immune system of animals have been intensively studied in recent years. Nevertheless, the physical response of cells to these small particles is poorly understood. This study aimed to provide new insights into the impact of MPs on the fish immune system at the cellular level. For this purpose, Atlantic salmon immune

cells from DI, blood and HK were exposed to high, medium and low concentrations of PE-MPs, PS-MPs, and PE+PS-MPs for 1, 24, 48, and 72 h. Here we report for the first-time quantitative data on the accumulation of different MP polymer types in fish leucocytes, analyzed by imaging flow cytometry. The present study shows that Atlantic salmon immune cells can phagocytose MPs, although to a relatively low degree. Less than 6% of the cells exposed to high MP concentrations phagocytosed them, and a maximum of 0.08% of cells was found to phagocytose the MPs at low concentrations. The concentration had a clear effect on the response of immune cells exposed to MP, as observed in other organisms, including humans (Kögel et al., 2019). Increased release of MPs into the environment can therefore have more serious consequences than previously thought.

From the results of the present study, it is clear that the phagocytic ability and capacity of the cells from the different tissues of salmon are influenced by the type of polymer to which it is exposed. Overall, salmon immune cells had a higher ability to phagocytose PE-MPs compared to PS-MPs. PE-MPs bioaccumulated in the cells, while the number of cells with PS-MPs decreased over time. This indicates that the cells are able to discriminate the MP types, as discussed below. The decrease in the ability of the cells to phagocytose PS-MPs may be attributed to the process of exocytosis. Macrophages have been shown to exocytose nanoparticles of different nature (Oh and Park, 2014). In human cells, a time dependent exocytosis was observed, and ∼66% of the endocytosed nanoparticles were exocytosed after 48 h of exposure (Asharani et al., 2009). However, exocytosis of particles, compared to endocytosis, is poorly understood and, to our knowledge, no studies are found that report exocytosis of MPs on fish. Nevertheless, a few recent studies have reported the excretion of PS-MPs by living organisms. Accumulated PS nanoparticles decreased after a few days of exposure of zebrafish embryos and larvae to the particles (Pitt et al., 2018). The authors suggested that 72 hpf larvae were able to excrete the PS through the gastrointestinal tract. Japanese medaka exposed to 10 μm PS-MPs was reported to egest 0.5–2% of the ingested microplastic per day (Zhu et al., 2020). Nevertheless, Japanese medaka exposed for 3 weeks to 2 μm PS-MPs still had significant amounts of particles in their intestine (Assas et al., 2020). A study carried out with Pacific oyster (*Crassostrea gigas*) found that PS-MPs did not accumulate in the gut and that the organism could egest these particles through the feces (Sussarellu et al., 2016). The shape of the microbeads was suggested to be the main factor determining the egestion of those particles by the oyster, and could, in turn, explain the higher phagocytic ability of the cells to PE-MPs than PS-MPs observed in this study. A study carried out on rat macrophages found that shape is the main factor regulating the phagocytosis, rather than the particle size. Spherical particles were phagocytosed less successfully than other shapes because the angle formed between the particle and the cell in the initial contact (Ω) was bigger (Champion and Mitragotri, 2006). The PS-MPs used in the present study had a uniform shape, with more than 80% of the particles showing a perfect sphericity, while the PE-MP particles were more irregularly shaped, with only 25% of the particles being spherical (**Supplementary File 1**). Hence, the higher ability of the cells to phagocytose PE-MPs could potentially be due to their lower Ω compared to PS-MPs. This would also explain the similar phagocytic response observed between cells exposed to PE+PS-MPs and PE-MPs. Cells exposed to PE+PS-MPs phagocytosed PE-MPs faster and more successfully than PS-MPs, relatively masking the effect of the latter particles on the cells. This hypothesis became evident after looking into the cells exposed to PE+PS-MPs at 1 h and comparing the number of phagocytic cells with internalized PE-MP with the ones with internalized PS-MPs (**Supplementary Figure**). The roughness and shape are hence important factors to consider when assessing the toxicity of MPs, as it has also been reported in *in vivo* studies (Choi et al., 2018). This is especially relevant since <1% of the MPs found in marine waters are spherical (Isobe, 2016). The higher phagocytic ability of the cells to PE-MPs could also be due to the less uniform size of these particles (1–5 μm) compared to the PS-MPs (2.1 μm). However, analyses of the images of the cells with internalized PE-MPs did not reveal any evidence of cells ingesting smaller particles (1 μm) compared to larger particles (5 μm). Similarly, mice macrophages exposed to 1.2 and 5.2 μm PS-MPs did not show differences in the phagocytic ability to these particles (Tomazic-Jezic et al., 2001). Nevertheless, the authors found differences when they assessed the ability of bigger particles (12.5 μm). Another important factor to consider when analyzing the results of the phagocytic ability is the number of particles/L. The concentration of microplastic added to each sample was calculated in mg/L, but since the density of PE and PS is different, cells incubated with PE-MP were not exposed to the same number of particles as cells exposed to PS-MP. An estimation of the number of particles/L was done to assess whether the higher phagocytic ability of the cells toward PE-MP could be due to the higher number of particles/L. Our calculation revealed that cells exposed to 50, 5, and 0.05 mg/L of PE-MP were exposed to 4.8×10^9, 4.8×10^8, and 4.8×10^6 particles/L respectively, while cells exposed to PS-MP were exposed to more particles/L in comparison (1.75×10^{10}, 1.75×10^9, and 1.75×10^7, respectively). These results underline that cells had a higher response to PE-MPs than to PS-MPs.

As for the phagocytic capacity, blood and HK cells generally ingested more PS-MP particles than PE-MPs. The higher phagocytic capacity of cells toward PS-MPs could be related to the polarity of the polymer, as previously suggested for PS and PC micro- and nanoparticles (Greven et al., 2016). PS tends to become positively charged when it is in contact with water, while PE is consider a non-polar polymer and its charges remain stable (Albrecht et al., 2009). The positively charged PS have higher ability to interact with the cells, stimulating its uptake by cells. Positively charged particles show higher interaction with the cells because of electrostatic attraction, given the negative charge of the cell plasma membrane. Moreover, the binding of PS could be associated to hydrogen bond formation between the charged particles and the hydrophilic region of the phospholipids from the plasma membrane, as suggested for cellulose (Bhattacharya et al., 2010; Ma et al., 2017). Thus, the higher phagocytic capacity of the cells toward the PS-MPs could partly be explained by the polarity of the polymer.

Immune cells from the three tissues responded differently to MPs. Blood and HK cells showed a higher ability to phagocytose MPs compared to DI cells (**Figure 3**). DI immune cells consisted of different leucocytes, and not all of them were phagocytic. On the other hand, the harvested adherent cells from blood and HK were mainly monocytes/macrophages, the main phagocytic cells composing the leucocytes along with the neutrophils (Delves and Roitt, 2000). This abundance in macrophages would explain the main difference in phagocytic ability observed between tissues. However, in Atlantic salmon, the percentage of monocytes/macrophages found in the leucocytes is only 10% lower than the percentage of monocytes/macrophages found in the adherent cells isolated with the protocol used in the present study (Park et al., 2020). Hence, differences in the phagocytic ability of cells from different tissues could also be explained by the principle of organ-specific innate immunity. This principle states that the activation of innate immunity differs between different organs, which have specialized mechanisms to achieve a more efficient response (Raz, 2007). The lower ability of fish DI cells to phagocytose MPs could be a beneficial adaptation to prevent or reduce the uptake of particles from the food. Several fish species ingest and accumulate MPs in the digestive tract (Bråte et al., 2017) as well as in several other tissues, such as the gills, liver and even in the brain (Ding et al., 2018). It has been suggested that MPs translocate from the gut into the circulatory system in fish, which in turn will transport them to other tissues and organs (Ding et al., 2018; Wang et al., 2020). The present study provides new understanding of the MP accumulation potential in Atlantic salmon immune cells and sheds light on potential entry routes for MP particles.

Cell mortality increased at 72 h in all the groups, including the control group. Hence inferences about the cytotoxicity of the MPs based on the mortality of the control group cannot be drawn. Nevertheless, **Figure 2** suggests that MP did not induce any clear polymer- or concentration-dependent signs of cytotoxicity in the studied salmon immune cells. A study on gilthead seabream and European sea bass leucocytes reported no decrease in viability of cells exposed to high concentrations (1, 10, and 100 g/L) of PE-MP and PVC-MP for 1 and 24 h. However, immune parameters such as respiratory burst and phagocytic capacity of the cells were affected (Espinosa et al., 2018). A sublethal effect of MP on fish immune cells was also observed in fathead minnow, where exposure of neutrophils to PS or PC micro- and nanoparticles induced cell degranulation as well as oxidative burst activity (Greven et al., 2016). In the present study, we used fluorescent-dyed MPs and therefore did not measure any additional parameters other than cell mortality. Earlier studies have shown that some fluorochromes can leach and accumulate in tissues (Catarino et al., 2019), and influence the superoxide production as well as other immune parameters directly (De Clerck et al., 1994). Our focus was to understand the physical impact of salmon immune cells to different types of MP polymers, by assessing the phagocytic ability and capacity of the cells.

In the present study, we found that MPs can impact the immune cells of Atlantic salmon even at relatively low concentrations and that different polymers can induce distinct responses on the cells. Exposure of farmed salmon to MP can have economic implications for this prime aquaculture industry (FAO, 2018), especially if the MP particles act as vectors for contaminants (Browne et al., 2013). Moreover, plastic is widely used in aquaculture and MP released from sources such as feeding pipes into the surrounding waters may potentially be ingested by the farmed fish (Lusher et al., 2017a; Gomiero et al., 2020). We found that PE-MP accumulates more easily than PS-MP in the immune cells of the Atlantic salmon. PE is the most abundant MP polymer in the marine environment (Enders et al., 2015; Ter Halle et al., 2017; Erni-Cassola et al., 2019; Pannetier et al., 2020) and, therefore, the most often ingested polymer by fishes (Rummel et al., 2016; Bråte et al., 2017). Hence, the impact of PE-MPs on wild populations of salmonid species cannot be ignored.

CONCLUSION

The present study used an imaging flow cytometry approach to understand how different MP polymers are taken up by fish cells. This technique enabled us to prove for the first time that a single cell can phagocytose two different microplastic polymer types simultaneously. In conclusion, Atlantic salmon immune cells isolated from different tissues phagocytosed microplastic polymers, even at relatively low concentrations. Polyethylene microparticles were more easily ingested than polystyrene ones. With increasing amounts of plastic debris in the marine environment, the study highlights how their uptake into the tissues can lead to damaging effects on aquatic life. The methodology adopted in the present study opens new possibilities for observing the impact of micro and nanoplastic on several species, including humans.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

Ethical review and approval was not required for the animal study because, in accordance the Norwegian regulation of the Research Animal Act (FOR-2015-06-18-761). The Approval of trials regulation §6 states that approval requirement does not apply to experiments involving only the killing of animals to use organs or tissues from them. Live fish was handled by personnel with FELASA-C course, based on the policies by the Federation of European Laboratory Animal Science Association.

AUTHOR CONTRIBUTIONS

IA-G, PO, and VK: conceptualization and design of the study. IA-G and YP: methodology, protocols, and experiment set up. IA-G: analysis of data and writing. IA-G, YP, VK, and

PO: review and editing. All authors contributed to the article and approved the submitted version.

ACKNOWLEDGMENTS

Bisa Saraswathy from Nord University and Nicolas Dupont from UiO (University of Oslo) are acknowledged for the suggestions and help provided for the statistical analysis of the data. The support from Virginia Abellán Zapata on the mathematic formulation is also appreciated.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fenvs.2020.560206/full#supplementary-material

REFERENCES

Albrecht, V., Janke, A., Németh, E., Spange, S., Schubert, G., and Simon, F. (2009). Some aspects of the polymers' electrostatic charging effects. *J. Electrostat.* 67, 7–11. doi: 10.1016/j.elstat.2008.10.002

Alimba, C. G., and Faggio, C. (2019). Microplastics in the marine environment: current trends in environmental pollution and mechanisms of toxicological profile. *Environ. Toxicol. Pharmacol.* 68, 61–74. doi: 10.1016/j.etap.2019.03.001

Arthur, C., Baker, J. E., and Bamford, H. A. (2009). *Proceedings of the International Research Workshop on the Occurrence, Effects, and Fate of Microplastic Marine Debris, September 9–11, 2008*. Tacoma, WA: University of Washington Tacoma.

Asharani, P., Hande, M. P., and Valiyaveettil, S. (2009). Anti-proliferative activity of silver nanoparticles. *BMC Cell Biol.* 10:65. doi: 10.1186/1471-2121-10-65

Assas, M., Qiu, X., Chen, K., Ogawa, H., Xu, H., Shimasaki, Y., et al. (2020). Bioaccumulation and reproductive effects of fluorescent microplastics in medaka fish. *Marine Pollut. Bullet.* 158, 111446. doi: 10.1016/j.marpolbul.2020.111446

Attaya, A., Wang, T., Zou, J., Herath, T., Adams, A., Secombes, C. J., et al. (2018). Gene expression analysis of isolated salmonid GALT leucocytes in response to PAMPs and recombinant cytokines. *Fish Shellfish Immunol.* 80, 426–436. doi: 10.1016/j.fsi.2018.06.022

Bhattacharya, P., Lin, S., Turner, J. P., and Ke, P. C. (2010). Physical adsorption of charged plastic nanoparticles affects algal photosynthesis. *J. Phys. Chem. C* 114, 16556–16561. doi: 10.1021/jp1054759

Bråte, I. L. N., Huwer, B., Thomas, K. V., Eidsvoll, D. P., Halsband, C., Almroth, B. C., et al. (2017). *Micro-and Macro-Plastics in Marine Species From Nordic Waters*. Copenhagen: Nordic Council of Ministers. doi: 10.6027/TN2017-549

Browne, M. A., Niven, S. J., Galloway, T. S., Rowland, S. J., and Thompson, R. C. (2013). Microplastic moves pollutants and additives to worms, reducing functions linked to health and biodiversity. *Curr. Biol.* 23, 2388–2392. doi: 10.1016/j.cub.2013.10.012

California Current Ecosystem LTER and Goldstein, M. (2017). Numerical (No m-3) concentrations (mg m-3) of subsurface microplastic debris collected by bongo net aboard the Scripps Environmental Accumulation of Plastic Expedition (SEAPLEX) cruise, August 2009. ver 2. *Environ. Data Initiative.* doi: 10.6073/pasta/67b4659a0fc883d40f12849090e1c011

Catarino, A. I., Frutos, A., and Henry, T. B. (2019). Use of fluorescent-labelled nanoplastics (NPs) to demonstrate NP absorption is inconclusive without adequate controls. *Sci. Total Environ.* 670, 915–920. doi: 10.1016/j.scitotenv.2019.03.194

Champion, J. A., and Mitragotri, S. (2006). Role of target geometry in phagocytosis. *Proc. Natl. Acad. Sci. U. S. A.* 103, 4930–4934. doi: 10.1073/pnas.0600997103

Choi, J. S., Jung, Y.-J., Hong, N.-H., Hong, S. H., and Park, J.-W. (2018). Toxicological effects of irregularly shaped and spherical microplastics in a marine teleost, the sheepshead minnow (*Cyprinodon variegatus*). *Marine Pollut. Bullet.* 129, 231–240. doi: 10.1016/j.marpolbul.2018.02.039

De Clerck, L. S., Bridts, C. H., Mertens, A. M., Moens, M. M., and Stevens, W. J. (1994). Use of fluorescent dyes in the determination of adherence of human leucocytes to endothelial cells and the effect of fluorochromes on cellular function. *J. Immunol. Method.* 172, 115–124. doi: 10.1016/0022-1759(94)90384-0

Delves, P. J., and Roitt, I. M. (2000). The immune system. *N. Engl. J. Med.* 343, 37–49. doi: 10.1056/NEJM200007063430107

Ding, J., Zhang, S., Razanajatovo, R. M., Zou, H., and Zhu, W. (2018). Accumulation, tissue distribution, and biochemical effects of polystyrene microplastics in the freshwater fish red tilapia (*Oreochromis niloticus*). *Environ. Pollut.* 238, 1–9. doi: 10.1016/j.envpol.2018.03.001

Enders, K., Lenz, R., Stedmon, C. A., and Nielsen, T. G. (2015). Abundance, size and polymer composition of marine microplastics $\geq$10μm in the Atlantic Ocean and their modelled vertical distribution. *Marine Pollut. Bullet.* 100, 70–81. doi: 10.1016/j.marpolbul.2015.09.027

Erni-Cassola, G., Zadjelovic, V., Gibson, M. I., and Christie-Oleza, J. A. (2019). Distribution of plastic polymer types in the marine environment: a meta-analysis. *J. Hazard. Mater.* 369, 691–698. doi: 10.1016/j.jhazmat.2019.02.067

Espinosa, C., Beltrán, J. M. G., Esteban, M. A., and Cuesta, A. (2018). *In vitro* effects of virgin microplastics on fish head-kidney leucocyte activities. *Environ. Pollut.* 235, 30–38. doi: 10.1016/j.envpol.2017.12.054

Espinosa, C., Cuesta, A., and Esteban, M. Á. (2017). Effects of dietary polyvinylchloride microparticles on general health, immune status and expression of several genes related to stress in gilthead seabream (*Sparus aurata* L.). *Fish Shellfish Immunol.* 68, 251–259. doi: 10.1016/j.fsi.2017.07.006

Esteban, M. Á., Cuesta, A., Chaves-Pozo, E., and Meseguer, J. (2015). Phagocytosis in teleosts. Implications of the new cells involved. *Biology* 4, 907–922. doi: 10.3390/biology4040907

FAO (2018). *The State of World Fisheries and Aquaculture 2018-Meeting the sustainable development goals*. Rome: FAO.

Foley, C. J., Feiner, Z. S., Malinich, T. D., and Höök, T. O. (2018). A meta-analysis of the effects of exposure to microplastics on fish and aquatic invertebrates. *Sci. Total Environ.* 631, 550–559. doi: 10.1016/j.scitotenv.2018.03.046

GESAMP (2015). "Sources, fate and effects of microplastics in the marine environment: a global assessment," in *IMO/FAO/UNESCO-IOC/UNIDO/WMO/IAEA/UN/UNEP/UNDP Joint Group of Experts on the Scientific Aspects of Marine Environmental Protection*, ed P. J. Kershaw. Rep. Stud. GESAMP No. 90, 96. Available online at: https://ec.europa.eu/environment/marine/good-environmental-status/descriptor-10/pdf/GESAMP_microplastics%20full%20study.pdf

Gewert, B., Plassmann, M. M., and Macleod, M. (2015). Pathways for degradation of plastic polymers floating in the marine environment. *Environ. Sci. Process. Impacts* 17, 1513–1521. doi: 10.1039/C5EM00207A

Gomiero, A., Haave, M., Kögel, T., Bjorøy, Ø., Gjessing, M., Lea, T. B., et al. (2020). *TRACKing of PLASTtic Emissions From Aquaculture Industry*. Bergen: TrackPlast.

Greven, A. C., Merk, T., Karagöz, F., Mohr, K., Klapper, M., Jovanović, B., et al. (2016). Polycarbonate and polystyrene nanoplastic particles act as stressors to the innate immune system of fathead minnow (*Pimephales promelas*). *Environ. Toxicol. Chem.* 35, 3093–3100. doi: 10.1002/etc.3501

Hartmann, N. B., HüFfer, T., Thompson, R. C., Hassellö, V. M., Verschoor, A., et al. (2019). Are we speaking the same language? recommendations for a definition and categorization framework for plastic debris. *Environ. Sci. Technol.* 53, 1039–1047. doi: 10.1021/acs.est.8b05297

Haugland, G. T., Jordal, A.-E. O., and Wergeland, H. I. (2012). Characterization of small, mononuclear blood cells from salmon having high phagocytic capacity and ability to differentiate into dendritic like cells. *PLoS ONE* 7:e49260. doi: 10.1371/journal.pone.0049260

Isobe, A. (2016). Percentage of microbeads in pelagic microplastics within Japanese coastal waters. *Marine Pollut. Bullet.* 110, 432–437. doi: 10.1016/j.marpolbul.2016.06.030

Kögel, T., Bjorøy, Ø., Toto, B., Bienfait, A. M., and Sanden, M. (2019). Micro- and nanoplastic toxicity on aquatic life: determining factors. *Sci. Total Environ.* 709:136050. doi: 10.1016/j.scitotenv.2019.136050

Lei, L., Wu, S., Lu, S., Liu, M., Song, Y., Fu, Z., et al. (2018). Microplastic particles cause intestinal damage and other adverse effects in zebrafish Danio rerio and nematode *Caenorhabditis elegans*. *Sci. Total Environ.* 619, 1–8. doi: 10.1016/j.scitotenv.2017.11.103

Lu, Y., Zhang, Y., Deng, Y., Jiang, W., Zhao, Y., Geng, J., et al. (2016). Uptake and accumulation of polystyrene microplastics in zebrafish (*Danio rerio*) and toxic effects in liver. *Environ. Sci. Technol.* 50, 4054–4060. doi: 10.1021/acs.est.6b00183

Lusher, A., Hollman, P., and Mendoza-Hill, J. (2017a). *Microplastics in Fisheries and Aquaculture: Status of Knowledge on Their Occurrence and Implications for Aquatic Organisms and Food Safety*. Rome: FAO Fisheries and Aquaculture Technical Paper.

Lusher, A., Welden, N., Sobral, P., and Cole, M. (2017b). Sampling, isolating and identifying microplastics ingested by fish and invertebrates. *Analyt. Method.* 9, 1346–1360. doi: 10.1039/C6AY02415G

Ma, Y., Poole, K., Goyette, J., and Gaus, K. (2017). Introducing membrane charge and membrane potential to T cell signaling. *Front. Immunol.* 8:1513. doi: 10.3389/fimmu.2017.01513

Oh, N., and Park, J.-H. (2014). Endocytosis and exocytosis of nanoparticles in mammalian cells. *Int. J. Nanomed.* 9:51. doi: 10.2147/IJN.S26592

Pannetier, P., Morin, B., Le Bihanic, F., Dubreil, L., Clérandeau, C., Chouvellon, F., et al. (2020). Environmental samples of microplastics induce significant toxic effects in fish larvae. *Environ. Int.* 134:105047. doi: 10.1016/j.envint.2019.105047

Park, Y., Abihssira-García, I. S., Thalmann, S., Wiegertjes, G., Barreda, D., Olsvik, P. A., et al. (2020). Imaging flow cytometry protocols for examining phagocytosis of microplastics and bioparticles by immune cells of aquatic animals. *Front. Immunol.* 11:203. doi: 10.3389/fimmu.2020.00203

Pitt, J. A., Kozal, J. S., Jayasundara, N., Massarsky, A., Trevisan, R., Geitner, N., et al. (2018). Uptake, tissue distribution, and toxicity of polystyrene nanoparticles in developing zebrafish (*Danio rerio*). *Aquatic Toxicol.* 194, 185–194. doi: 10.1016/j.aquatox.2017.11.017

Raz, E. (2007). Organ-specific regulation of innate immunity. *Nat. Immunol.* 8:3. doi: 10.1038/ni0107-3

Rummel, C. D., Löder, M. G. J., Fricke, N. F., Lang, T., Griebeler, E.-M., Janke, M., et al. (2016). Plastic ingestion by pelagic and demersal fish from the North Sea and Baltic Sea. *Marine Pollut. Bullet.* 102, 134–141. doi: 10.1016/j.marpolbul.2015.11.043

Strand, J., Tairova, Z., Danielsen, J., Hansen, J. W., Magnusson, K., Naustvoll, L.-J., et al. (2015). *Marine Litter in Nordic Waters*. Copenhagen: Nordisk Ministerråd. doi: 10.6027/TN2015-521

Suaria, G., Avio, C. G., Mineo, A., Lattin, G. L., Magaldi, M. G., Belmonte, G., et al. (2016). The Mediterranean Plastic Soup: synthetic polymers in Mediterranean surface waters. *Sci. Rep.* 6:37551. doi: 10.1038/srep37551

Sussarellu, R., Suquet, M., Thomas, Y., Lambert, C., Fabioux, C., Pernet, M. E. J., et al. (2016). Oyster reproduction is affected by exposure to polystyrene microplastics. *Proc. Natl. Acad. Sci. U. S. A.* 113, 2430–2435. doi: 10.1073/pnas.1519019113

Ter Halle, A., Jeanneau, L., Martignac, M., Jardé, E., Pedrono, B., Brach, L., et al. (2017). Nanoplastic in the North Atlantic subtropical gyre. *Environ. Sci. Technol.* 51, 13689–13697. doi: 10.1021/acs.est.7b03667

Tomazic-Jezic, V. J., Merritt, K., and Umbreit, T. H. (2001). Significance of the type and the size of biomaterial particles on phagocytosis and tissue distribution. *J. Biomed. Mater. Res.* 55, 523–529. doi: 10.1002/1097-4636(20010615)55:4<523::AID-JBM1045>3.0.CO;2-G

Underhill, D. M., and Goodridge, H. S. (2012). Information processing during phagocytosis. *Nat. Rev. Immunol.* 12, 492–502. doi: 10.1038/nri3244

UNEP (2016). *UNEP Frontiers 2016 Report: Emerging Issues of Environmental Concern*. Nairobi: United Nations Environment Programme.

Von Moos, N., Burkhardt-Holm, P., and KöHler, A. (2012). Uptake and effects of microplastics on cells and tissue of the blue mussel *Mytilus edulis* L. after an experimental exposure. *Environ. Sci. Technol.* 46, 11327–11335. doi: 10.1021/es302332w

Wang, W., Ge, J., and Yu, X. (2020). Bioavailability and toxicity of microplastics to fish species: a review. *Ecotoxicol. Environ. Safety* 189:109913. doi: 10.1016/j.ecoenv.2019.109913

Whyte, S. K. (2007). The innate immune response of finfish–a review of current knowledge. *Fish Shellfish Immunol.* 23, 1127–1151. doi: 10.1016/j.fsi.2007.06.005

Wright, S. L., and Kelly, F. J. (2017). Plastic and human health: a micro issue? *Environ. Sci. Technol.* 51, 6634–6647. doi: 10.1021/acs.est.7b00423

Zapata, A., Diez, B., Cejalvo, T., Gutierrez-De Frias, C., and Cortes, A. (2006). Ontogeny of the immune system of fish. *Fish Shellfish Immunol.* 20, 126–136. doi: 10.1016/j.fsi.2004.09.005

Zhu, M., Chernick, M., Rittschof, D., and Hinton, D. E. (2020). Chronic dietary exposure to polystyrene microplastics in maturing Japanese medaka (*Oryzias latipes*). *Aquatic Toxicol.* 220:105396. doi: 10.1016/j.aquatox.2019.105396

Zitouni, N., Bousserrhine, N., Belbekhouche, S., Missawi, O., Alphonse, V., Boughatass, I., et al. (2020). First report on the presence of small microplastics ($\leq$3 μm) in tissue of the commercial fish *Serranus scriba* (Linnaeus. 1758) from Tunisian coasts and associated cellular alterations. *Environ. Pollut.* 236:114576. doi: 10.1016/j.envpol.2020.114576

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Supplementary file 1

Table of Contents

Agglomeration rate

To estimate the agglomeration rate of the microplastic particles we use the Imaging Flow Cytometer (IFC) software IDEAS®.

First, we plotted all the events detected by the IFC in a scatterplot where the Y axis represented the Aspect Ratio of the particles (cell morphology) and the X axis, their Area (cell size). From there, we excluded the cells and selected the particles that were smaller in size, corresponding to the microplastic particles and other debris (Figure 1).

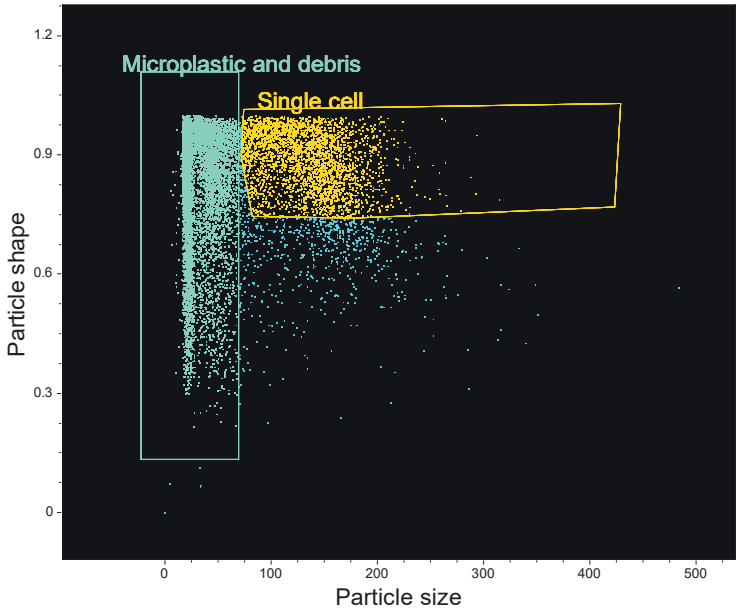


Figure 1: Scatterplot showing the size (X axis) and shape (Y axis) of the particles detected by IFC. In blue are selected the particles smaller in size, which consist on microplastics and debris.

To select only the microplastics from those small particles, we made a histogram with the fluorescence intensity on the X axis. Particles with no fluorescence were considered as debris and only the fluorescent particles were analysed (Figure 2, R3).

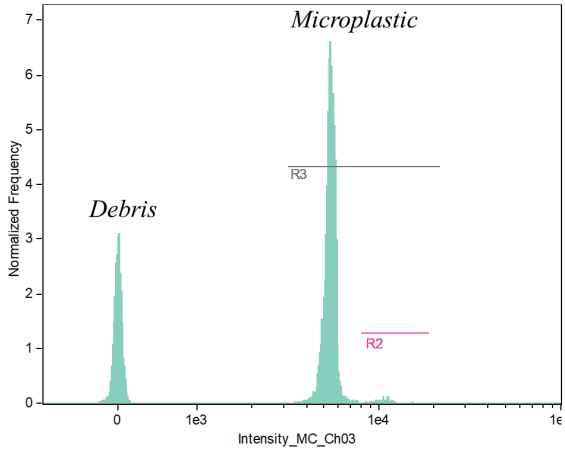


Figure 2: Histogram representing the fluorescence of the small particles previously selected.

After the detection of the microplastic particles, two different process were done to estimate the agglomeration rate of the microplastic, for PE and PS.

PS-MP size was very uniform. Hence, agglomeration of particles was detected through a second peak with higher fluorescence intensity in the same histogram mentioned before (Figure 2, R2). To ensure that the number of events corresponded to actual agglomerated particles and not to two particles detected at the same time by the IFC, particles of R3 (Figure 2) were plotted in a histogram which X axis corresponded to the Aspect Ratio of the image. The Aspect Ratio shows the sphericity of the particle detected (Figure 3): ratio close to 1 correspond to spherical particles (Figure 3, R6) and ratio closer to 0 show elongated particles (Figure 3, R4). Events with aspect ratio around 0.6 corresponded to agglomerated particles (Figure 3, R5).

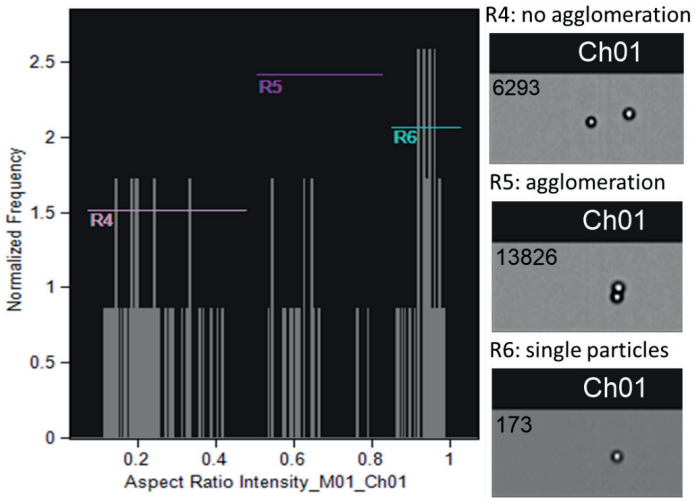


Figure 1

In the case of PE microparticles, the detection of agglomerated particles was more challenging due to the size range of these particles (1 to 5 µm). As shown in figure 4, the PE fluorescence was not detected as a narrow peak as it happened with the PS, but in a wide peak (Figure 4, R3).

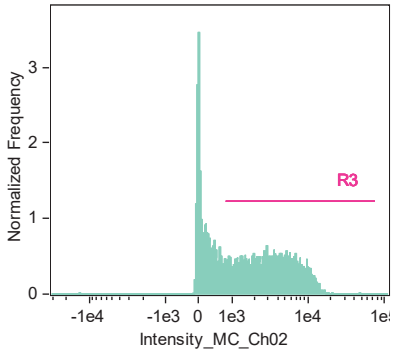
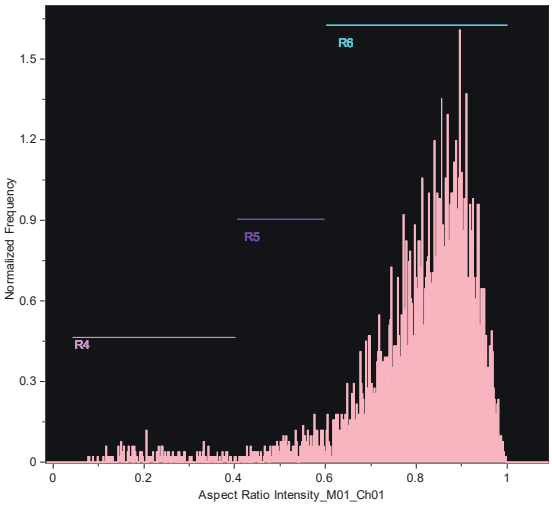


Figure 2

Hence, agglomeration could not be detected by a second peak of higher fluorescence. To estimate the agglomeration ratio of the PE particles, a histogram of the particles detected in R3 (Figure 4) was done with the Aspect Ratio of the particles represented on the X axis (Figure 5). Events with Aspect Ratio between 0.4 and 0.6 were considered agglomerated particles (Figure 5, R5). However, data of agglomeration of PE-MP are less reliable than those of PS-MP because false positives might be detected due to the less sphericity of the PE particles (see next section below for more information on the sphericity of the microplastic particles).



Aspect Ratio Intensity_M01_Ch01

Population	Count	%Gated
R3 & R1 & Focus	5107	100
R6 & R3 & R1 & Focus	4740	92.8
R4 & R3 & R1 & Focus	137	2.68
R5 & R3 & R1 & Focus	223	4.37

Figure 3

The agglomeration rate was then calculated as percentage of events where particles were agglomerated (Figures 3 and 5, R5) from the total amount of microplastic particles (Figures 2 and 4, R3).

PS particles had an agglomeration average rate of 2.04 ± 2.12 %, and PE particles, 3.22 ± 1.32 %.

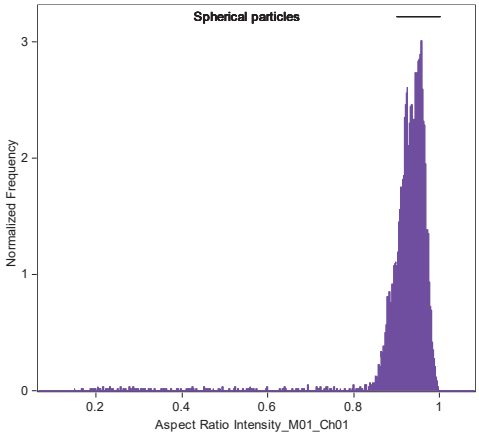
Sphericity of the particles

To estimate the sphericity of the particles by IFC we used the Aspect Ratio feature, a measurement calculated directly by the IFC software that provides information about the shape of the particles: particles which Aspect Ratio is close to 1 are spherical while particles which Aspect Ratio is closer to 0 are more elongated.

To estimate the sphericity of the PS and PE microplastics, single microplastic particles were isolated with the IFC software as mentioned above. Then single microplastic particles were plotted in a histogram against their aspect ratio on the X axis. We considered that particles with an Aspect Ratio over 0.9 are perfectly spherical (Figure 6).

83% of PS-MP were spherical, while only 25% of PE-MP were in that category (Figures 6 and 7).

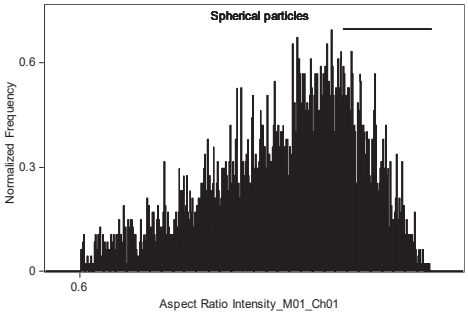
PS-MP particles



Aspect Ratio Intensity_M01_Ch01

Population	Count	%Gated
R4 & R1 & Focus	6212	100
Spherical particles & R4 & R1 & Focus	5158	83

PE-MP particles



Aspect Ratio Intensity_M01_Ch01

Population	Count	%Gated
PE single & R3 & R1 & Focus	4744	100
Spherical particles & PE single & R3 & R1 & Focus	1189	25.1

Figure 4

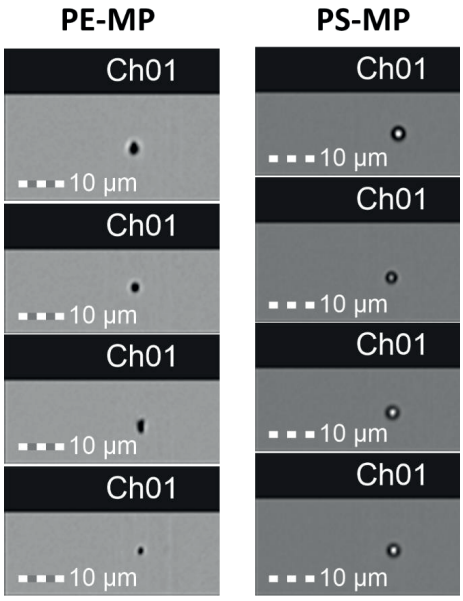


Figure 5: Pictures of PE-MP (left) and PS-MP (right) capture by Imaging Flow Cytometry

Supplementary file 2

Table of contents

Mortality

Table S1: Results of Poisson regression analyses for the mortality of intestinal (DI) cells. Comparisons between the four timepoints are shown for each treatment. P-values (right column) under 0.05, 0.01 and 0.001 (, **, *** respectively) show statistical differences in the cell mortality of two timepoints. Negative estimates show that the mortality at the timepoints in bold are significantly lower than the timepoints in parenthesis, while positive estimates show that the mortality at the timepoints in bold are significantly higher. Degrees of freedom are shown at the end of the table.*

Treatment	Time (h)	Estimate	Standard error	P-value
C	(1-) **24**	8.353e-01	1.996e-01	2.84e-05 ***
	(1-) **48**	8.109e-01	2.003e-01	5.16e-05 ***
	(1-) **72**	2.877e-01	2.205e-01	0.19196
	(24-) **48**	-0.02439	0.15619	0.87590
	(24-) **72**	-0.54764	0.18133	0.00253 **
	(48-) **72**	-0.52325	0.18215	0.00407 **
PE-0.05	(1-) **24**	0.56531	0.17710	0.00141 **
	(1-) **48**	0.55389	0.17747	0.00180 **
	(1-) **72**	0.24686	0.18875	0.19091
	(24-) **48**	-0.01143	0.15119	0.939743
	(24-) **72**	-0.31845	0.16428	0.052568
	(48-) **72**	-0.30703	0.16468	0.062268
PE-5	(1-) **24**	1.143869	1.914e-01	2.27e-09 ***
	(1-) **48**	6.360e-01	2.061e-01	0.00203 **
	(1-) **72**	2.231e-01	2.236e-01	0.31831
	(24-) **48**	-0.507880	0.153478	0.000936 ***
	(24-) **72**	-0.920725	0.176272	1.76e-07 ***
	(48-) **72**	-0.41285	0.19217	0.031685 *
PE-50	(1-) **24**	0.40547	0.26352	0.123894
	(1-) **48**	1.09861	0.23570	3.15e-06 ***
	(1-) **72**	0.15415	0.27817	0.579475
	(24-) **48**	0.69315	0.20412	0.000684 ***
	(24-) **72**	-0.25131	0.25198	0.318583
	(48-) **72**	-0.94446	0.22272	2.23e-05 ***
PS-0.05	(1-) **24**	0.326397	0.173787	0.060363
	(1-) **48**	0.684337	0.162461	2.53e-05 ***
	(1-) **72**	-0.035718	0.189012	0.850115
	(24-) **48**	0.35794	0.14666	0.014659 *
	(24-) **72**	-0.36211	0.17561	0.039208 *
	(48-) **72**	-0.720055	0.164412	1.19e-05 ***
PS-5	(1-) **24**	0.48380	0.18953	0.010691 *
	(1-) **48**	0.72594	0.18159	6.40e-05 ***
	(1-) **72**	0.88469	0.17719	5.95e-07 ***
	(24-) **48**	0.24214	0.15637	0.121498
	(24-) **72**	0.40089	0.15124	0.008032 **

	(48-) **72**	0.15875	0.14116	0.260769
PS-50	(1-) **24**	6.061e-01	2.072e-01	0.00344 **
	(1-) **48**	4.595e-01	2.129e-01	0.03088 *
	(1-) **72**	1.022e+00	1.944e-01	1.47e-07 ***
	(24-) **48**	-1.466e-01	1.808e-01	0.417494
	(24-) **72**	4.155e-01	1.586e-01	0.008792 **
	(48-) **72**	0.56212	0.16596	0.000707 ***
PE+PS-0.05	(1-) **24**	1.147883	0.199796	9.18e-09 ***
	(1-) **48**	0.693147	0.213201	0.001149 **
	(1-) **72**	-0.062520	0.250122	0.802619
	(24-) **48**	-0.454736	0.157375	0.003858 **
	(24-) **72**	-1.210404	0.204630	3.32e-09 ***
	(48-) **72**	-0.75567	0.21774	0.000519 ***
PE+PS-5	(1-) **24**	0.91629	0.19194	1.81e-06 ***
	(1-) **48**	0.567106	0.203079	0.005230 **
	(1-) **72**	0.581922	0.202538	0.004064 **
	(24-) **48**	-0.34918	0.15954	0.028615 *
	(24-) **72**	-0.33437	0.15885	0.035293 *
	(48-) **72**	0.014815	0.172137	0.931414
PE+PS-50	(1-) **24**	1.05416	0.24214	1.34e-05 ***
	(1-) **48**	1.20831	0.23762	3.68e-07 ***
	(1-) **72**	1.22121	0.23727	2.65e-07 ***
	(24-) **48**	1.542e-01	1.677e-01	0.358118
	(24-) **72**	1.671e-01	1.672e-01	0.317873
	(48-) **72**	0.012903	0.160647	0.93598

d.f. = 238

Table S2: Results of Poisson regression analyses for the mortality of blood cells. Comparisons between the four timepoints are shown for each treatment. P-values (right column) under 0.05, 0.01 and 0.001 (, **, *** respectively) show statistical differences in the cell mortality of two timepoints. Negative estimates show that the mortality at the timepoints in bold are significantly lower than the timepoints in parenthesis, while positive estimates show that the mortality at the timepoints in bold are significantly higher. Degrees of freedom are shown at the end of the table.*

Treatment	Time (h)	Estimate	Standard error	P-value
C	(1-) **24**	-0.48445	0.16235	0.002845 **
	(1-) **48**	-0.36819	0.15765	0.019516 *
	(1-) **72**	-0.20020	0.15155	0.186491
	(24-) **48**	0.1163	0.1609	0.4700
	(24-) **72**	0.28425	0.15494	0.06657
	(48-) **72**	0.1680	0.1500	0.2628
PE-0.05	(1-) **24**	-0.55748	0.15321	0.000274 ***
	(1-) **48**	-0.45811	0.14853	0.002040 **
	(1-) **72**	0.224169	0.129397	0.083201
	(24-) **48**	0.09937	0.16864	0.555684
	(24-) **72**	0.78165	0.15206	2.74e-07 ***
	(48-) **72**	0.68228	0.14734	3.65e-06 ***
PE-5	(1-) **24**	-0.123614	0.157435	0.4324
	(1-) **48**	-0.047628	0.154347	0.7576
	(1-) **72**	0.227784	0.144524	0.1150
	(24-) **48**	7.599e-02	0.1592	0.6332
	(24-) **72**	0.3514	0.1497	0.0189 *
	(48-) **72**	0.2754	0.1465	0.0601
PE-50	(1-) **24**	-0.234193	0.157726	0.13759
	(1-) **48**	-0.193371	0.155955	0.21501
	(1-) **72**	0.407661	0.140572	0.00373 **
	(24-) **48**	4.082e-02	0.1650	0.8046
	(24-) **72**	0.6419	0.1505	2.01e-05 ***
	(48-) **72**	0.601032	0.148675	5.29e-05 ***
PS-0.05	(1-) **24**	-0.24604	0.15970	0.1234
	(1-) **48**	-0.35726	0.16447	0.0298 *
	(1-) **72**	0.20235	0.14404	0.1601
	(24-) **48**	-0.1112	0.1669	0.50520
	(24-) **72**	0.4484	0.1468	0.00226 **
	(48-) **72**	0.559616	0.152015	0.000232 ***
PS-5	(1-) **24**	-0.013423	0.163849	0.9347
	(1-) **48**	0.039221	0.161721	0.8084
	(1-) **72**	0.319181	0.157990	0.0434 *
	(24-) **48**	0.052644	0.162278	0.7456
	(24-) **72**	0.332604	0.158560	0.0359 *
	(48-) **72**	0.279960	0.156359	0.0734
PS-50	(1-) **24**	-0.081346	0.180483	0.652198
	(1-) **48**	-0.133531	0.182981	0.465539

	(1-) **72**	0.242946	0.174158	0.163022
	(24-) **48**	-0.052186	0.186564	0.7797
	(24-) **72**	0.324292	0.177919	0.0683
	(48-) **72**	0.37648	0.18045	0.0370 *
PE+PS-0.05	(1-) **24**	0.064539	0.1607	0.68802
	(1-) **48**	0.1369	0.1580	0.38636
	(1-) **72**	0.2593	0.1602	0.10566
	(24-) **48**	0.072321	0.155331	0.64151
	(24-) **72**	0.194744	0.157625	0.21665
	(48-) **72**	0.122423	0.154834	0.42913
PE+PS-5	(1-) **24**	-8.004e-02	0.1634	0.62430
	(1-) **48**	-0.1978	0.1687	0.24082
	(1-) **72**	0.3582	0.1535	0.01964 *
	(24-) **48**	-0.1178	0.1718	0.49297
	(24-) **72**	0.4383	0.1570	0.00524 **
	(48-) **72**	0.556038	0.162409	0.000618 ***
PE+PS-50	(1-) **24**	-0.3137	0.1742	0.07184
	(1-) **48**	-6.625e-02	0.1628	0.68414
	(1-) **72**	-0.1490	0.1752	0.39482
	(24-) **48**	0.24741	0.17675	0.1616
	(24-) **72**	0.16462	0.18814	0.3816
	(48-) **72**	-0.08279	0.17764	0.641190

d.f.=229

Table S3: Results of Poisson regression analyses for the mortality of head kidney cells. Comparisons between the four timepoints are shown for each treatment. P-values (right column) under 0.05, 0.01 and 0.001 (, **, *** respectively) show statistical differences in the cell mortality of two timepoints. Negative estimates show that the mortality at the timepoints in bold are significantly lower than the timepoints in parenthesis, while positive estimates show that the mortality at the timepoints in bold are significantly higher. Degrees of freedom are shown at the end of the table.*

Treatment	Time (h)	Estimate	Standard error	P-value
C	(1-) **24**	-0.302281	0.184663	0.101645
	(1-) **48**	-0.075223	0.173544	0.664685
	(1-) **72**	0.484392	0.153039	0.001550 **
	(24-) **48**	0.22706	0.18770	0.226409
	(24-) **72**	0.786673	0.168926	3.21e-06 ***
	(48-) **72**	0.55962	0.15669	0.000355 ***
PE-0.05	(1-) **24**	0.09309	0.21590	0.66634
	(1-) **48**	0.53492	0.19666	0.00653 **
	(1-) **72**	0.70527	0.19089	0.00022 ***
	(24-) **48**	0.441833	0.191070	0.020755 *
	(24-) **72**	0.612178	0.185122	0.000943 ***
	(48-) **72**	0.170345	0.162276	0.29385
PE-5	(1-) **24**	0.332134	0.228127	0.145416
	(1-) **48**	0.780159	0.210219	0.000206 ***
	(1-) **72**	0.9461	0.2051	3.97e-06 ***
	(24-) **48**	0.448025	0.188754	0.017616 *
	(24-) **72**	0.614010	0.183038	0.000795 ***
	(48-) **72**	0.165985	0.160165	0.300044
PE-50	(1-) **24**	0.16127	0.21512	0.453451
	(1-) **48**	0.60158	0.19672	0.002228 **
	(1-) **72**	0.82198	0.18971	1.47e-05 ***
	(24-) **48**	0.440312	0.187014	0.018551 *
	(24-) **72**	0.660712	0.179623	0.000235 ***
	(48-) **72**	0.2204	0.1571	0.16070
PS-0.05	(1-) **24**	-0.27193	0.19159	0.15579
	(1-) **48**	0.09097	0.17426	0.60163
	(1-) **72**	0.51083	0.15936	0.00135 **
	(24-) **48**	0.362905	0.187952	0.0535
	(24-) **72**	0.782759	0.174232	7.03e-06 ***
	(48-) **72**	0.419854	0.154972	0.00674 **
PS-5	(1-) **24**	-0.7178	0.1929	0.000198 ***
	(1-) **48**	-0.1872	0.1640	0.253692
	(1-) **72**	0.1151	0.1519	0.448642
	(24-) **48**	0.530628	0.199263	0.007746 **
	(24-) **72**	0.832909	0.189392	1.09e-05 ***
	(48-) **72**	0.30228	0.15992	0.058736
PS-50	(1-) **24**	-0.44731	0.20502	0.02913 *
	(1-) **48**	0.19319	0.17293	0.26394

	(1-) **72**	0.38894	0.16584	0.01902 *
	(24-) **48**	0.640503	0.197875	0.00121 **
	(24-) **72**	0.836248	0.191708	1.29e-05 ***
	(48-) **72**	0.1957	0.1569	0.2123
PE+PS-0.05	(1-) **24**	-0.154151	0.210280	0.46351
	(1-) **48**	0.412245	0.184178	0.02520 *
	(1-) **72**	0.490206	0.181406	0.00689 **
	(24-) **48**	0.566395	0.193192	0.003370 **
	(24-) **72**	0.644357	0.190550	0.000721 ***
	(48-) **72**	7.796e-02	0.1613	0.62883
PE+PS-5	(1-) **24**	-0.09393	0.19992	0.63847
	(1-) **48**	0.24362	0.17537	0.16478
	(1-) **72**	0.43937	0.16838	0.00907 **
	(24-) **48**	0.33755	0.19037	0.07620
	(24-) **72**	0.53330	0.18395	0.00374 **
	(48-) **72**	0.1957	0.1569	0.2123
PE+PS-50	(1-) **24**	-0.145712	0.191199	0.44600
	(1-) **48**	0.212922	0.175065	0.22389
	(1-) **72**	0.365114	0.169451	0.03119 *
	(24-) **48**	0.3586	0.1825	0.04940 *
	(24-) **72**	0.5108	0.1771	0.00393 **
	(48-) **72**	0.1522	0.1596	0.3402

d.f. = 238

Phagocytic ability

Table S4: Poisson regression results of the analyses of the phagocytic ability of all the three tissues. The estimates of the coefficients in the columns DI, Blood and HK indicate the differences in phagocytic ability between timepoints. Negative numbers in red indicate that the phagocytic ability at the timepoints in bold are significantly lower than the timepoints in parenthesis, while positive numbers in green indicate that the phagocytic ability at the timepoints in bold are significantly higher. The numbers in black indicate no significant differences. X indicates missing data for the given parameter. D.f. show the degrees of freedom for each tissue.

Polymer	Concentration (mg/L)	Time (h)	DI *d.f.=107*	Blood *d.f.=160*	HK *d.f.=161*
PE-MP	0.05	(1-) **24**	X	1.3863	-0.58779
		(1-) **48**	X	1.0986	0.44183
		(24-) **48**	X	-0.28768	1.02962*
	5	(1-) **24**	0.72744***	0.59876***	0.28227***
		(1-) **48**	-0.56253**	0.86963***	0.19555**
		(24-) **48**	-1.28996***	0.27087***	-0.08672
	50	(1-) **24**	6.586e-01***	0.29745***	0.32356***
		(1-) **48**	2.157e-01***	0.57833***	0.42953***
		(24-) **48**	-4.429e-01***	0.28088***	0.10576 ***
PS-MP	0.05	(1-) **24**	X	X	-2.124e-06
		(1-) **48**	X	-0.1823	-2.124e-06
		(24-) **48**	X	X	5.439e-14
	5	(1-) **24**	-0.4838*	1.88965**	0.102857
		(1-) **48**	-1.40009***	0.91203***	-0.298855*
		(24-) **48**	-0.9163**	-0.9776***	-0.401713**
	50	(1-) **24**	-0.05688	0.74904***	-0.12148**
		(1-) **48**	-0.60574***	0.56689***	-0.65716***
		(24-) **48**	-0.54886***	-0.18215***	-0.53569***
PE+PS-MP	0.05	(1-) **24**	X	1.6094*	-2.398e-10
		(1-) **48**	X	1.2993*	-2.231e-01
		(24-) **48**	X	-0.31015	-2.231e-01
	5	(1-) **24**	-1.961e-01	0.70476***	0.096768
		(1-) **48**	-7.095e-02	0.42092***	-0.32393***
		(24-) **48**	0.1252	-0.28384***	-0.31845***
	50	(1-) **24**	0.666152***	-0.05263	0.59551***
		(1-) **48**	0.568226***	0.49693***	0.57417***
		(24-) **48**	-0.09793	0.549559***	-0.02324

*, ** and *** indicate statistical differences at significance levels of 5, 1 and 0.1 %, respectively

Table S5: Poisson regression results of the analysis of the phagocytic ability of all the three tissues. The estimates of the coefficients provided in the columns DI, Blood and HK indicate the differences in the ability of cells to ingest different polymers. Negative numbers in red indicate that the phagocytic ability of the polymer in bold is significantly lower than the one of the polymer in parenthesis, while positive numbers in green indicate that the phagocytic ability of the polymer in bold is significantly higher. The number in black indicate no significant differences. X indicates the missing data for the given parameter. D.f. show the degrees of freedom for each tissue.

Time (h)	Concentration (mg/L)	Polymer	DI *d.f.=107*	Blood *d.f.=160*	HK *d.f.=161*
1	0.05	(PE-) **PS**	X	1.6864*	-1.50408
		(PE-) **PE+PS**	X	0.4055	-5.878e-01
		(PS-) **PE+PS**	X	-1.2809	9.163e-01
	5	(PE-) **PS**	-0.16389	-1.75565***	-1.35024***
		(PE-) **PE+PS**	-0.16389	-0.23983**	-0.21347**
		(PS-) **PE+PS**	-3.434e-15	1.51583***	1.136764***
	50	(PE-) **PS**	2.957e-01***	-0.97442***	-0.65257***
		(PE-) **PE+PS**	-4.047e-01***	0.08448*	-0.41929***
		(PS-) **PE+PS**	-0.700385***	1.05890***	0.23328***
24	0.05	(PE-) **PS**	X	X	-9.163e-01
		(PE-) **PE+PS**	X	0.62861	-1.132e-13
		(PS-) **PE+PS**	X	X	9.163e-01
	5	(PE-) **PS**	-1.37512***	-0.46476***	-1.529646***
		(PE-) **PE+PS**	-1.0874***	-0.13382*	-0.39897***
		(PS-) **PE+PS**	0.2877	0.33094***	1.130674***
	50	(PE-) **PS**	-4.198e-01***	-0.52283***	-1.09358***
		(PE-) **PE+PS**	-3.971e-01***	-0.26559***	-0.14512***
		(PS-) **PE+PS**	0.02264	0.25724***	0.94847***
48	0.05	(PE-) **PS**	X	4.055e-01	-1.94591*
		(PE-) **PE+PS**	X	0.60614	-1.25276*
		(PS-) **PE+PS**	X	0.20067	6.931e-01
	5	(PE-) **PS**	-1.00145***	-1.71326	-1.84464***
		(PE-) **PE+PS**	0.32769	-0.68854	-0.63619***
		(PS-) **PE+PS**	1.32914***	1.02472***	1.20846***
	50	(PE-) **PS**	-0.52569***	-0.98586	-1.73926***
		(PE-) **PE+PS**	-0.05212	0.003085	-0.27464***
		(PS-) **PE+PS**	0.47358***	0.98895***	1.46461***

*, ** and *** indicate statistical differences at significance levels of 5, 1 and 0.1 %, respectively

Table S6: Poisson regression results of the analysis of the phagocytic ability of all the three tissues. The estimates of the coefficients given in the columns DI, Blood and HK show the differences between concentrations. Positive numbers in green indicate that the phagocytic ability at the concentration in bold is significantly higher than the concentration in parenthesis. The numbers in black indicate no significant differences. X indicates missing data for the given parameter. D.f. show the degrees of freedom for each tissue.

Polymer	Time (h)	Concentration (mg/L)	DI *d.f.=107*	Blood *d.f.=160*	HK *d.f.=161*
PE-MP	1	(0.05-) **5**	X	4.9127***	3.94050***
		(0.05-) **50**	X	6.58755***	5.47553***
		(5-) **50**	1.97376***	1.67490***	1.53503***
	24	(0.05-) **5**	X	4.12512***	4.81056***
		(0.05-) **50**	X	5.49870***	6.387e+00***
		(5-) **50**	1.90495***	1.37359***	1.57632***
	48	(0.05-) **5**	X	4.68367***	3.69422***
		(0.05-) **50**	X	6.067e+00***	5.46323***
		(5-) **50**	2.75197***	1.38359***	1.76900***
PS-MP	1	(0.05-) **5**	X	1.4706***	4.094342***
		(0.05-) **50**	X	3.9267***	6.32704***
		(5-) **50**	2.43337***	2.45613***	2.23270***
	24	(0.05-) **5**	X	X	4.197202***
		(0.05-) **50**	X	X	6.206e+00
		(5-) **50**	2.8603***	1.31552***	2.008365***
	48	(0.05-) **5**	X	2.56495***	3.795e+00***
		(0.05-) **50**	X	4.67594***	5.670e+00***
		(5-) **50**	3.22773***	2.11099***	1.87439***
PE+PS-MP	1	(0.05-) **5**	X	4.2674***	4.315e+00***
		(0.05-) **50**	X	6.26657***	5.644e+00***
		(5-) **50**	1.733e+00***	1.99920***	1.329215***
	24	(0.05-) **5**	X	3.36269***	4.411585***
		(0.05-) **50**	X	4.60450***	6.241e+00***
		(5-) **50**	2.5953***	1.24182***	1.829859***
	48	(0.05-) **5**	X	3.38900***	4.310799***
		(0.05-) **50**	X	5.464217***	6.44135***
		(5-) **50**	2.37217***	2.075215***	2.13055***

*, ** and *** indicate statistical differences at significance levels of 5, 1 and 0.1 %, respectively

Phagocytic capacity

Table S7: Poisson regression results of the analysis of the phagocytic capacity of all the three tissues. Here the differences between the time points are inferred based on the estimates of the coefficients in columns DI, Blood, and HK. Positive number in green indicates that the phagocytic capacity at the timepoint in bold is significantly higher than the timepoint in parenthesis. The numbers in black indicate no significant differences. X indicates missing data for the given parameter. D.f. show the degrees of freedom for each tissue.

Polymer	Concentration (mg/L)	Time (h)	DI *d.f.=80*	Blood *d.f.=124*	HK *d.f.=134*
PE-MP	0.05	(1-) **24**	X	-4.223e-15	1.224e-14
		(1-) **48**	X	-4.422e-15	1.227e-14
		(24-) **48**	X	7.102e-16	2.169e-16
	5	(1-) **24**	2.676e-15	0.011085	-3.145e-03
		(1-) **48**	2.601e-15	-0.003190	-1.264e-02
		(24-) **48**	-7.692e-17	-0.014275	-0.009494
	50	(1-) **24**	-0.037617	0.060003	0.04426
		(1-) **48**	0.116534	0.022372	0.03161
		(24-) **48**	1.542e-01*	-0.037631	-0.012650
PS-MP	0.05	(1-) **24**	X	X	-1.648e-15
		(1-) **48**	X	-1.732e-15	-3.205e-16
		(24-) **48**	X	X	3.149e-16
	5	(1-) **24**	1.608e-16	-0.001450	0.026922
		(1-) **48**	-7.393e-17	-0.068993	-0.016182
		(24-) **48**	2.277e-16	-0.067543	-0.043103
	50	(1-) **24**	-9.985e-02	0.054533	0.008186
		(1-) **48**	-9.985e-02	0.065091	-0.08428
		(24-) **48**	-1.182e-16	0.01056	-0.092461
PE+PS-MP	0.05	(1-) **24**	X	-3.253e-16	1.262e-18
		(1-) **48**	X	1.645e-16	-1.617e-17
		(24-) **48**	X	2.668e-16	-3.020e-17
	5	(1-) **24**	-0.048790	0.030724	-0.012638
		(1-) **48**	-0.048790	-0.038160	-0.023830
		(24-) **48**	-5.318e-17	-0.068885	-0.011191
	50	(1-) **24**	-0.019268	-0.023359	-1.438e-03
		(1-) **48**	0.107267	0.015748	1.711e-15
		(24-) **48**	0.12654	0.03911	0.001438

*, ** and *** indicate statistical differences at significance levels of 5, 1 and 0.1 %, respectively

Table S8: Poisson regression results of the analysis of the phagocytic capacity of all the three tissues. The estimates of the coefficients provided in the columns DI, Blood and HK indicate the differences in the capacity of cells to ingest different polymers. Negative number in red indicates that the phagocytic capacity of the polymer in bold is significantly lower than the polymer in parenthesis, while positive number in green indicates that the phagocytic capacity of the polymer in bold is significantly higher. The numbers in black indicate no significant differences. X indicates missing data for the given parameter. D.f. show the degrees of freedom for each tissue.

Time (h)	**Concentration (mg/L)**	**Polymer**	**DI** *d.f.=80*	**Blood** *d.f.=124*	**HK** *d.f.=134*
		(PE-) **PS**	X	-3.530e-15	1.226e-14
	0.05	(PE-) **PE+PS**	X	-4.167e-15	1.219e-14
		(PS-) **PE+PS**	X	-8.747e-16	-2.617e-16
		(PE-) **PS**	2.722e-15	0.094151	-0.022223
1	**5**	(PE-) **PE+PS**	4.879e-02	0.020489	-2.046e-15
		(PS-) **PE+PS**	0.048790	-0.073662	0.022223
		(PE-) **PS**	0.062228	0.022372	0.06363
	50	(PE-) **PE+PS**	0.009267	0.044255	0.01593
		(PS-) **PE+PS**	-0.052962	0.021883	-4.769e-02
		(PE-) **PS**	X	X	6.956e-16
	0.05	(PE-) **PE+PS**	X	5.713e-16	1.334e-16
		(PS-) **PE+PS**	X	X	4.820e-16
		(PE-) **PS**	-4.327e-17	0.081616	0.007843
24	**5**	(PE-) **PE+PS**	-3.044e-18	0.040129	-0.009494
		(PS-) **PE+PS**	1.772e-16	-0.041487	-0.017337
		(PE-) **PS**	9.878e-17	0.016902	0.027550
	50	(PE-) **PE+PS**	2.762e-02	-0.039107	-0.029768
		(PS-) **PE+PS**	2.762e-02	-0.05601	-0.057318
		(PE-) **PS**	X	-3.229e-16	-5.111e-18
	0.05	(PE-) **PE+PS**	X	-4.380e-16	9.092e-18
		(PS-) **PE+PS**	X	1.046e-15	-2.994e-16
		(PE-) **PS**	1.043e-17	0.02835	-0.025766
48	**5**	(PE-) **PE+PS**	-1.088e-16	-0.014481	-0.011191
		(PS-) **PE+PS**	7.065e-17	-0.042830	0.014575
		(PE-) **PS**	-1.542e-01*	0.065091	-0.05226
	50	(PE-) **PE+PS**	2.081e-17	0.037631	-1.568e-02
		(PS-) **PE+PS**	1.542e-01*	-0.02746	3.658e-02

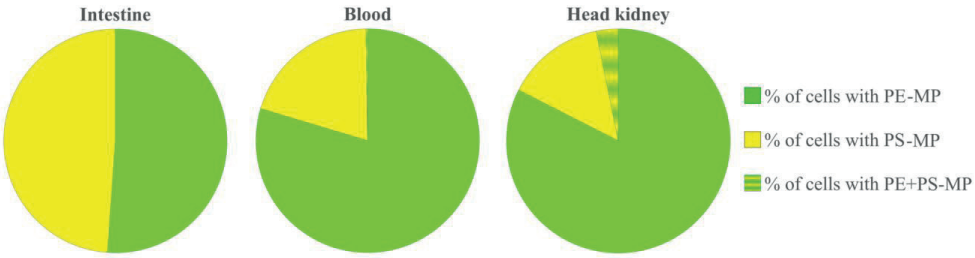
*, ** and *** indicate statistical differences at significance levels of 5, 1 and 0.1 %, respectively

Table S9: Poisson regression results of the analysis of the phagocytic capacity of all the three tissues. The estimates of the coefficients given in the columns DI, Blood and HK show the differences between concentrations. Positive numbers in green indicate that the phagocytic capacity at the concentrations in bold are significantly higher than the concentrations in parenthesis. The numbers in black indicate no significant differences. X indicates missing data for the given parameter. D.f. show the degrees of freedom for each tissue.

Polymer	Time (h)	Concentration (mg/L)	DI *d.f.=80*	Blood *d.f.=124*	HK *d.f.=134*
PE-MP	1	(0.05-) **5**	X	4.561e-02	5.984e-02
		(0.05-) **50**	X	9.985e-02	1.325e-01*
		(5-) **50**	3.762e-02	0.054235	7.265e-02
	24	(0.05-) **5**	X	5.670e-02	5.670e-02
		(0.05-) **50**	X	0.159849*	0.176751**
		(5-) **50**	-4.768e-17	0.103153	0.120055*
	48	(0.05-) **5**	X	0.04242	0.047202
		(0.05-) **50**	X	0.122218	0.164101**
		(5-) **50**	1.542e-01	0.079797	0.116899*
PS-MP	1	(0.05-) **5**	X	1.398e-01	3.762e-02
		(0.05-) **50**	X	1.222e-01	1.961e-01
		(5-) **50**	9.985e-02	-0.017544	0.158498**
	24	(0.05-) **5**	X	X	0.064539
		(0.05-) **50**	X	X	0.204300
		(5-) **50**	2.459e-16	0.038439	0.139762**
	48	(0.05-) **5**	X	0.070769	0.021435
		(0.05-) **50**	X	0.18731*	0.11184
		(5-) **50**	-2.898e-16	0.11654*	0.09040
PE+PS-MP	1	(0.05-) **5**	X	6.610e-02	5.984e-02
		(0.05-) **50**	X	1.441e-01	1.484e-01*
		(5-) **50**	-0.001907	-0.017544	8.858e-02
	24	(0.05-) **5**	X	0.096824	0.047202
		(0.05-) **50**	X	0.12074	0.146982*
		(5-) **50**	0.02762	0.02392	0.099781
	48	(0.05-) **5**	X	0.027939	0.036010
		(0.05-) **50**	X	0.159849*	1.484e-01*
		(5-) **50**	1.542e-01	0.131909*	1.124e-01*

*, ** and *** indicate statistical differences at significance levels of 5, 1 and 0.1 %, respectively

Supplementary figure



Supplementary figure: Cells exposed to high concentrations (50 mg/L) of the MP mixture after 1 h of exposure. The pie charts show, for each tissue, the percentage of cells that only phagocytosed PE (green), only PS (yellow) or that phagocytosed both polymer types (green and yellow).

Paper III

Distinct polymer dependent sorption of persistent pollutants associated with Atlantic salmon farming to microplastics

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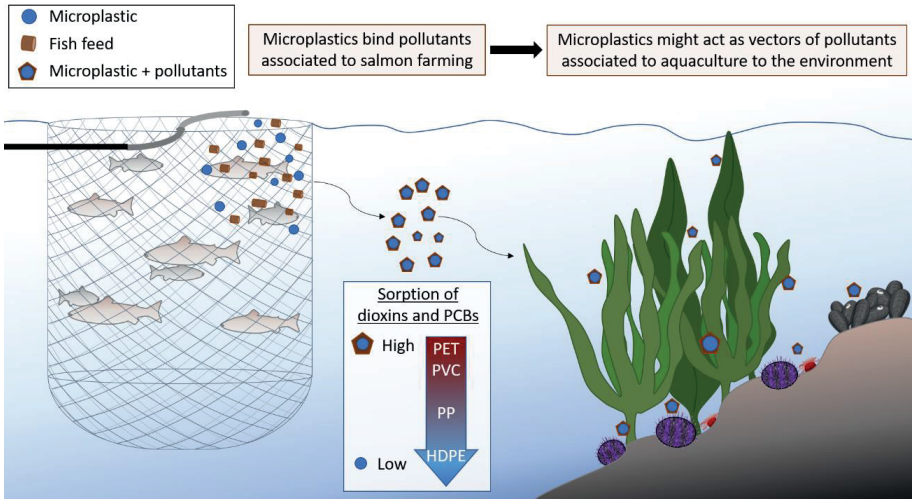
Abstract

Fish feed used in Atlantic salmon aquaculture contains quantifyable levels of persistent organic pollutants, POPs, that are released along with the organic waste into the water near fish farms. Plastics used in fish farming, such as feeding pipes, are sources of microplastics to the surrounding environment due to wear and tear. In this study we placed caged HDPE, PP, PET and PVC microplastics close to two salmon farms for three months, and analysed them for sorbed POPs. For comparison, additional microplastics were deployed in a non-polluted marine reference site and in a polluted marine urban harbour. As a positive control, microplastics were incubated with fish feed under laboratory conditions. Furthermore, blue mussels were caged next to the microplastics placed in the sea and analysed for POPs. After three months in the sea, the composition of POPs adsorbed on the microplastics placed next to the fish farms were similar to that of the positive control and different from the reference and harbour sites. PET and PVC sorbed significantly higher levels of dioxins and PCBs compared to HDPE, while the levels sorbed to PP were intermediate and did not differ statistically from PET, PVC or HDPE. In addition,composition of dioxins accumulated in blue mussels did not reflect the patterns observed on the microplastics probably due to polymer-specific affinity of POPs. In conclusion, the results of this study show that microplastics sorb POPs associated with salmon farming differentially, depending on the polymer type, and that they can potentially be vectors of such chemicals in the marine environment.



Graphical abstract.

Keywords: Microplastic (MP), Atlantic salmon farming, aquaculture, persistent organic pollutant (POP), environmental impact, chemical interaction

Abbreviations

BTBPE	1,2-bis(2,4,6-tribromophenoxy)ethane
DDT	Dichlorodiphenyltrichloroethane
EQS	Environmental Quality Standards
FF	Fish farm
GC-ECD	Gas Chromatography with Electron Capture Detector
GC-MS	Gas chromatography-mass spectrometry
HCH	Hexachlorocyclohexane
HDPE	High density polyethylene
HxCDD/F	Hexachlorodibenzo-p-dioxin/furan
HpCDD/F	Heptachlorodibenzo-para-dioxin/furan
HRGC/HRMS	High resolution gas chromatography/high resolution mass spectrometry
MP	Microplastic
LDPE	Low density polyethylene
LOD	Level of Detection
LOQ	Level of Quantification
OCDD/F	Octachlorodibenzodioxin/furan

PAH	Polycyclic Aromatic Hydrocarbon
PBCCH	Pentabromochorocyclohexane
PBDE	Polybrominated diphenyl ethers
PBT	2,3,4,5,6-pentabromotoluene
PCB	Polychlorinated biphenyl
PCDD/F	Polychlorinated dibenzodioxins/furans
PE	Polyethylene
PeCDD/F	Pentachlorodibenzo-P-dioxin/furan
PET	Polyethylene terephthalate
PP	Polypropylene
POP	Persistent organic pollutant
pTBX	2,3,5,6-tetrabromo-p-xylene
PVC	Polyvinyl chloride
TBPhA	Tetrabromophthalic anhydride
TBoCT	Tetrabromo-o-chlorotoluene
TCDD/TCDF	Tetrachlorodibenzo-p-dioxin/furan
TEQ	Toxic Equivalency
TEF	Toxic Equivalency Factor
uPVC	Unplasticized polyvinyl chloride

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1. Introduction

Microplastics (MPs), commonly defined as plastic particles ranging 1 µm to 5 mm (Hartmann et al., 2019), are ubiquitous. In the marine environment, they have been reported in coastal waters, surface waters, the water column, deep-sea sediments, estuaries and fjords (Lusher, 2015, Harris, 2020). MPs can have a wide range of negative effects on biota, from reduced feeding to immune system alterations (Lusher, 2015). Depending on size and shape, MPs can cross body barriers, e.g. be translocated from the digestive tract to other tissues and organs, and be taken up into cells (Abihssira-García et al., 2020). For instance, MPs have been detected in liver and fillet of both farmed and wild salmonids (Gomiero et al., 2020a). Plastic microfibers have also been detected in several organs from blue mussels exposed to concentrations of 2000 particles/L, including mantle and foot (Kolandhasamy et al., 2018). Accumulation of MPs in edible tissues might constitute a health risk for human consumers. Negative effects of MPs may derive from plastic additives as well as the plastic polymer particles themselves (Campanale et al., 2020, Kögel et al., 2020). MPs can also sorb persistent organic pollutants (POPs) due the hydrophobic nature these chemicals, and act as vectors for POPs in the marine environment (Hirai et al., 2011). For example, several studies have documented sorption of PCBs, PAHs, pesticides and other POPs, on MPs collected from marine environments (Hirai et al., 2011, Rodrigues et al., 2019, Rochman et al., 2013a, Ziccardi et al., 2016, Chen et al., 2019). POPs sorbed to MPs may be transferred to the organisms after ingestion, increasing and complicating the impact of such particles and pollutants (Rochman et al., 2013b, Chua et al., 2014). Even though it has been argued that the amount of pollutant transferred from MPs to organisms might be irrelevant compared to dietary or environmental exposure, and that MPs could pass through the gastrointestinal tract without leaching chemicals (Koelmans, 2015, Lohmann, 2017), translocation of small MPs into tissues, organs and cells of non-polluted organisms could be a pathway for uptake of POPs in marine organisms (Koelmans, 2015, Bakir et al., 2016), potentially leading to bioaccumulation. In addition, interaction of microplastics with certain POPs such as dioxins should be studied more in depth. To our knowledge, there is no record of levels of dioxins adhered to MPs in the environment except for the levels found in charred MPs (Saliu et al., 2018). Dioxins are chemical substances formed as byproduct during combustion reactions in the presence of chlorine (Altarawneh et al., 2009). In vertebrates, dioxins are carcinogenic and can disrupt the immune, nervous and

endocrine systems, and affect reproduction and development (Schecter et al., 2006). Considering the increasing numbers of wildfires as result of climate change (Higuera and Abatzoglou, 2021, Calheiros et al., 2021), and that dioxins in polar regions tend to accumulate due to slower degradation in cold areas (Kobusińska et al., 2020), more attention should be paid to the impact of dioxins in the environment together with microplastics.

Atlantic salmon (*Salmo salar*) is one of the most consumed fish species in the world and the commercially most important farmed marine fish species (FAO, 2020). However, the levels of pollutants associated with Atlantic salmon faming has long been a concern. Salmon feed contains about 10% of fish oil (Ytrestøyl et al., 2015), which is the main source of POPs to farmed salmon. Analysis of 20 salmon feed samples randomly collected from salmon farms in Norway in 2017 showed concentrations of polychlorinated biphenyls (PCB_6) of 3 µg/kg, sum of dioxins (PCDD/PCDF) and dioxin-like PCBs of 0.6 ng TEQ/kg, and concentrations of polybrominated diphenyl ethers ($PBDE_7$) of 0.38 µg/kg (Sele et al., 2019). In addition, fish feed also contained traces of organochlorine pesticides such as DDT (and degradation products), toxaphene and endosulfan, and organophosphorus pesticides such as chlorpyrifos-methyl and pirimiphos-methyl. In recent years, replacement of fish oils with plant oils has led to reduced levels of POPs in farmed Atlantic salmon in Norway (Berntssen et al., 2010, Nøstbakken et al., 2015, Lundebye et al., 2017). However, with the increased use of plant ingredients, agricultural pesticides and PAHs have been detected in higher concentrations in the feed (Berntssen et al., 2010, Nácher-Mestre et al., 2014).

For each kg of salmon produced, about 0.5 kg of feces and unconsumed feed pellets are generated (Grefsrud et al., 2018). This waste slips through the open-cage net pens and spreads in the environment, depending on local physical, chemical and biological factors. Open fish farms thus represent local point-sources of pollution. Yet little is known about how MPs might contribute to the spreading of POPs near fish farms. Fish farms benefit from the lightweight, strong and flexible plastic in permanent installations. Net pens, ropes, floats and pontoons are some of the framework structure made of plastic polymers such as HDPE, PP, PET or PVC (Lusher et al., 2017). Abrasion and loss of plastic items inevitably lead to the release of MPs into the environment. A recent study showed the presence of MP polymers such as PP, PE, PVC and PET in sediment, seawater and suspended matter around Atlantic salmon farms of Norway. Moreover, feeding pipes used

in fish farming, mainly made of HDPE, have been shown to actively release MPs along with the fish feed, in which PE, PP and PET MPs have also been reported (Gomiero et al., 2020b). In Norway, about 1000 commercial salmonid farm facilities are placed in the marine environment (BarentsWatch, 2021), and the amount of Atlantic salmon produced annually is about 1.3 million metric tons (Ytrestøyl et al., 2015). Thus, understanding the potential role of MPs as vector of pollutants from this activity is of vital importance for countries such as Norway.

The objective of this study was to evaluate whether MPs can significantly sorb POPs coming from fish feed and consequently act as potential vectors of those chemicals to the surrounding environment. In addition, to better understand the impact of different MP polymers in the environment, we assessed the sorption capacity of different polymer types to POPs. For this purpose, four types of MP polymers commonly found in the environment and detected in the vicinity of salmon farming facilities (Suaria et al., 2016, Bråte et al., 2017, Gomiero et al., 2020b) were placed close to two salmon farms for three months. For comparison, MPs were additionally deployed in two sites not influenced by salmon farming; one low-polluted and one polluted location. Furthermore, a positive control for fish feed pollutants was set up under laboratory conditions. POPs sorbed to MPs were then qualitatively and quantitatively analysed. Blue mussels (*Mytilus edulis*), often used in water monitoring studies (Beyer et al., 2017), were collected from the reference site and placed next to the MPs at all stations, with the objective of documenting the pattern of POPs in the environment.

2. Materials and methods

2.1. Blue mussels

Adult blue mussels (*Mytilus edulis*) were collected from an unpolluted location in Åselistraumen in Bodø Municipality, Norway (67º12'01"N 14º37'56"E) in October 2018. This site was also used as reference station (negative control) in the experiment. The mussels weighed on average 6.4 ±2.1 g and had a length of 3.7 ±0.2 cm (n=700, mean ±st.dev.). The mussels were transported to the laboratory in aerated water, divided into four groups and used in the field experiment as described below.

2.2. Plastic polymers

Commercial unplasticized and uncoloured MPs were purchased from GoodFellow (Huntingdon, UK). PP (3 mm), HDPE (3 mm), uPVC (250 µm) and PET (3-5 mm) were

selected for this study due to their high occurrence in the marine environment. The three first mentioned polymers have been reported to be among the most abundant plastics in the sea (Suaria et al., 2016), while the last one is the main polymer found in some demersal fish, such as as Atlantic cod, probably due to its high density (Bråte et al., 2016).

2.3. Field experiment

To assess the behaviour of POPs from salmon farming with respect to MPs, four stainless steel cages (40 x 40 cm) containing MPs and mussels were deployed in early October 2018 in four different sites of the Salten district of Nordland, Norway: two at two different Atlantic salmon farms operated by the Salten Aqua AS Group, located in Morsdalsfjorden (FF1) (67º01'37.859N 14º07'26.76 E) and Saltdalsfjorden (FF2) (67°12'10.3"N 15°16'32.3"E); one in a polluted area, Bodø Harbour (67º17'46"N 14º23'43"E); and one in a non-polluted reference station situated in Åselistraumen (67º12'05"N 14º37'14"E; Figure 1).

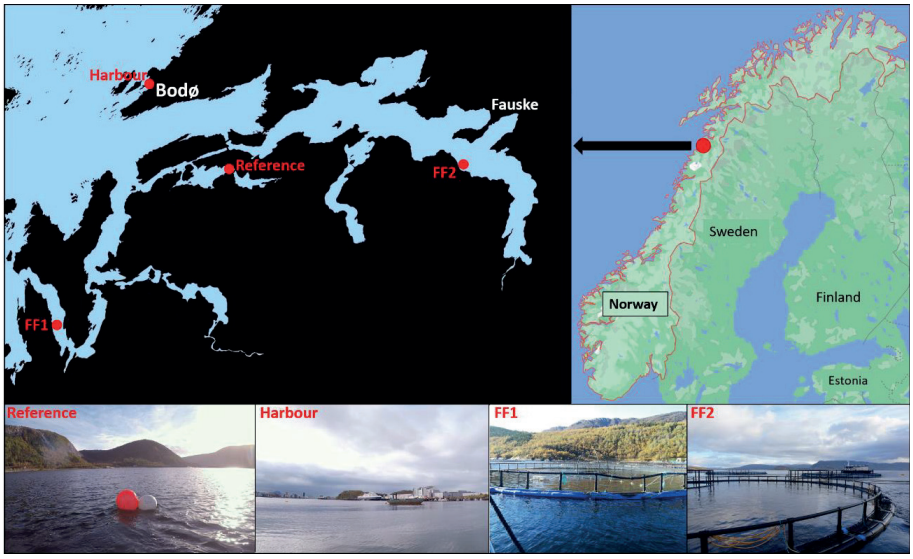

Figure 1: Maps of the studied area (top). Cages were deployed in the Salten district of Nordland, Norway (top right, red point) in four different sites (top left, red points): Åselistraumen as non-polluted control (Reference), Bodø harbour as polluted control, and two fish farms located in Morsdalsfjorden and Saltdalsfjorden (FF1 and FF2 respectively). Bottom: The four studied sites. Pictures obtained on the day the cages were deployed. Maps were extracted from Google Maps; top left map was modified for better visualization.

157 Each cage contained 50 g each of pristine HDPE, PP, PET and uPVC MPs, individually
158 enclosed between two stainless steel sieves of 150 µm mesh size to avoid polymers to
159 mix, and were randomly placed in the cage (Figure 2). Blue mussels (n = 175) were added
160 to each cage to monitor chemicals present in the surrounding water. The four cages were
161 deployed at 5 to 8 meter depth at the fish farms, in approximately 5 meters horizontal
162 distance from the net pens. In January 2019, plastic polymers and mussels were collected
163 after 3 months in the sea and stored at -20ºC for chemical analyses. Cages were deployed
164 in autumn/winter, when the hours of light are at a minimum, to avoid excess biofilm
165 growth on the cages, which would block the water flow to the MPs. Water temperature
166 during the 3 months experiment was obtained from public data recorded at the fish farms.
167 Average temperatures at FF1 and FF2 were 8.7±1.1ºC and 7.3±1.0ºC respectively, and
168 decreased gradually from 10.7 ºC to 6.6 ºC at FF1 and from 8.3 ºC to 5.5 ºC at FF2 from
169 October to January (BarentsWatch, 2021). Water salinity was measured during the
170 experiment with a CTD deployed at 7 m depth in Saltfjorden and Saltdalsfjorden. The
171 recorded practical salinities were 31.5 and 28.8 respectively.

172 2.4. Positive controls

173 To evaluate to which degree feed-based POPs sorb to MPs placed close to fish farms,
174 positive controls were made by mixing fish feed with each of the four types of plastic
175 polymers separately (Figure 2). Twelve beakers of 1 liter, three for each polymer (n=3),
176 were filled with 800 mL of seawater previously filtered through a 10 µm mesh and
177 autoclaved. A concentration of 2 g/L of MPs and 4 g/L of fish feed, previously dried at
178 80 ºC for 19 hours and mashed until powder with a mortar, were added to each flask. The
179 mixtures of feed and MPs were kept in rotation for 3 days at room temperature on a
180 magnetic stirrer at 600 rpm, a speed selected to ensure thorough mixing and to avoid the
181 plastic to float. The MPs were then collected with a small stainless steel spatula and stored
182 at -20ºC until further analyses.

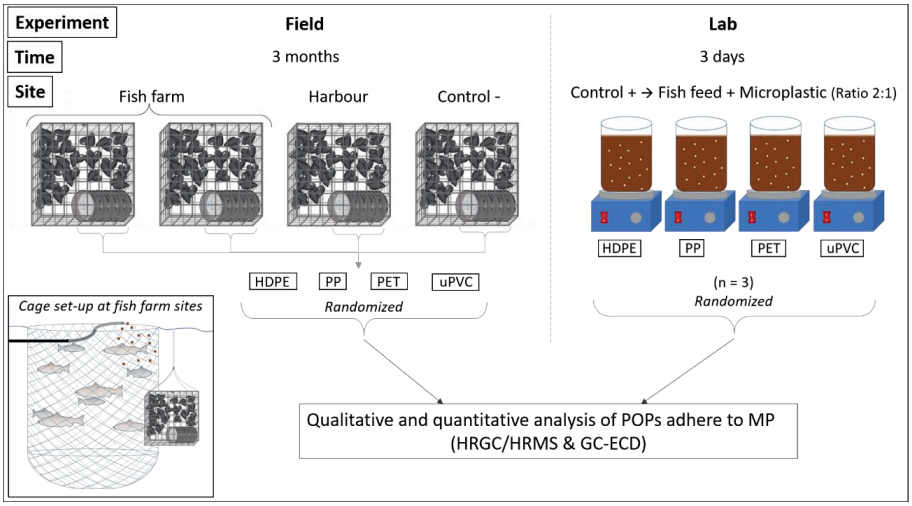

Figure 2: Schematic overview of the experimental design. The field experiment is shown on the left, while the laboratory-made positive controls are shown on the right.

2.5. Chemical analysis

2.5.1. Mussel analysis

Chemical analyses of the soft tissue of whole blue musselswere carried out in the Chemistry and Undesirables Laboratory of the Institute of Marine Research, Bergen, Norway. The methods used to analyse polychlorinated dibenzo-para-dioxins (dioxins/ PCDD), polychlorinated dibenzo- para- furans (furans/PCDF), dioxin-like polychlorinated biphenyls (dl- PCBs (non- ortho and mono-ortho PCBs)), non- dioxin-like polychlorinated biphenyls (ndl- PCBs) and polybrominated diphenyl ethers (PBDEs) are accredited for seafood. Wet or freeze-dried samples were mixed with hydromatrix, and internal standards for dioxins, furans, PCBs and PBDEs were added. The samples underwent extraction with hexane using an Accelerated Solvent Extractor (ASE) instrument. The fat content in the sample was extracted by using sulfuric acid impregnated silica gel. The extract was further purified chromatographically using four consecutive columns, packed with $AgNO_3$-silica gel, H_2SO_4- silica gel, carbon and alumina, respectively, on a GO-HT instrument. Two fractions were collected in the GO-HT process: fraction 1, which contains mono-ortho PCBs, ndl-PCBs and PBDEs, and fraction 2, which contains dioxins, furans and non-ortho PCBs. Dioxins, furans and non-ortho PCBs were analysed using HRGC/HRMS and quantified using an isotope dilution/internal standard method. Mono-ortho PCBs, ndl-PCBs and tri-hepta PBDEs were analysed using GC/MSMS and were quantified using an isotope dilution/intern

standard method. Octa-deca PBDEs were analysed using GC-MS (NCI) and were quantified using an isotope dilution/internal standard method. For dioxins, furans and dl-PCB the toxic equivalent values (TEQ) were calculated by multiplying the quantified concentrations of each congener with the toxic equivalent factor (TEF) of the associated congener.

For pesticide analysis, all samples were weighed in wet. Isotope labelled internal standard was added to the sample material and followed by liquid-liquid extraction using the Citrate QuEChERs method by adding acetonitrile, water and citrate salts (Merck). Extraction was followed by low-temperature precipitation by freezing-out at -20°C over night. For clean-up, two rounds of dispersive solid phase extraction (dSPE) were carried out by using Z-sep (Merck). Sample was upconcentrated and solvent exchanged to isooctane before analysis by GC/MSMS. The area of the peaks in the chromatogram were used for quantification by the help of a multi-standard curve. The EURL criteria for pesticide analysis (described in the SANTE document) was followed.

The limit of quantification (LOQ) was set to three times the value of limit of detection (LOD). LOD was calculated either by "paired observations", the software's noise calculation or by determining the minimum in the calibration curve that is part of the linear area. Average response factors of all points in the calibration curve were used to quantify the amount of analyte. The upperbound approach was used for samples <LOQ (EC, 2017), i.e. analysis results measured to be less than LOQ were given as <LOQ in numeral value and calculated as LOQ in sums

2.5.2. Microplastic analysis

Two replicates of each of the PP, HDPE, PET and uPVC polymers incubated at each site were used for chemical analysis. Frozen samples were treated gently to remove the biofilm on the MPs through an enzymatically and strong alkali driven cleaning process. Each sample (1 g) was sequentially incubated at room temperature with a mixture 1:20 (v:v) of Celluclast and 1mL Viscozyme enzymes (Sigma Aldrich) in 0.1 M PBS at pH 6.0 for 24 h and with 10% KOH for 6h at RT. Plastic beads were gently flushed and rinsed with millipore water through a vacuum system to remove the degraded biofilm and were allowed to dry at room temperature for 1 h. Samples were then sonicated three times for 20 min with 5 mL of dichloromethane, HPLC grade, and the supernatant was collected after each sonication step. Extracts were preconcentrated by a Rotavapor system and

238 analysed using the following HRGC/HRMS methods: USEPA Method 1668B, USEPA
239 Method 8290A and USEPA Method 1614A for polychlorinated biphenyls, dioxins and
240 brominated flame retardants respectively; and the following GC-ECD method for
241 chlorinates pesticides: USEPA Method 508.1.

242 2.6. Statistical analysis
243 All the statistical analyses were carried out using R (1.1.463). To test our main hypothesis
244 of whether MPs bind pollutants associated with fish farming, permutational multivariate
245 analysis of variance (PERMANOVA) was carried out. PERMANOVA was applied to
246 assess whether the composition of POPs sorbed to the MPs was similar in samples from
247 the fish farms and positive control, and to assess whether the composition of POPs in
248 MPs from the harbour was different to the other sites and positive control. Chemical
249 measurements of dioxins, PCBs, pesticides and brominated flame retardants sorbed to the
250 four polymers were used in this analysis. The homogeneity of multivariate dispersion was
251 previously checked using betadisper and pairwise permutation MANOVA was used for
252 post-hoc testing. The reference station was excluded from these analyses due to the excess
253 of data below the detection limits.

254 To get a better understanding of the sorption capacity of the four studied MP polymers to
255 POPs, Kruskal-Wallis test with Dunn's post-hoc test was carried out for each dioxin and
256 PCB congener individually using polymer type (HDPE, PET, PP, uPVC) as the
257 independent variable. For this analysis, only dioxin and PCB data were used from the two
258 fish farms and the positive control (n=6), since the PERMANOVA test showed that
259 compositions of POPs sorbed to MPs from these samples were statistically similar.

260 Correlation analysis was carried out to assess the relationship between the POPs sorbed
261 to MPs and the bioaccumulated levels in mussels, the latter assumed to reflect the
262 background level of POPs in the water. These analyses were used as a proxy to evaluate
263 affinity and competitive binding of POPs to MPs. Spearman's rank correlation analysis
264 was done for each polymer type individually using the levels of dioxins bound to MPs
265 and bioaccumulated in mussels from all four stations. For this analysis, 2,3,7,8-TCDD;
266 1,2,3,7,8-PeCDD; 1,2,3,4,7,8-HxCDD; 1,2,3,6,7,8-HxCDD; 1,2,3,7,8,9-HxCDD;
267 1,2,3,4,6,7,8-HpCDD data were used. For additional information, one way ANOVA, or
268 Kruskal-Wallis test in case of non-parametric data, with Tukey or Dunn's post-hoc tests,
269 respectively, were done to compare the levels of all POPs analysed in the mussels from

the four different sites. Normality and homogeneity of variance were previously tested with Shapiro test and Bartlett test, respectively. These results are shown in Supplementary Table S1.

3. Results

3.1. MPs sorb POPs from fish farming

The levels of all analysed POPs were below the detection limit in the MP polymers placed at the reference station. For the harbour, the two fish farms and the positive control, the levels of the majority of pesticides (dieldrin, endrin, α-endosulfan, β-endosulfan, HCB and heptachlor) and half of the novel brominated flame retardants (PBCCH, TBoCT and pTBX) analysed were also below the detection limit in all polymer types. Hence, only data for dioxins and PCBs were used in the PERMANOVA analyses. The concentrations of all studied POPs sorbed into the four MP polymer types are shown in Supplementary Table S2.

The levels of POPs sorbed to the MPs were quantitatively assessed to understand whether MPs can bind POPs associated with fish farming. PERMANOVA was used to compare the composition of POPs sorbed to MPs from the different sites and the positive control (Figure 3). The results of the PERMANOVA analysis showed that the composition of POPs in MPs from the harbour was statistically different from the composition of POPs in MPs from FF1 and FF2, and the positive control, ($p<0.05$). However, there were no significant differences in the composition of POPs sorbed to MPs from FF1, FF2 and the positive control.

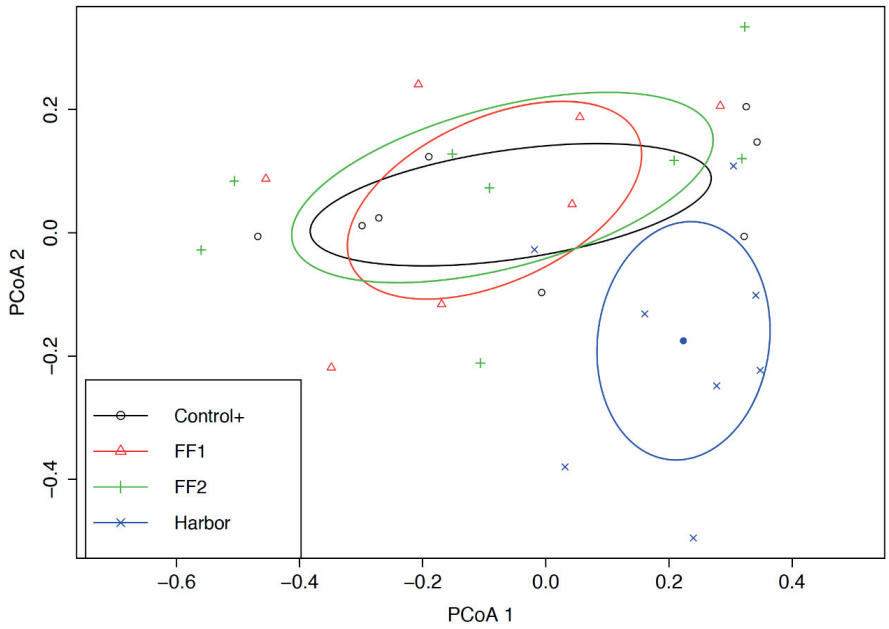

Figure 3: Principal Coordinate Analysis (PCoA) ordination of quantitative data of POPs sorbed to MPs collected from the positive control (black), fish farm 1 (red), fish farm 2 (green) and Bodø harbour (blue). Overlapping circles show that the composition of POPs bound to the MPs from the two salmon farms and the positive control are statistically similar.

3.2. Sorption capacity of MP polymers to POPs

To evaluate if binding affinities of POPs differ among the studied MP polymers, a Kruskal-Wallis test was done for each dioxin congener and each PCB Aroclor (Figure 4 and 5). Only data obtained from MPs placed at the two fish farms and the positive control were used, since no differences were found in the composition of POPs these MPs in the previous analysis.

Overall, dioxins and PCBs showed low affinity for HDPE and high affinity for PET and uPVC. The dioxin 2,3,7,8-TCDD (Figure 4A) and the Aroclor 1248 (Figure 5B) bound significantly more to uPVC MPs than to HDPE MPs ($p<0.01$), while no statistical differences were detected between uPVC and HDPE MPs, and PET and PP MPs. The Aroclors 1060 and 1254 (Figure 5A and C respectively) bound significantly more to PET MPs than to HDPE MPs ($p<0.01$ and 0.05 respectively), and no differences were evident among the other polymer types. The dioxin congeners 1,2,3,4,7,8-HxCDD, 1,2,3,7,8-PeCDD, and the PCB Aroclor 1260 bound significantly more to both PET and uPVC MPs than to HDPE ($p<0.05$ and 0.01) (Figure 4B, 4E, and 5D respectively). The levels of

dioxins and PCBs in PP MPs were not statistically different to the levels of the other polymer types. The dioxins 1,2,3,6,7,8-HxCDD, 1,2,3,7,8,9-HxCDD and 1,2,3,4,6,7,8-HpCDD did not show a significant binding affinity towards any MP polymer.

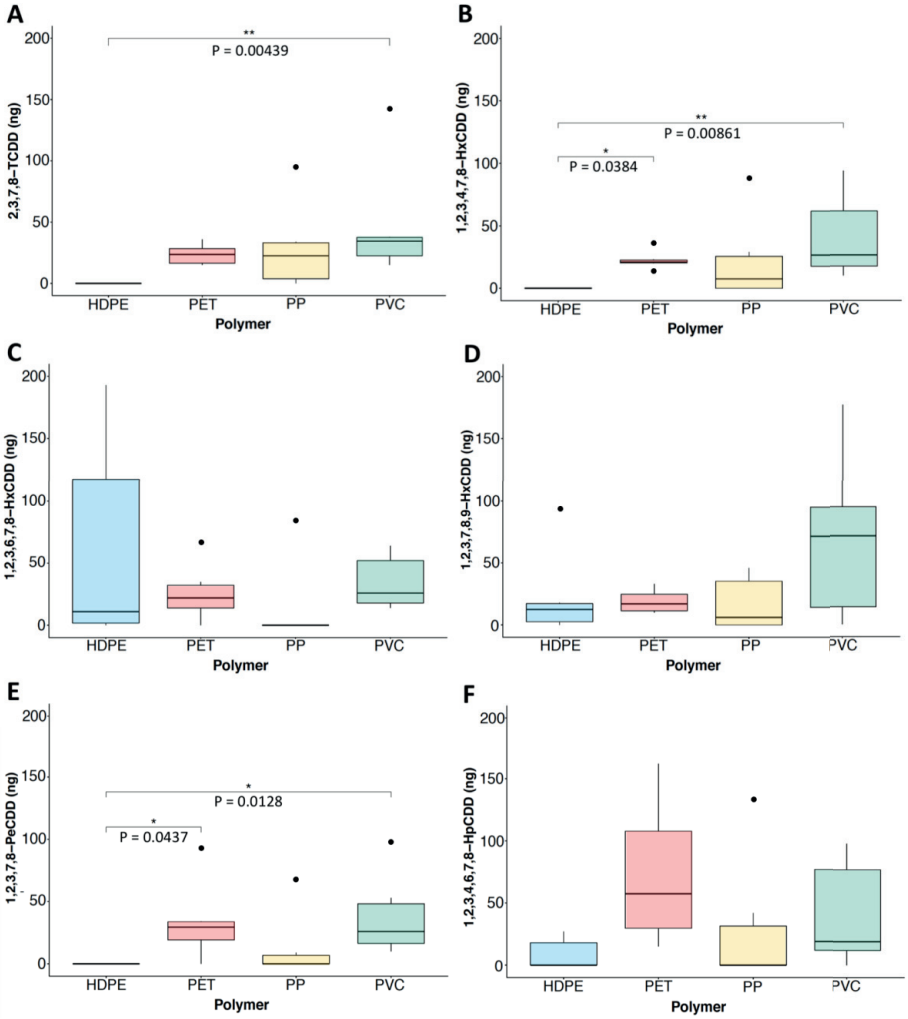

*Figure 4: Boxplot showing the levels of six different dioxin congeners associated with salmon farming sorbed to four MP polymer types: high density polyethylene (HDPE, blue), polyethylene terephthalate (PET, red), polypropylene (PP, yellow) and unplasticized polyvinyl chloride (PVC, green). The median of the data (n=6) is represented by a line in the center of the box. The top and bottom of the box represent the upper and lower quartiles, and the dots show outliers. * and ** show statistical differences (p<0.05 and 0.01 respectively) among polymers; exact p-values are written underneath.*

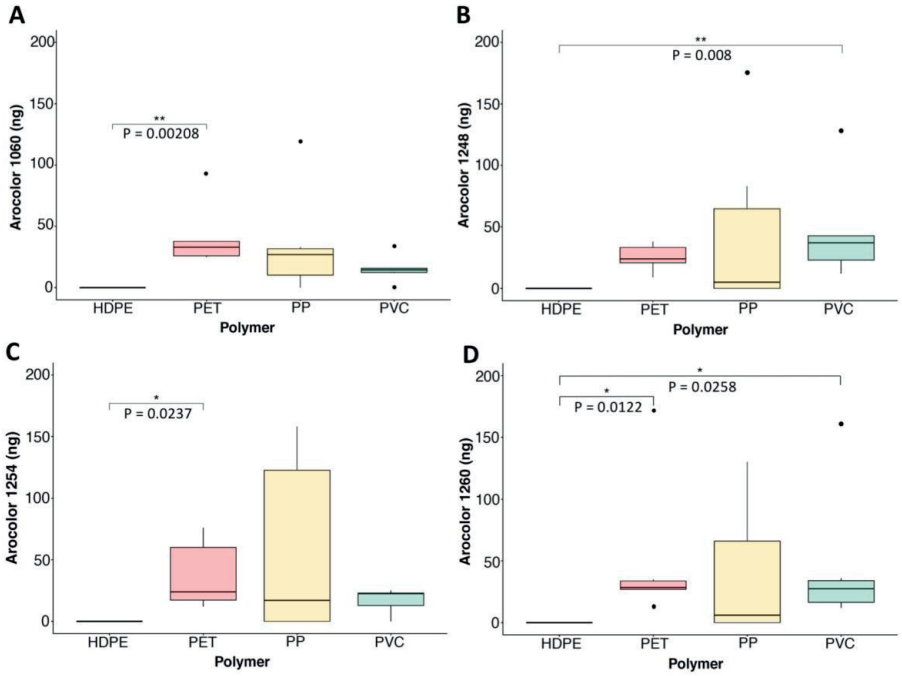

*Figure 5: Boxplot showing the levels of four PCB Aroclors associated to salmon farming sorbed to four MP polymer types: high density polyethylene (HDPE, blue), polyethylene terephthalate (PET, red), polypropylene (PP, yellow) and unplasticized polyvinyl chloride (PVC, green). The median of the data (n=6) is represented by a line in the center of the box. The top and bottom of the box represent the upper and lower quartiles, and the dots show outliers. * and ** show statistical differences (p<0.05 and 0.01 respectively) among polymers; the exact p-values are written underneath.*

3.3. Competitive behaviour of POPs to sorb MPs

To evaluate whether the composition of POPs sorbed to the MPs after three months in the sea reflected the levels of POPs in the surrounding water, the levels of dioxins bound to the MPs were compared to the levels of dioxins bioaccumulated in the blue mussels from the four sites. No significant correlations were found between the dioxins sorbed to the four MP polymers and the dioxins bioaccumulated in the mussels (Figure 6).

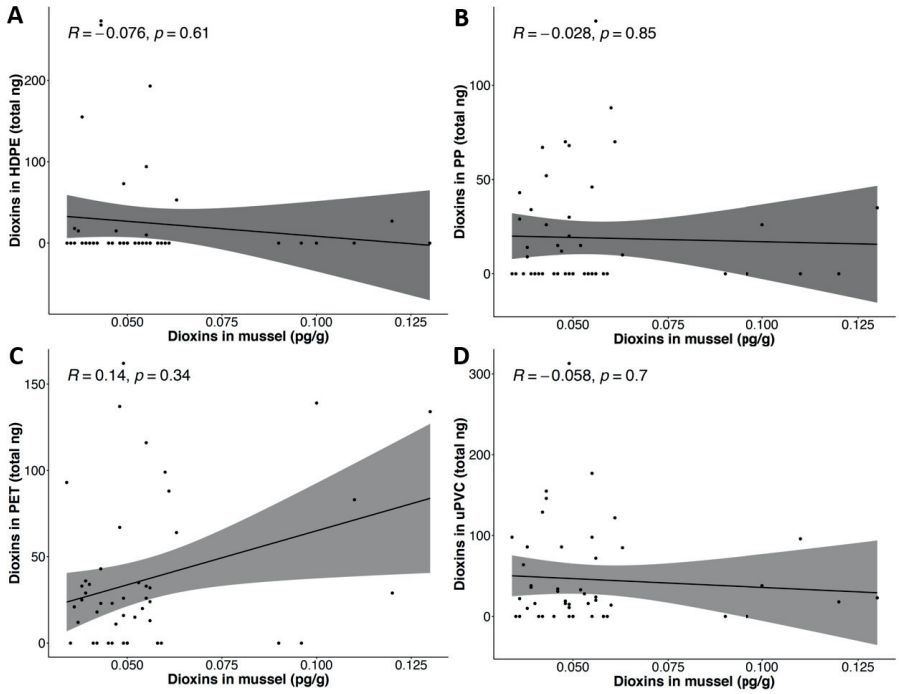

Figure 6: Spearman\s rank correlations comparing dioxin levels accumulated in blue mussels and dioxin levels sorbed to (A) HDPE MPs, (B) PP MPs, (C) PET MPs and (D) uPVC MPs from the reference station, Bodø harbour and both fish farms. On the top left of each graph are the results of the correlation analyses. Positive or negative R values indicate positive or negative correlations, respectively.

4. Discussion

This study suggests that MPs can sorb POPs associated with Atlantic salmon farming, and documents that such pollutants have polymer-specific binding affinities. Polymer type and pollutant type, in addition to background pollution in the water, are therefore determining factors that should be considered when assessing the potential role of MPs as vectors of pollutants from aquaculture in the marine environment.

In this study, four types of MP polymers (PE, PP, PET and uPVC) were either incubated for three days with fish feed or placed in the sea for three months next to two marine fish farms, in an urban harbour and in a non-polluted fjord. MP polymers collected from the two fish farms, the harbour and the lab control sorbed dioxins, PCBs and some brominated flame retardants (TBPhA, BTBPE and PBT), whereas pesticides were barely detected. However, MPs placed at the reference station did not have quantifiable levels of any of the analysed POPs. It is well known that MPs can sorb pollutants in the

environment. At the beginning of this century, plastic pellets sampled in waters from all five continents were reported to be polluted with PCBs and pesticides (Ogata et al., 2009). Since then, several studies have reported the presence of POPs bound to MPs in the marine environment (Ziccardi et al., 2016, Rodrigues et al., 2019). Nevertheless, the role of MPs as vector of such pollutants to organisms or other environments is still a controversial matter (Koelmans et al., 2022). Some studies have shown that pollutants sorbed to MPs can be transferred to organisms under specific conditions. For instance, Murray River rainbow fish (*Melanotaenia fluviatilis*) exposed to MPs spiked with PBDEs bioaccumulated greater amount of such pollutants compared to individuals exposed to virgin MPs (Wardrop et al., 2016). However, other studies have reported that exposure of organisms to pollutants sorbed to MPs is insignificant compared to exposure of pollutants through other pathways, such as diet or environmental exposure. Furthermore, it has been suggested that MPs might act as a buffer by sorbing pollutants in the water and, thereby, reducing bioavailability of such compounds in polluted environments (Sørensen et al., 2020, Koelmans et al., 2022). The aim of this study was to evaluate the potential of MPs to sorb POPs associated with fish farming and, consequently, to act as a possible vector of such pollutants. Our results show that the composition of POPs sorbed to MPs placed for three months next to two fish farms were similar to that of MPs incubated with fish feed for three days, but were significantly different to MPs placed for three months in the harbour and the reference station. This suggests that MPs found in the surroundings of salmon farms can sorb POPs present in the fish feed. However, the ability of MPs to transfer POPs from fish farms to organisms depends on several factors that were not addressed in this study. Pollution levels in the surrounding environments, current dynamics in the area, species affected or even size and shape of MPs are some important factors to assess when studying the transfer of pollutants from MPs to organisms. Therefore, the role of MPs in transfering POPs from salmon farming remains uncertain based only on our results.

PET and PVC MPs placed for 3 months close to salmon farms showed significantly higher levels of POPs than HDPE MPs. Sorption of hydrophobic organic components (HOCs) to MPs depends on several factors. Size, shape and color of the MPs as well as pH, salinity, temperature of the water and biofilm formation around the MPs are important factors in the interaction with HOCs and MPs (Ziccardi et al., 2016, Rummel et al., 2017). MPs used in this study were non-coloured, and, although uPVC MPs was

somewhat smaller (1/6), PE, PP, and PET MPs were similar in size and shape. The crystallinity of MPs, by contrast, varied among polymers. Crystallinity of polymers is an important factor that affects the adsorption of POPs. Crystalline polymers have a well-ordered and firm structure that does not favor adsorption of chemicals. Amorphous polymers have larger surface area that allows a higher sorption of HOCs (Endo and Koelmans, 2016, Rodrigues et al., 2019). HDPE is characterized by a high crystallinity, while PET and PP are considered semi-crystalline polymers, and PVC has amorphous structure (Wang et al., 2020). Thus, differences in the sorption of POPs to the four polymers studied could be explained by the degree of crystallinity. However, the level of crystallinity of a polymer can vary considerably as a result of the production process (Endo and Koelmans, 2016), which could explain differences observed between our study and earlier reports. A study carried out in California, USA, found that HDPE, LDPE and PP MPs deployed for several months in San Diego Bay had significantly higher levels of PCBs and PAHs than PET and PVC MPs (Rochman et al., 2013a). In another study, PE MPs collected in Japanese coastal areas had higher amount of PCBs adsorbed than PP MPs, although the concentrations of PCBs in single pellets from same locations had a high variability (Endo et al., 2005). Differences in water temperature, salinity and biofilm formation could also explain the discrepancies with those studies, which are carried out at lower latitudes. Our study was carried out north of the Arctic Polar Circle during the winter, with low seawater temperatures and when the lack of light redices biofilm growth (Aure and Skjoldal, 2003, NOAA, 2009).

This study focused on the sorption of dioxins and PCBs onto MPs and, to our knowledge, this is the first report to show that MPs can bind relatively high levels of dioxins close to salmon farms. The group of POPs evaluated in this work might, therefore, explain the differences obtained between this and other reports. Previous studies have mainly analysed the sorption of PCBs, brominated flame retardants, pesticides and PAHs on MPs (Hirai et al., 2011, Rodrigues et al., 2019, Rochman et al., 2013a, Ziccardi et al., 2016). Very few reports are available on the levels of dioxins bound to MP polymers in the sea. To our knowledge, only one study has reported levels of PCDD/PCDFs on MPs and such pollutants were only detected in charred MPs collected at the coast of the Maldives (Saliu et al., 2018). Different types of pollutants can have different affinities to polymers. For instance, PAHs and chlorinated benzenes were reported to sorb stronger to PE than PP, while PP had higher sorption capacity than PE for hexachlorocyclohexanes (HCHs) (Lee

et al., 2014). Thus, pollutants with higher affinities to polymers may outcompete other pollutants. For example, in a mixture of DDT and phenanthrene it was observed that the first chemical outcompeted the latter in terms of MP adsorption (Bakir et al., 2012). This process could potentially explain the non-correlation observed between the composition of POPs found in the mussels and the MPs. Bioaccumulated levels of dioxins in mussels placed next to the MPs for three months in the sea were quantitatively different. Mussels are sentinel species often used to biomonitor aquatic pollution since they are regarded to generally accumulate pollutants present in the water and have low biotransformation capacity (Beyer et al., 2017). Thus, pollutants found in their tissues tend to reflect those found the surrounding environment. One possible explanation for the different levels of POPs in MPs and mussels in waters with a cocktail of pollutants could therefore be the competitive binding of pollutants to plastic.

In terms of fish farming, this study suggests that PET and PVC MPs could have a higher environmental impact than HDPE MPs. PET and PVC are high-density polymers (1.1 to 1.5 g/cm^3). Since their densities are higher than seawater (1.02 g/ cm^3), these MP polymers tend to sink and accumulate in benthic sediments (Bråte et al., 2017, Gomiero et al., 2019). Benthic areas beneath fish farms are usually enriched with organic waste, resulting from fish faeces and non-eaten feed pellets, and associated pollutants. Sediment beneath fish farms could, therefore, be potential sources of polluted MPs. However, the impact of polluted MPs on resident organisms is probably insignificant compared to the background exposure (Koelmans, 2015), although polluted MPs could represent a environmental threat if carried to non-polluted areas by ocean currents.

PP MPs also sorbed significant amount of POPs associated to fish farming. PP has a lower density (0.9 g/cm^3 approx.) than seawater and PP MPs will therefore remain for longer periods in the pelagic zone (Bråte et al., 2017). Thus, PP MPs could have higher capacity to transport POPs from aquaculture facilities to the surrounding areas than other MP polymer types (Koelmans, 2015). Furthermore, PP is used in fish farming materials such as mooring ropes, which eventually release MPs as a result of wear and tear (Lusher et al., 2017). Thus, the impact of this polymer in the environment and in relation to aquaculture could be more important than previously expected. Based on this study, HDPE MPs might play a less important role as vector of POPs from aquaculture facilities to the environment. However, because feeding pipes in salmon farms are a known source of HDPE MPs to the environment (Gomiero et al., 2020b), and because the vast majority

of materials used in fish farming are made of PE, this MP type might still play a significant role in spreading pollutants from fish farms. Moreover, weathering of plastic and changes in the degree of crystallinity of polymers in the environment could modify the sorption patterns observed in this study (Rodrigues et al., 2019). Considering all the above, the role of MPs as potential vectors of pollutants from aquaculture facilities should be studied more in depth and considered in future assessments of the environmental impact of fish farms with open-nets.

5. Conclusion

To our knowledge, this is the first study to assess the potential role of MPs in the spreading of pollutants from marine aquaculture. Our results show that the potential of MPs to transport POPs depends on the type of polymer, and indicate that PET and PVC MPs might contribute to spreading pollutants from Atlantic salmon farming to a larger extent than HDPE. Overall, this study proposes that MPs could potentially act as vectors of POPs associated with fish feed and might play a part in the environmental impact of Atlantic salmon farming.

6. Acknowledgements

The Institute of Marine Research and Nord University funded this study. Fish feeds were kindly provided by the Gifas and Wenberg companies in the Salten Aqua AS group. We want to thank the staff at the fish farms for the help they provided during deployment and sampling of the cages. We thank the technical staff at IMR, Anne Margrethe Aase, Aina Bruvik, Nawaraj Gautam, Siren Hatland and Mannfred Torsvik, for sample reception and preparation, and the staff at the laboratory of Chemistry and Undesirables, Jannicke Bakkejord Alling, Marianne Hauglid Flågeng, Annie Fürstenberg, Kjersti Kolås, Dargmar-Betty Nordgård, Franziska Randers, Kari Breistein Sæle, Amarjargal Sengee, Andreas Linge Tomren, Teclu Habtemariam Weldegebriel under leadership of Bergitte Reisersen, for the analysis of the POPs in mussels. We also thank Jannicke Bakkejord Alling for critical comments on the method description. The help of colleagues from Nord University, and the technical staff at NORCE, especially Kjell Birger Øysæd, is also highly appreciated.

7. References

Abihssira-García, I.S., Park, Y., Kiron, V. & Olsvik, P.A. (2020). Fluorescent microplastic uptake by immune cells of Atlantic salmon (*Salmo salar* L.). *Front Environ Sci,* 8**:** 233. DOI https://doi.org/10.3389/fenvs.2020.560206

Altarawneh, M., Dlugogorski, B.Z., Kennedy, E.M. & Mackie, J.C. (2009). Mechanisms for formation, chlorination, dechlorination and destruction of polychlorinated dibenzo-p-dioxins and dibenzofurans (PCDD/Fs). *Prog Energy Combust Sci,* 35**:** 245-274. DOI https://doi.org/10.1016/j.pecs.2008.12.001

Aure, J. & Skjoldal, H.R. (2003). *OSPAR Common Procedure for Identification of Eutrophication Status: Application of the Screening Procedure for the Norwegian coast north of 62oN (Stad–Russian border)* (Report No. TA-1997). Miljødirectoratet. https://nettarkiv.miljodirektoratet.no/hoeringer/tema.miljodirektoratet.no/no/Publikasjoner/Publikasjoner/2004/Februar/OSPAR_Common_Procedure_for_Identification_of_Eu/index.html

Bakir, A., O'connor, I.A., Rowland, S.J., Hendriks, A.J. & Thompson, R.C. (2016). Relative importance of microplastics as a pathway for the transfer of hydrophobic organic chemicals to marine life. *Environ Pollut,* 219**:** 56-65. DOI https://doi.org/10.1016/j.envpol.2016.09.046

Bakir, A., Rowland, S.J. & Thompson, R.C. (2012). Competitive sorption of persistent organic pollutants onto microplastics in the marine environment. *Mar Pollut Bull,* 64**:** 2782-2789. DOI https://doi.org/10.1016/j.marpolbul.2012.09.010

Barentswatch (2021). Fish Health. https://www.barentswatch.no/fiskehelse/

Berntssen, M.H., Julshamn, K. & Lundebye, A.-K. (2010). Chemical contaminants in aquafeeds and Atlantic salmon (*Salmo salar*) following the use of traditional-versus alternative feed ingredients. *Chemosphere,* 78**:** 637-646. DOI https://doi.org/10.1016/j.chemosphere.2009.12.021

Beyer, J., Green, N.W., Brooks, S., Allan, I.J., Ruus, A., Gomes, T., Bråte, I.L.N. & Schøyen, M. (2017). Blue mussels (*Mytilus edulis* spp.) as sentinel organisms in coastal pollution monitoring: a review. *Mar Environ Res,* 130**:** 338-365. DOI https://doi.org/10.1016/j.marenvres.2017.07.024

Bråte, I.L.N., Eidsvoll, D.P., Steindal, C.C. & Thomas, K.V. (2016). Plastic ingestion by Atlantic cod (*Gadus morhua*) from the Norwegian coast. *Mar Pollut Bull,* 112**:** 105-110. DOI https://doi.org/10.1016/j.marpolbul.2016.08.034

Bråte, I.L.N., Huwer, B., Thomas, K.V., Eidsvoll, D.P., Halsband, C., Almroth, B.C. & Lusher, A. (2017). *Micro-and macro-plastics in marine species from Nordic waters.* Nordic Council of Ministers, Copenhagen, Denmark.

Calheiros, T., Pereira, M. & Nunes, J.P. (2021). Assessing impacts of future climate change on extreme fire weather and pyro-regions in Iberian Peninsula. *Sci Total Environ,* 754**:** 142233. DOI https://doi.org/10.1016/j.scitotenv.2020.142233

Campanale, C., Massarelli, C., Savino, I., Locaputo, V. & Uricchio, V.F. (2020). A detailed review study on potential effects of microplastics and additives of concern on human health. *Int J Env Res Public Health,* 17**:** 1212. DOI https://doi.org/10.3390/ijerph17041212

Chen, Q., Zhang, H., Allgeier, A., Zhou, Q., Ouellet, J.D., Crawford, S.E., Luo, Y., Yang, Y., Shi, H. & Hollert, H. (2019). Marine microplastics bound dioxin-like chemicals: Model explanation and risk assessment. *J Hazard Mater,* 364**:** 82-90. DOI https://doi.org/10.1016/j.jhazmat.2018.10.032

Chua, E.M., Shimeta, J., Nugegoda, D., Morrison, P.D. & Clarke, B.O. (2014). Assimilation of polybrominated diphenyl ethers from microplastics by the marine amphipod, *Allorchestes compressa*. *Environ Sci Technol,* 48**:** 8127-8134. DOI https://doi.org/10.1021/es405717z

Ec. (2017). *Commission regulation (EU) 2017/644 of 5 April 2017 laying down methods of sampling and analysis for the control of levels of dioxins, dioxin-like PCBs and non-dioxin-like PCBs in certain foodstuffs and repealing Regulation (EU) No 589/2014*. Retrieved from https://eur-lex.europa.eu/legal-content/EN/TXT/PDF/?uri=CELEX:32017R0644&from=EN

Endo, S. & Koelmans, A.A. (2016). Sorption of hydrophobic organic compounds to plastics in the marine environment: equilibrium. In: Takada, H. & Karapanagioti, H.K. (Eds.) *Hazardous chemicals associated with plastics in the marine environment.* Springer, Switzerland.

Endo, S., Takizawa, R., Okuda, K., Takada, H., Chiba, K., Kanehiro, H., Ogi, H., Yamashita, R. & Date, T. (2005). Concentration of polychlorinated biphenyls (PCBs) in beached resin pellets: variability among individual particles and regional differences. *Mar Pollut Bull,* 50**:** 1103-1114. DOI https://doi.org/10.1016/j.marpolbul.2005.04.030

Fao (2020). The State of World Fisheries and Aquaculture 2020. Sustainability in action. In: *The State of World Fisheries and Aquaculture (SOFIA).* FAO, Rome, Italy.

Gomiero, A., Haave, M., Bjorøy, Ø., Herzke, D., Kögel, T., Nikiforov, V. & Øysæd, K.B. (2020a). *Quantification of microplastic in fillet and organs of farmed and wild salmonids-a comparison of methods for detection and quantification* (Report No. 8/2020). NORCE Environment. https://norceresearch.brage.unit.no/norceresearch-xmlui/bitstream/handle/11250/2687619/NORCE%20Report%208_2020%20SalmoDetect%20project.pdf?sequence=1&isAllowed=y

Gomiero, A., Haave, M., Kögel, T., Bjorøy, Ø., Gjessing, M., Lea, T.B., Horve, E., Martins, C. & Olafsen, T. (2020b). *TRACKing of PLASTtic emissions from aquaculture industry* (Report No. 4/2020). NORCE Environment. https://norceresearch.brage.unit.no/norceresearch-xmlui/bitstream/handle/11250/2649891/Rapport%20NORCE%20milj%C3%B8%204-2020.pdf?sequence=4

Gomiero, A., Øysæd, K.B., Agustsson, T., Van Hoytema, N., Van Thiel, T. & Grati, F. (2019). First record of characterization, concentration and distribution of microplastics in coastal sediments of an urban fjord in south west Norway using a thermal degradation method. *Chemosphere,* 227**:** 705-714. DOI https://doi.org/10.1016/j.chemosphere.2019.04.096

Grefsrud, E.S., Glover, K., Grøsvik, B.E., Husa, V., Karlsen, Ø., Kristiansen, T., Kvamme, B.O., Mortensen, S., Samuelsen, O.B. & Stien, L.H. (2018). *Risk report Norwegian fish farming 2018* (Report No. 1-2018). Institute of Marine Research, Norway. http://hdl.handle.net/11250/2584600

Harris, P.T. (2020). The fate of microplastic in marine sedimentary environments: A review and synthesis. *Mar Pollut Bull,* 158**:** 111398. DOI https://doi.org/10.1016/j.marpolbul.2020.111398

Hartmann, N.B., HüFfer, T., Thompson, R.C., HassellöV, M., Verschoor, A., Daugaard, A.E., Rist, S., Karlsson, T., Brennholt, N. & Cole, M. (2019). Are we speaking the same language? Recommendations for a definition and categorization framework for plastic debris. *Environ Sci Technol*. DOI https://doi.org/10.1021/acs.est.8b05297

Higuera, P.E. & Abatzoglou, J.T. (2021). Record-setting climate enabled the extraordinary 2020 fire season in the western United States. *Global Change Biol,* 27**:** 1-2. DOI https://doi.org/10.1111/gcb.15388

Hirai, H., Takada, H., Ogata, Y., Yamashita, R., Mizukawa, K., Saha, M., Kwan, C., Moore, C., Gray, H. & Laursen, D. (2011). Organic micropollutants in marine plastics debris from the open ocean and remote and urban beaches. *Mar Pollut Bull,* 62**:** 1683-1692. DOI https://doi.org/10.1016/j.marpolbul.2011.06.004

Kobusińska, M.E., Lewandowski, K.K., Panasiuk, A., Łęczyński, L., Urbaniak, M., Ossowski, T. & Niemirycz, E. (2020). Precursors of polychlorinated dibenzo-p-dioxins and dibenzofurans in Arctic and Antarctic marine sediments: Environmental concern in the face of climate change. *Chemosphere,* 260**:** 127605. DOI https://doi.org/10.1016/j.chemosphere.2020.127605

Koelmans, A.A. (2015). Modeling the role of microplastics in bioaccumulation of organic chemicals to marine aquatic organisms. A critical review. In: Melanie Bergmann, Lars Gutow & Klages, M. (Eds.) *Marine Anthropogenic Litter.* Springer, Cham Switzerland, pp 309-324.

Koelmans, A.A., Diepens, N.J. & Mohamed Nor, N.H. (2022). Weight of evidence for the microplastic vector effect in the context of chemical risk assessment. *Microplastic in the Environment: Pattern and Process.* Springer, Cham, Switzerland.

Kögel, T., Bjorøy, Ø., Toto, B., Bienfait, A.M. & Sanden, M. (2020). Micro-and nanoplastic toxicity on aquatic life: Determining factors. *Sci Total Environ,* 709**:** 136050. DOI https://doi.org/10.1016/j.scitotenv.2019.136050

Kolandhasamy, P., Su, L., Li, J., Qu, X., Jabeen, K. & Shi, H. (2018). Adherence of microplastics to soft tissue of mussels: a novel way to uptake microplastics beyond ingestion. *Sci Total Environ,* 610**:** 635-640. DOI https://doi.org/10.1016/j.scitotenv.2017.08.053

Lee, H., Shim, W.J. & Kwon, J.-H. (2014). Sorption capacity of plastic debris for hydrophobic organic chemicals. *Sci Total Environ,* 470**:** 1545-1552. DOI https://doi.org/10.1016/j.scitotenv.2013.08.023

Lohmann, R. (2017). Microplastics are not important for the cycling and bioaccumulation of organic pollutants in the oceans—but should microplastics be considered POPs themselves? *Integr Environ Assess Manag,* 13**:** 460-465. DOI https://doi.org/10.1002/ieam.1914

Lundebye, A.-K., Lock, E.-J., Rasinger, J.D., Nøstbakken, O.J., Hannisdal, R., Karlsbakk, E., Wennevik, V., Madhun, A.S., Madsen, L. & Graff, I.E. (2017). Lower levels of persistent organic pollutants, metals and the marine omega 3-fatty acid DHA in farmed compared to wild Atlantic salmon (*Salmo salar*). *Environ Res,* 155**:** 49-59. DOI https://doi.org/10.1016/j.envres.2017.01.026

Lusher, A. (2015). Microplastics in the marine environment: distribution, interactions and effects. *Marine Anthropogenic Litter.* Springer, Cham, Switzerland, pp 245-307.

Lusher, A., Hollman, P. & Mendoza-Hill, J. (2017). *Microplastics in fisheries and aquaculture: status of knowledge on their occurrence and implications for aquatic organisms and food safety* (Report No 615). FAO Fisheries and Aquaculture Technical Paper, Rome, Italy.

Nácher-Maestre, J., Serrano, R., Portolés, T., Berntssen, M.H., Pérez-Sánchez, J. & Hernández, F.L. (2014). Screening of pesticides and polycyclic aromatic hydrocarbons in feeds and fish tissues by gas chromatography coupled to high-resolution mass spectrometry using atmospheric pressure chemical ionization. *J Agric Food Chem,* 62**:** 2165-2174. DOI https://doi.org/10.1021/jf405366n

Noaa (2009). World Ocean Atlas 2009 Figures. National Oceanic and Atmospheric Administarion. https://www.nodc.noaa.gov/OC5/WOA09F/pr_woa09f.html

Nøstbakken, O.J., Hove, H.T., Duinker, A., Lundebye, A.-K., Berntssen, M.H., Hannisdal, R., Lunestad, B.T., Maage, A., Madsen, L. & Torstensen, B.E. (2015). Contaminant levels in Norwegian farmed Atlantic salmon (*Salmo salar*) in the 13-year period from 1999 to 2011. *Environ Int,* 74**:** 274-280. DOI https://doi.org/10.1016/j.envint.2014.10.008

Ogata, Y., Takada, H., Mizukawa, K., Hirai, H., Iwasa, S., Endo, S., Mato, Y., Saha, M., Okuda, K. & Nakashima, A. (2009). International Pellet Watch: Global monitoring of persistent organic pollutants (POPs) in coastal waters. 1. Initial phase data on PCBs, DDTs, and HCHs. *Mar Pollut Bull,* 58**:** 1437-1446. DOI https://doi.org/10.1016/j.marpolbul.2009.06.014

Rochman, C.M., Hoh, E., Hentschel, B.T. & Kaye, S. (2013a). Long-term field measurement of sorption of organic contaminants to five types of plastic pellets: implications for plastic marine debris. *Environ Sci Technol,* 47**:** 1646-1654. DOI https://doi.org/10.1021/es303700s

Rochman, C.M., Hoh, E., Kurobe, T. & Teh, S.J. (2013b). Ingested plastic transfers hazardous chemicals to fish and induces hepatic stress. *Scientific reports,* 3**:** 3263.

Rodrigues, J.P., Duarte, A.C., Santos-Echeandía, J. & Rocha-Santos, T. (2019). Significance of interactions between microplastics and POPs in the marine environment: a critical overview. *TrAC,* 111**:** 252-260. DOI https://doi.org/10.1016/j.trac.2018.11.038

Rummel, C.D., Jahnke, A., Gorokhova, E., KüHnel, D. & Schmitt-Jansen, M. (2017). Impacts of biofilm formation on the fate and potential effects of microplastic in the aquatic environment. *Environ Sci Technol Lett,* 4**:** 258-267. DOI https://doi.org/10.1021/acs.estlett.7b00164

Saliu, F., Montano, S., Garavaglia, M.G., Lasagni, M., Seveso, D. & Galli, P. (2018). Microplastic and charred microplastic in the Faafu Atoll, Maldives. *Mar Pollut Bull,* 136**:** 464-471. DOI https://doi.org/10.1016/j.marpolbul.2018.09.023

Schecter, A., Birnbaum, L., Ryan, J.J. & Constable, J.D. (2006). Dioxins: an overview. *Environ Res,* 101**:** 419-428. DOI https://doi.org/10.1016/j.envres.2005.12.003

Sele, V., Sanden, M., Berntssen, M., Storesund, J.E., Lie, K.K., Espe, M., Lundebye, A.-K., Hemre, G.I., Waagbø, R. & Ørnsrud, R. (2019). *Program for overvåking av fiskefôr-Årsrapport for prøver innsamlet i 2018*. Institute of Marine Research. https://www.mattilsynet.no/dyr_og_dyrehold/for/rapport_overvaakingsprogram_fiskefor_2018.35512/binary/Rapport:%20Overv%C3%A5kingsprogram%20fiskef%C3%B4r%202018

Sørensen, L., Rogers, E., Altin, D., Salaberria, I. & Booth, A.M. (2020). Sorption of PAHs to microplastic and their bioavailability and toxicity to marine copepods under co-exposure conditions. *Environ Pollut,* 258**:** 113844. DOI https://doi.org/10.1016/j.envpol.2019.113844

Suaria, G., Avio, C.G., Mineo, A., Lattin, G.L., Magaldi, M.G., Belmonte, G., Moore, C.J., Regoli, F. & Aliani, S. (2016). The Mediterranean Plastic Soup: synthetic polymers in Mediterranean surface waters. *Sci Rep,* 6**:** 37551. DOI https://doi.org/10.1038/srep37551

Wang, C., Zhao, J. & Xing, B. (2020). Environmental Source, Fate, and Toxicity of Microplastics. *J Hazard Mater***:** 124357. DOI https://doi.org/10.1016/j.jhazmat.2020.124357

Wardrop, P., Shimeta, J., Nugegoda, D., Morrison, P.D., Miranda, A., Tang, M. & Clarke, B.O. (2016). Chemical Pollutants Sorbed to Ingested Microbeads from Personal Care Products Accumulate in Fish. *Environ Sci Technol,* 50**:** 4037-4044. DOI 10.1021/acs.est.5b06280

Ytrestøyl, T., Aas, T.S. & Åsgård, T. (2015). Utilisation of feed resources in production of Atlantic salmon (*Salmo salar*) in Norway. *Aquaculture,* 448**:** 365-374. DOI https://doi.org/10.1016/j.aquaculture.2015.06.023

Ziccardi, L.M., Edgington, A., Hentz, K., Kulacki, K.J. & Driscoll, S.K. (2016). Microplastics as vectors for bioaccumulation of hydrophobic organic chemicals in the marine environment: A state-of-the-science review. *Environ Toxicol Chem,* 35**:** 1667-1676. DOI https://doi.org/10.1002/etc.3461

Supplementary tables

Table S1: Concentrations of persistent organic pollutants (POPs) (mean ± standard deviation) in blue mussels (n=3) from the four studied sites after three months in the sea. For each congener, statistical differences between sites are shown in bold and with different letters; sites sharing the same letters are not significantly different. Congeners where letters are not present did not show statistical differences.

POP	Congener	Reference station	Harbour	FF1	FF2
Dioxins *(pg/g ww (TEQ))*	*2378-TCDD*	0.043 ± 0.002	0.04 ± 0.003	0.04 ± 0	0.046 ± 0.008
	12378-PeCDD	0.04 ± 0.004	0.042 ± 0.001	0.039 ± 0.006	0.04 ± 0.001
	123478-HxCDD	**0.0054 ± 4e-04** ***ab***	**0.006 ± 0.001** ***a***	**0.0054 ± 1e-04** ***ab***	**0.0041 ± 5e-04** ***b***
	123678-HxCDD	0.0054 ± 4e-04	0.006 ± 0.001	0.0055 ± 1e-04	0.0042 ± 6e-04
	123789-HxCDD	0.0054 ± 4e-04	0.006 ± 0.001	0.0055 ± 6e-05	0.0041 ± 6e-04
	1234678-HpCDD	**9.1e-04 ± 5e-05** ***ab***	**0.0011 ± 2e-04** ***ab***	**0.0012 ± 6e-05** ***a***	**5e-04 ± 9e-05** ***b***
	OCDD	**8.1e-05 ± 5e-06** ***ab***	**9.7e-05 ± 5e-06** ***ab***	**1e-04 ± 8e-06** ***a***	**3.9e-05 ± 2e-06** ***b***
	2378-TCDF	0.011 ± 0.002	0.012 ± 0.001	0.0117 ± 6e-04	0.0093 ± 4e-04
	12378-PeCDF	**0.0024 ± 4e-04** ***ab***	**0.0029 ± 6e-04** ***a***	**0.0023 ± 3e-05** ***ab***	**0.0018 ± 2e-04** ***b***
	23478-PeCDF	0.024 ± 0.004	0.03 ± 0.007	0.024 ± 0.001	0.017 ± 0.002
	123478-HxCDF	**0.0025 ± 3e-04** ***a***	**0.0037 ± 4e-04** ***b***	**0.0023 ± 3e-04** ***a***	**0.0019 ± 0.0002** ***a***
	123678-HxCDF	**0.0023 ± 3e-04** ***ab***	**0.0035 ± 4e-04** ***a***	**0.0022 ± 2e-04** ***ab***	**0.0019 ± 2e-04** ***b***
	123789-HxCDF	**0.0032 ± 3e-04** ***ab***	**0.0051 ± 6e-04** ***a***	**0.0029 ± 3e-04** ***ab***	**0.0023 ± 1e-04** ***b***
	234678-HxCDF	**0.0025 ± 3e-04** ***ab***	**0.0038 ± 5e-04** ***a***	**0.0026 ± 2e-04** ***ab***	**0.0019 ± 2e-04** ***b***
	1234678-HpCDF	2.3e-04 ± 5e-05	4.7e-04 ± 2e-05	2.5e-04 ± 1e-05	2.8e-04 ± 9e-05
	1234789-HpCDF	2.3e-04 ± 3e-05	3.2e-04 ± 5e-05	2.1e-04 ± 3e-05	3e-04 ± 1e-04
	OCDF	**1.8e-06 ± 3e-07** ***a***	**6.8 e-06 ± 3e-07** ***b***	**3.8e-06 ± 6e-07** ***c***	**1.1e-06 ± 2e-07** ***a***
Dioxin-like PCBs *(pg/g ww (TEQ))*	***PCB-77***	**6.7e-05 ± 9e-06** ***a***	**2.8e-04 ± 4e-05** ***b***	**8.6e-05 ± 7e-06** ***a***	**1e-04 ± 1e-05** ***a***
	PCB-81	**6e-06 ± 3e-07** ***a***	**1.4e-05 ± 3e-06** ***b***	**6.9e-06 ± 8e-07** ***a***	**9.1e-06 ± 6e-07** ***a***
	PCB-126	**0.017 ± 0.004** ***a***	**0.06 ± 0.02** ***b***	**0.025 ± 0.003** ***ab***	**0.025 ± 0.001** ***ab***
	PCB-169	**0.001 ± 2e-04** ***ab***	**0.0015 ± 3e-19** ***a***	**9e-04 ± 7e-05** ***b***	**0.0013 ± 6e-05** ***ab***
	PCB-105	**7.5e-04 ± 5e-05** ***a***	**0.0049 ± 7e-04** ***b***	**0.0011 ± 1e-04** ***ab***	**0.0011 ± 1e-04** ***ab***
	PCB-114	**0.0022 ± 5e-05** ***a***	**0.0052 ± 8e-04** ***b***	**0.0023 ± 2e-04** ***a***	**0.0025 ± 2e-04** ***a***
	PCB-118	**0.0023 ± 4e-04** ***a***	**0.013 ± 0.002** ***b***	**0.0033 ± 3e-04** ***ab***	**0.0033 ± 2e-04** ***ab***
	PCB-123	**4.5e-04 ± 1e-05** ***a***	**0.001 ± 1e-04** ***b***	**4.7e-04 ± 3e-05** ***a***	**5e-04 ± 3e-05** ***a***
	PCB-156	**0.0022 ± 5e-05** ***a***	**0.011 ± 0.001** ***b***	**0.0023 ± 2e-04** ***a***	**0.0025 ± 2e-04** ***a***
	PCB-157	**0.0022 ± 5e-05** ***a***	**0.0052 ± 8e-04** ***b***	**0.0023 ± 2e-04** ***a***	**0.0025 ± 2e-04** ***a***
	PCB-167	**4.5e-05 ± 1e-06** ***a***	**1.7e-04 ± 1e-05** ***b***	**4.7e-05 ± 3e-06** ***a***	**5e-05 ± 3e-06** ***a***
	PCB-189	**4.5e-04 ± 1e-05** ***a***	**0.001 ± 1e-04** ***b***	**4.7e-04 ± 3e-05** ***a***	**5e-04 ± 3e-05** ***a***
	PCB-101	**0.024 ± 0.004**	**0.07 ± 0.01**	**0.03 ± 0.003**	**0.032 ± 0.002**

		a	***b***	***ab***	***ab***
PCBs *(ng/g ww)*	***PCB-138***	**0.043 ± 0.005** ***a***	**0.32 ± 0.02** ***b***	**0.066 ± 0.002** ***c***	**0.07 ± 0.003** ***c***
	PCB-153	**0.065 ± 0.006** ***a***	**0.42 ± 0.06** ***b***	**0.093 ± 0.005** ***ac***	**0.103 ± 0.006** ***c***
	PCB-180	**0.009 ± 0** ***a***	**0.054 ± 0.007** ***b***	**0.0093 ± 6e-04** ***ab***	**0.0097 ± 6e-04** ***ab***
	PCB-28	**0.009 ± 0** ***a***	**0.021 ± 0.003** ***b***	**0.0093 ± 6e-04** ***ab***	**0.0097 ± 6e-04** ***ab***
	PCB-52	**0.009 ± 0** ***a***	**0.021 ± 0.003** ***b***	**0.011 ± 0.001** ***ab***	**0.011 ± 0.002** ***ab***
Brominated flame retardants *(ng/g ww)*	*PBDE 28*	9e-04 ± 2e-05	0.0021 ± 3e-04	9.4e-04 ± 7e-05	9.7e-04 ± 5e-05
	PBDE 47	**0.0097 ± 5e-04** ***a***	**0.063 ± 0.004** ***b***	**0.0102 ± 7e-04** ***ab***	**0.025 ± 0.001** ***ab***
	PBDE 66	**9e-04 ± 2e-05** ***a***	**0.0024 ± 1e-04** ***b***	**9.4e-04 ± 7e-05** ***a***	**0.0011 ± 1e-04** ***a***
	PBDE 99	**0.0063 ± 2e-04** ***ab***	**0.052 ± 0.004** ***a***	**0.004 ± 5e-04** ***b***	**0.013 ± 0.001** ***ab***
	PBDE 100	**0.0047 ± 6e-05** ***ab***	**0.025 ± 0.001** ***a***	**0.0042 ± 2e-04** ***b***	**0.0078 ± 2e-04** ***ab***
	PBDE 119	9e-04 ± 2e-05	0.0021 ± 3e-04	9.4e-04 ± 7e-05	9.7e.04 ± 5e-05
	PBDE 138	0.0027 ± 1e-04	0.006 ± 0.001	0.0028 ± 2e-04	0.003 ± 2e-04
	PBDE 153	**0.0015 ± 6e-05** ***a***	**0.0036 ± 6e-04** ***b***	**0.0016 ± 1e-04** ***ab***	**0.0017 ± 1e-04** ***ab***
	PBDE 154	**0.001± 1e-04** ***a***	**0.005 ± 0.001** ***b***	**0.0015 ± 4e-04** ***a***	**0.0017 ± 2e-04** ***a***
	PBDE 183	0.0063 ± 2e-04	0.015 ± 0.002	0.0066 ± 4e-04	0.007 ± 5e-04
	PBDE 49	**0.0021 ± 1e-04** ***a***	**0.0101 ± 8e-04** ***b***	**0.0029 ± 6e-05** ***ab***	**0.0044 ± 6e-05** ***ab***

Table S2: Concentrations of persistent organic pollutants (POPs) (mean ± standard deviation) on microplastic (MP) polymers from all four sites after three months in the sea and the positive control. Data show ng of pollutant per g of MP polymer (n=3). Samples below LOD are shown in the table as <d.l. (i.e. below detection limit).

MP Polymer	POP	Positive control	Reference station	Harbour	FF1	FF2
HDPE	*2378-TCDD*	< d.l.	< d.l.	214 ± 83.4	< d.l.	< d.l.
	12378-PeCDD	< d.l.	< d.l.	134 ± 189.5	< d.l.	< d.l.
	123478-HxCDD	< d.l.	< d.l.	63 ± 14.1	< d.l.	< d.l.
	123678-HxCDD	79 ± 101.8	< d.l.	< d.l.	96.5 ± 136.5	7.5 ± 10.6
	123789-HxCDD	< d.l	< d.l.	< d.l.	52 ± 59.4	16.5 ± 2.1
	1234678-HpCDD	12 ± 17	< d.l.	< d.l.	13.5 ± 19.1	< d.l.
	Aroclor 1260	< d.l.	< d.l.	< d.l.	< d.l.	< d.l.
	Aroclor 1254	< d.l.	< d.l.	199.5 ± 27.6	< d.l.	< d.l.
	Aroclor 1248	< d.l.	< d.l.	90.5 ± 128	< d.l.	< d.l.
	Aroclor 1060	< d.l.	< d.l.	< d.l.	< d.l.	< d.l.
	Aldrin	< d.l.	< d.l.	< d.l.	< d.l.	< d.l.
	Dieldrin	< d.l.	< d.l.	< d.l.	< d.l.	< d.l.
	Endrin	< d.l.	< d.l.	< d.l.	< d.l.	< d.l.
	α–endosulfan	< d.l.	< d.l.	< d.l.	< d.l.	< d.l.
	β-endosulfan	< d.l.	< d.l.	< d.l.	< d.l.	< d.l.
	HCB	< d.l.	< d.l.	< d.l.	< d.l.	< d.l.
	Heptachlor	< d.l.	< d.l.	< d.l.	< d.l.	< d.l.
	PBCCH	< d.l.	< d.l.	< d.l.	< d.l.	< d.l.
	TBoCT	< d.l.	< d.l.	< d.l.	< d.l.	< d.l.
	TBPhA	< d.l.	< d.l.	140.5 ± 150.6	< d.l.	< d.l.
	BTBPE	< d.l.	< d.l.	< d.l.	321 ± 56.6	190 ± 193.7
	pTBX	< d.l.	< d.l.	< d.l.	< d.l.	< d.l.
	PBT	136.5 ± 55.9	< d.l.	38.2 ± 129.5	155.6 ± 214	292 ± 84.8
PP	*2378-TCDD*	47.5 ± 67.2	< d.l.	20 ± 8.5	17 ± 24	22.5 ± 10.6
	12378-PeCDD	34 ± 48.1	< d.l.	59.5 ± 10.6	< d.l.	4.5 ± 6.4
	123478-HxCDD	44 ± 62.2	< d.l.	15 ± 7.1	< d.l.	22 ± 9.9
	123678-HxCDD	42.5 ± 60.1	< d.l.	79 ± 12.7	< d.l.	< d.l.
	123789-HxCDD	< d.l.	< d.l.	69 ± 1.4	23 ± 32.5	27.5 ± 21.9
	1234678-HpCDD	21 ± 29.7	< d.l.	30.5 ± 6.4	< d.l.	67 ± 94. 7
	Aroclor 1260	107 ± 32.5	< d.l.	156 ± 9.9	6 ± 8.5	< d.l.
	Aroclor 1254	< d.l.	< d.l.	176.5 ± 99.7	76 ± 107.5	96 ± 87.7
	Aroclor 1248	< d.l.	< d.l.	146.5 ± 116.7	87.5 ± 123.7	46.5 ± 51.6
	Aroclor 1060	59.5 ± 84.1	< d.l.	34.5 ± 33.2	15.5 ± 14.8	30.5 ± 3.5
	Aldrin	< d.l.	< d.l.	< d.l.	< d.l.	< d.l.
	Dieldrin	< d.l.	< d.l.	< d.l.	< d.l.	< d.l.
	Endrin	< d.l.	< d.l.	< d.l.	< d.l.	< d.l.
	α–endosulfan	< d.l.	< d.l.	< d.l.	< d.l.	< d.l.
	β-endosulfan	< d.l.	< d.l.	< d.l.	< d.l.	< d.l.
	HCB	< d.l.	< d.l.	< d.l.	< d.l.	< d.l.
	Heptachlor	< d.l.	< d.l.	< d.l.	< d.l.	< d.l.
	PBCCH	< d.l.	< d.l.	< d.l.	< d.l.	< d.l.
	TBoCT	< d.l.	< d.l.	< d.l.	< d.l.	< d.l.
	TBPhA	< d.l.	< d.l.	182 ± 257.4	< d.l.	< d.l.
	BTBPE	< d.l.	< d.l.	< d.l.	< d.l.	< d.l.
	pTBX	< d.l.	< d.l.	< d.l.	< d.l.	< d.l.
	PBT	177 ± 35.3	< d.l.	129.5 ± 146.4	214 ± 70.7	266.5 ± 137.9
PET	*2378-TCDD*	18 ± 4.2	< d.l.	34 ± 12.7	32.5 ± 4.9	20.5 ± 7.8
	12378-PeCDD	21.5 ± 6.4	< d.l.	20.5 ± 3.5	46.5 ± 65.8	33.5 ± 0.7
	123478-HxCDD	28.5 ± 10.6	< d.l.	40 ± 33.9	16.5 ± 4.9	22 ± 1.4
	123678-HxCDD	10 ± 14.1	< d.l.	118 ± 26.9	29.5 ± 7.8	39.5 ± 38.9
	123789-HxCDD	11.5 ± 2.1	< d.l.	125 ± 52.3	29.5 ± 4.9	16 ± 7.1
	1234678-HpCDD	88.5 ± 103.9	< d.l.	136.5 ± 3.5	56 ± 38.2	74 ± 59.4
	Aroclor 1260	31 ± 5.7	< d.l.	128 ± 29.7	21.5 ± 12	99.5 ± 102.5
	Aroclor 1254	41 ± 41	< d.l.	98.5 ± 26.2	17.5 ± 0.7	53 ± 32.5
	Aroclor 1248	28 ± 11.3	< d.l.	102.5 ± 38.9	31.5 ± 0.2	16 ± 9.9

	Aroclor 1060	61 ± 45.2	< d.l.	16 ± 2.8	31.5 ± 9.2	31 ± 8.5
	Aldrin	< d.l.	< d.l.	114.5 ± 20.5	< d.l.	< d.l.
	Dieldrin	< d.l.	< d.l.	< d.l.	< d.l.	< d.l.
	Endrin	< d.l.	< d.l.	< d.l.	< d.l.	< d.l.
	α–endosulfan	< d.l.	< d.l.	< d.l.	< d.l.	< d.l.
	β-endosulfan	< d.l.	< d.l.	< d.l.	< d.l.	< d.l.
	HCB	< d.l.	< d.l.	< d.l.	< d.l.	< d.l.
	Heptachlor	< d.l.	< d.l.	< d.l.	< d.l.	< d.l.
	PBCCH	< d.l.	< d.l.	< d.l.		< d.l.
	TBoCT	< d.l.	< d.l.	< d.l.	< d.l.	< d.l.
	TBPhA	17 ± 24	< d.l.	135 ± 165.5	< d.l.	17 ± 18.4
	BTBPE	< d.l.	< d.l.	< d.l.	< d.l.	< d.l.
	pTBX	< d.l.	< d.l.	< d.l.	< d.l.	< d.l.
	PBT	< d.l.	< d.l.	< d.l.	50 ± 59.4	151 ± 135.8
uPVC	*2378-TCDD*	80.5 ± 87	< d.l.	116 ± 42.4	37 ± 1.4	24 ± 12.7
	12378-PeCDD	35.5 ± 24.7	< d.l.	142 ± 18.4	66 ± 45.2	13 ± 4.2
	123478-HxCDD	52 ± 59.4	< d.l.	199 ± 161.2	44 ± 39.6	26.5 ± 6.4
	123678-HxCDD	37 ± 32.5	< d.l.	16.5 ± 3.5	26 ± 2.8	40 ± 33.9
	123789-HxCDD	77.5 ± 29	< d.l.	66.5 ± 78.5	88.5 ± 125.2	43 ± 60.8
	1234678-HpCDD	5 ± 7.1	< d.l.	30.5 ± 10.6	57 ± 55.1	59 ± 55.1
	Arocolor 1260	12.5 ± 0.7	< d.l.	119 ± 29.7	27.5 ± 0.7	98.5 ± 88.4
	Aroclor 1260	11 ± 15.6	< d.l.	41 ± 19.8	16.5 ± 9.2	24 ± 1.4
	Aroclor 1254	31.5 ± 16.3	< d.l.	168 ± 18.4	85 ± 60.8	22 ± 14.1
	Aroclor 1248	17 ± 24	< d.l.	151 ± 50.9	13 ± 1.4	15.5 ± 0.7
	Aroclor 1060	< d.l.	< d.l.	< d.l.	< d.l.	50 ± 70.7
	Dieldrin	< d.l.	< d.l.	< d.l.	< d.l.	< d.l.
	Endrin	< d.l.	< d.l.	< d.l.	< d.l.	< d.l.
	α–endosulfan	< d.l.	< d.l.	< d.l.	< d.l.	< d.l.
	β-endosulfan	< d.l.	< d.l.	< d.l.	< d.l.	< d.l.
	HCB	< d.l.	< d.l.	< d.l.	< d.l.	< d.l.
	Heptachlor	< d.l.	< d.l.	< d.l.	< d.l.	< d.l.
	PBCCH	< d.l.	< d.l.	< d.l.	< d.l.	< d.l.
	TBoCT	< d.l.	< d.l.	< d.l.	< d.l.	< d.l.
	TBPhA	< d.l.	< d.l.	< d.l.	< d.l.	< d.l.
	BTBPE	< d.l.	< d.l.	< d.l.	< d.l.	< d.l.
	pTBX	< d.l.	< d.l.	< d.l.	< d.l.	< d.l.
	PBT	98 ± 138.6	< d.l.	62.5 ± 88.4	217± 93.3	< d.l.

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Microplastics are ubiquitous in the marine environment and their impact on living organisms have raised concern in the recent years. Microplastic research field is relatively young. Novel methods have been establish for its study but many uncertainties remains on the impact of microplastics in the environment and organisms, for which new protocols are constantly being developed. In this thesis, we established a novel protocol for studying the cell response to diverse microplastics, where cell mortality and microplastic accumulation in cells was quantified by imaging flow cytometry. Through this protocol, we found that immune cells from very different species of economic and ecological importance, Atlantic salmon and blue mussel, had a significantly different response to different microplastic polymers. In addition, this thesis uncover the potential of different microplastic polymers to bind pollutants associated to Atlantic salmon farming, and suggests that microplastic could act as vector of pollutants from this fish farming facilities to non-polluted environments, increasing the environmental impact of aquaculture. Overall, this thesis provides new techniques for studying the impact of microplastics in living organisms, information about the microplastic impact in salmon and mussel, and shed light on the role of microplastics to impact the environmental risk of fish farming.

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ISBN: 978-82-93165-40-8
Trykk: Trykkeriet, Nord universitet
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