# Microplastic Pathways in Marine Pelagic Systems

A thesis submitted to the National University of Ireland, Galway

for the degree of Doctor of Philosophy

by

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## Declaration

I, Alina Madita Wieczorek, certify that this thesis is my own work and that the results presented here are to best of my knowledge correct. I have not obtained a degree in this University or elsewhere on the basis of any of this work. Contributions by co-authors and colleagues to each chapter are outlined below.

Signature: _____

Date: _____

**Chapter 1 – Mesopelagic Fish**
I designed and conducted the experiments, carried out FTIR analysis, analysed the data and wrote the manuscript. I also supervised undergraduate student Hannah Brownlow in the lab who assisted in microplastic extraction. Peter Croot provided access to reagents and lab facilities for microplastic extraction. Hannah Brownlow and Liam Morrison carried out SEM analysis. FTIR analysis was made available through Liam Morrison and technical support provided by Olivier Savard. Louise Allcock (chief scientist) and Eoin MacLoughlin collected the samples. Thomas Doyle and Eoin MacLoughlin conceptualised the study; Thomas Doyle oversaw the design of the experiment and the writing of the manuscript. All co-authors provided comments on the manuscript.

**Chapter 2 – Salps**
I designed and conducted the experiments, analysed the data and wrote the manuscript. Thomas Doyle oversaw the study design and the writing of the manuscript. Fabien Lombard contributed to the study design. Fabien Lombard and Peter Croot provided access to equipment and lab facilities. Jerome Sheahan helped to carry out the statistical analysis. All co-authors provided comments on the manuscript.

**Chapter 3 – Microzooplankton**
I designed and conducted the experiments, analysed the data and wrote the manuscript. Complementary data was made available by Wiebke Mohr (chlorophyll), Helena Osterholz (dissolved organic nitrogen and carbon), Gabriele Klockgether and Gaute Lavik (nutrients). The data was collected during a cruise led by Tim Ferdelman. Peter Croot oversaw the experimental design, analysis of the data and the writing of the manuscript.

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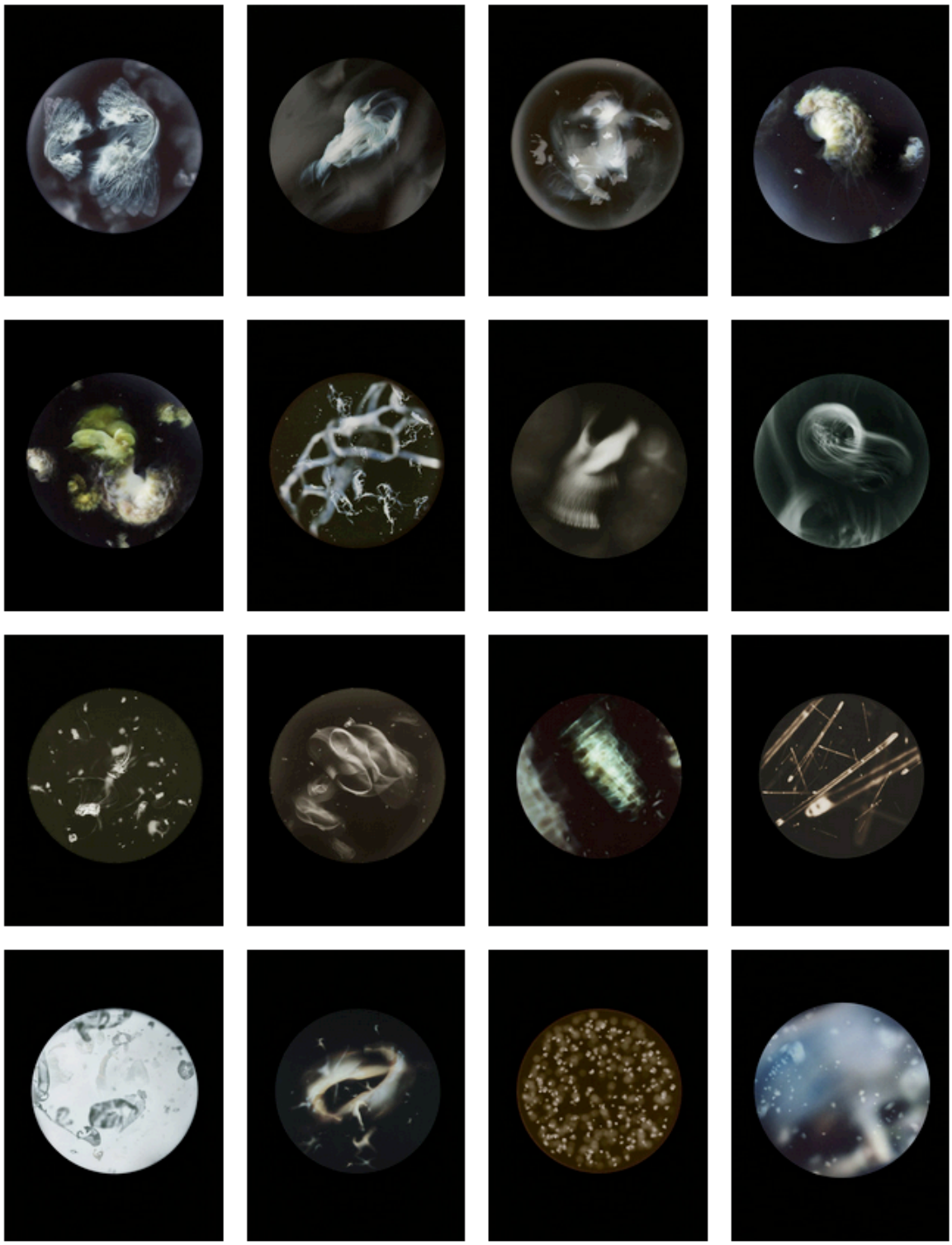
For my family, Ricarda, Jürgen, Renana and Dawid.



*Beyond Drifting*

Images of microplastics which resemble planktonic creatures

“The problem of environmental degradation is nearly as old as humanity, but never before has public concern been as deep and wide-spread as it is today. For the first time, general anxiety transcends personal and national boundaries. The well-being of the earth as we know it is at stake. The media reverberate with ominous premonitions about the future habitability of the globe, and the problem is high on the agenda of the top world leaders. How long will we last, and what can we do? Is the environment as ill as it seems, or are we entangled in a collective delusion?”

**Peter Westbroek, 1991** in *Life as a Geological Force*

## Abstract

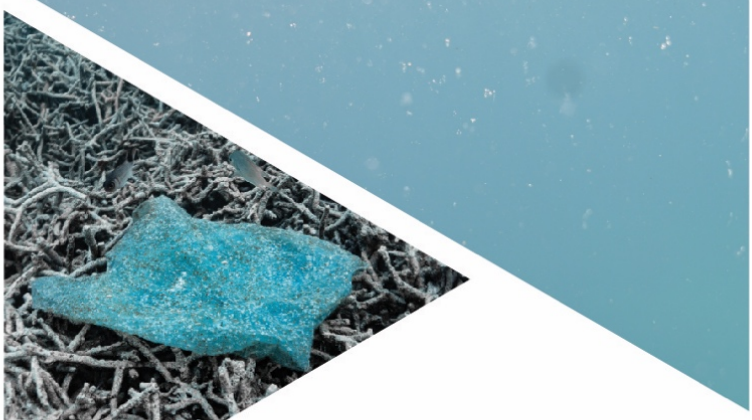
Marine litter and microplastics in particular have for some time now been postulated to be of environmental concern. Despite the ever-increasing amount of microplastic studies, conclusive evidence of the adverse effects of microplastics in the pelagic realm is currently deficient. Specifically, there is a need to look at contamination of key species in the environment and to conduct well-constructed cause and effect studies in order to make tangible conclusions about the potential environmental risks of microplastics. This type of study proves to be particularly challenging for the pelagic realm due to its diversity and dynamic nature. The work presented within this thesis aimed to assess how key ecological players interact with microplastics in the pelagic realm and what potential adverse effects these interactions present to the environment. Study organisms were chosen along two gradients, being organism size (macro to micro) and environmental nutrient saturation (eutrophic to oligotrophic). By doing so I was able to gain important insights into the pathways of microplastics in the pelagic realm.

Microplastic contamination of seven species of mesopelagic fishes, sampled from a nutrient rich warm-core eddy in the Northwest Atlantic, was investigated. With 73% of all of 233 assessed fish containing microplastics these fish seem to be particularly prone to microplastic contamination. Internalisation of microplastics could have happened either through direct ingestion from the sea water or through transfer from their planktivorous prey. Similarity in microplastic colour, size and shape to those sampled from the seawater indicates direct ingestion. Nevertheless, through the application of small mesh sizes of microplastic-retaining filters, this is the first study reporting microplastics in the gut contents of these fishes which overlap in size with food targeted by their planktivorous prey. Such high rates of microplastic contamination may have knock-on effects on individual fish health and those of their predators. For the gelatinous filter-feeding salps, which were exposed to microplastics in the laboratory, an effect on an individual level is unlikely as microplastics were simply in- and subsequently egested through incorporation into their faecal pellets. The incorporation of microplastics into salp faecal pellets did, however, slow down the pellets' sinking speed. High sinking velocities are a pivotal characteristic of this type of particulate organic matter as these allow it to bypass recycling in the upper water column and thus enable carbon sequestration to the ocean floor. Therefore, I show that microplastics may pose a threat to the distribution of organic matter in the water column and the cycling of carbon within

the biological pump. Lastly, microplankton communities sampled in the oligotrophic waters of the subtropical South Pacific were exposed to nanoplastic particles and ingestion by microzooplankton was investigated. Through experimental observations and encounter rate calculations it was shown that ingestion by microzooplankton is unlikely. For waters containing higher amounts of organic matter, coagulation of nanoplastics with such matter was demonstrated. This re-packages nanoplastics in a way that allows for their dispersal in the water column and increases their bioavailability. In waters where such matter is not sufficiently present a likely pathway could be filter-feeding ingestion by mucous grazers such as larvaceans and salps.

# INTRODUCTION

- (Micro-)Plastics – An Environmental Concern?
- The Marine Pelagic Environment
- Key Species
- So What?
- What If?
- Aims and Objectives



# Introduction

### (Micro-)Plastics – An Environmental Concern?

The birth of plastics (as defined in Table I. 1) took place on the 18th of June 1907 and was documented by Leo Baekeland in his laboratory notebook with the following words: “Yet the surface of the blocks of wood does not feel hard although a small part of gum that has oozed out is very hard.” (American Chemical Society, 1993). With these words he described the formation of what later became known as Bakelite - the first synthetic, mouldable polymer. Bakelite’s discovery was far from being a fortunate stroke of luck but more so a discovery which met the demand for an inert and durable substance. It quickly became a successful product and was marketed as “the material of a thousand uses” (Figure I. 1). Later, followed the discovery of many more synthetic polymers, collectively referred to as plastics (Table I.1). Today, the marketing slogan of “the material of a thousand uses” does not do plastic justice anymore. A life without them has become unthinkable to us (Feinkel, 2011).


**Figure I. 1:** Bakelite “The material of a Thousand Uses” advertisement, ©Ken Powell, reproduced with permission.

But how did plastics become an environmental concern? It may have been when the plastic industry realised that the production of a durable product was somewhat limiting economic growth and started to promote, what are now largely known as single-use plastics (Liboiron, 2013). Despite their durable nature it is estimated that only 30% of all plastics produced are still in use (Geyer et al., 2017). Since the introduction of large-scale manufacture of short-lived, single-use plastics, collection and disposal technologies have failed to meet the demand of production streams (Gourmelon, 2015). As a consequence, about 4,900 million metric tonnes, 60% of all plastics ever produced, can now be found in landfills and nature (Geyer et al., 2017).

In the marine environment, first records of plastics were those of ingestion by large charismatic species such as albatrosses and seals (Ryan, 2015). In 1972 Carpenter and Smith describe, for the first time, what has now been defined as a microplastic among their neuston samples of the Sargasso Sea (Carpenter and Smith, 1972) (Table I. 1). Today, a minimum of 5.25 trillion particles are thought to be present in the environment (Eriksen et al., 2014) and recent predictions estimate a 50-fold increase by 2100 (Everaert et al., 2018).

**Table I. 1:** Definitions of plastic, microplastic, nanoplastic, contaminant and pollutant.

| | **Definition** |
|---|---|
| **Plastic** | "A synthetic material made from a wide range of organic polymers such as polyethylene, PVC, nylon, etc., that can be moulded into shape while soft, and then set into a rigid or slightly elastic form." (*Source*: www.lexico.com, accessed 22.08.2019) |
| **Microplastic** | "Microplastics are any synthetic solid particle or polymeric matrix, with regular or irregular shape and with size ranging from 1 µm to 5 mm, of either primary or secondary manufacturing origin, which are insoluble in water." (Frias and Nash, 2019) |
| **Nanoplastic** | "Plastic particle within a size ranging from 1 to 1000 nm resulting from the degradation of industrial plastic objects and that can exhibit a colloidal behaviour." (Gigault et al., 2018) |
| **Contaminant** | "Inputs of alien and potentially toxic substances into the environment." (Stengel et al., 2006) |
| **Pollutant** | "Anthropogenically-introduced substances that have harmful effects on the environment." (Stengel et al., 2006) |

Once plastics enter our seas they are exposed to a series of physical and chemical stressors including ultraviolet radiation, wave action, temperature fluctuations, salinity, oxidising conditions, chemical pollutants and colonisation by microorganisms (Jahnke et al., 2017). These stressors drive the breakdown of

(macro-)plastics into microplastics and nanoplastics (for definition see Table I. 1)[1]. In the marine environment plastics are transported by currents (Van Sebille et al., 2015) while biologically driven processes are also thought to contribute to their distribution throughout the water column (Cozar et al., 2014; Cózar et al., 2015; Gorokhova, 2015; Song et al., 2018). Microplastics have been reported to be present in all oceans (Barnes et al., 2009), from the surface (Van Sebille et al., 2015) to the deepest point on earth (Peng et al., 2018). Up until 2016 some 220 species have been documented to have ingested microplastics from the environment (GESAMP Joint Group of Experts on the Scientific Aspects of Marine Environmental Protection, 2016).

However, does their ubiquitous nature make them a substance of environmental concern? It appears that serious concern exists, to the point that weathering plastic debris has been proposed as a potential planetary boundary threat alongside climate change and biodiversity loss (Jahnke et al., 2017). The authors do, however, acknowledge that thus far no disruptive effects of plastic on Earth system processes has been observed. Additionally, Borja and Elliot (2019) importantly highlight that in the context of microplastics, a distinction needs to be made between contaminants and pollutants; a contaminant being an alien substance in a given environment which may or may not cause harm and a pollutant being an anthropogenically-introduced substance that causes environmental harm (Stengel et al., 2006) (Table I. 1). Thus far many microplastic studies have merely described the presence of microplastic contamination, rather than pollution, in a given environment (Galgani et al., 2017; Borja and Elliott, 2019; Galloway et al., 2017; SAPEA, 2019). As a consequence, editors such as Borja and Elliot call for more studies addressing the “So what?” and “What if?” questions. “So what?” being those addressing the ecological effects of microplastics and potential solutions. “What if?” being those addressing the consequences of a scenario under which plastic levels cannot be and are not reduced.

While I largely agree with this point of view I think one adaptation in setting the future direction of microplastic studies is necessary. This is to add a third category aimed at providing empirical evidence of microplastic contamination of economically, culturally and/or ecologically important species in the wild. While desirable, it is not always possible to address the “So what?” question of

[1] To avoid overcomplication within the general introduction and discussion of this thesis the term “microplastics” will be used to loosely refer to micro- and nanoplastics.

microplastic ingestion by an organism. This is particularly true for very elusive (e.g. migrating) and deep sea species. Nevertheless, findings of microplastic contamination of culturally and/or ecologically important species may enable one to engage with and provide essential data to stakeholders and key actors. Their importance should not be neglected and a third category of “Key species” studies should also be given consideration. Important for these studies is the correct identification of a key species (see “Key species” section), and to acknowledge limitations of the insight gained by these studies, while also putting them in the context of “So what” and “What if” studies.

Within the remainder of this introductory chapter I will thus refrain from giving, yet another, extensive overview of the numerous studies of microplastics in marine species. Rather I will firstly provide a brief overview of the marine pelagic environment and then aspire to highlight some microplastic studies on (mostly) marine pelagic species which meet any of the three categories (Key species; So what?; What if?) and pinpoint knowledge gaps. I will then introduce the three research chapters of this PhD thesis and explain how these chapters contribute to the three categories of studies which help to address whether microplastics are of environmental concern.

## The Marine Pelagic Environment

The marine pelagic environment is defined as the free-flowing water of our oceans away from the coast and the sea benthos. This water mass makes up 99% of our biosphere by volume (Verity et al., 2002). Some 8% of this environment includes the water above the continental shelf (neritic zone), whereas the remaining 92% are the waters over our ocean basins (oceanic zone) (Kaiser et al., 2005). Vertically, the pelagic environment is divided into four zones: the epipelagic (0 – 200 m), the mesopelagic (200 – 1,000 m), the bathypelagic (1,000 m – 4,000 m) and the abyssopelagic (4,000 – 10,994 m) (Figure I. 2).

In comparison to the more static benthic and coastal environments, the pelagic environment is highly dynamic. Here the movement of large water masses is facilitated by physical factors such as planetary forcing, imbalances of solar radiation (due to climate cycles) and ocean basin morphology, as well as seasonal differences on a regional scale (Angel, 1993). These abiotic factors lead to circulation features such as currents, gyres, fronts, upwelling and eddies. Each of

these have their own spatial and temporal scale with some currents persisting for centuries and covering distances of thousands of kilometres and others, such as eddies, covering tens of kilometres and only lasting for a few days (Gubbay, 2006). Waters contained within any of these features have very different physical, chemical and biological characteristics (Angel, 1993). In high energy systems, such as eddies, fronts and upwelling areas, nutrients are commonly plentiful supporting short food webs dominated by large phytoplankton, (largely crustacean) zooplankton and fish (Sommer and Stibor, 2002). These high energy systems are very changeable and productive regions which are superimposed on an otherwise stable, low energy system characterised by low levels of nutrients and high nutrient recycling rates. In low energy systems food webs are more complex and commonly include the bacterial loop, small phytoplankton (due to higher surface area : volume ratio, allowing for increased nutrient absorption), microzooplankton, mesozooplankton, micronekton, fish and large predators (Sommer and Stibor, 2002).

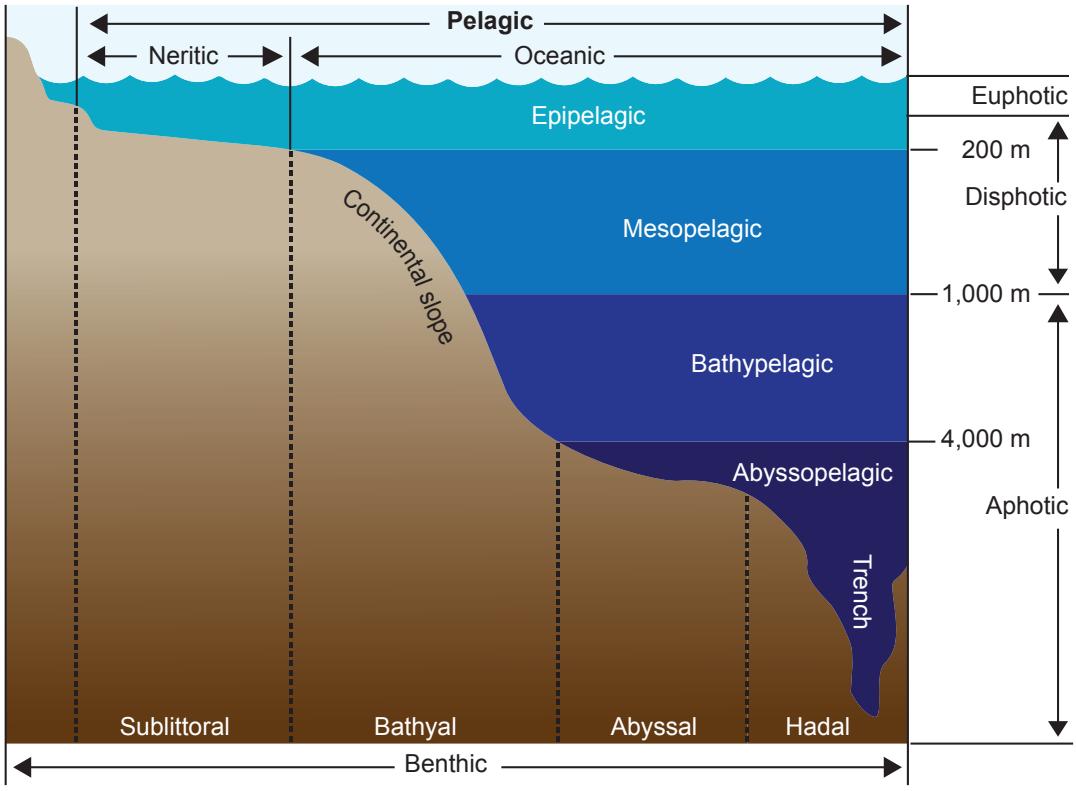



**Figure I. 2:** Graphical representation of the pelagic environment and its different zones. ©Alina M. Wieczorek

Pelagic ecosystems are estimated to account for 46% of our global net primary productivity (Field et al., 1998). Carbon which is captured this way and sequestered to depth through the pathways of the biological pump, is thought to account for the uptake of 1/6$^{th}$ – 1/3$^{rd}$ of all anthropogenic carbon released during the past two

centuries (Le Quéré et al., 2010; Passow and Carlson, 2012; Sabine, 2004). Sinking of organic matter, together with biomass input from chemoautotrophic processes at hydrothermal vents, make up the main supply of energy to deeper (bathy and abyssal) pelagic systems (Kaiser et al., 2005). As the concentration of sinking matter decreases with depth it has long been thought that deep pelagic zones are generally low in biomass and species richness. This view has recently been challenged and it is suggested that the deep pelagic may have been under-sampled and falsely assumed to be a desert of marine life (Webb et al., 2010). In recent years it has also been suggested that mesopelagic fish biomass has been underestimated by at least one order of magnitude making them a target for future exploitation (Irigoien et al., 2014; Kaartvedt et al., 2012; St. John et al., 2016). Pelagic fish from shallower waters are already thought to supply over 80% of fish consumed by humans (Pauly et al., 2002). Along with over-fishing, pelagic ecosystems are under threat from climate change, pollution, eutrophication and species introduction (Game et al., 2009). Whether microplastics pose another threat to the pelagic realm is an important question many marine researchers are currently trying to address.

## Key Species

Within this context I define key species as those which are valued for their economic, cultural and/or ecological importance and thus may be perceived as most relevant by stakeholders. What this essentially means is that humans need to have some sort of benefit from the investigated species. Such benefits are often listed within the concept of “ecosystem services” (Ehrlich and Mooney, 1983. Ecosystem services can be more obvious such as provision of food, or more abstract and subjective, such as an appreciation of an organism for its beauty. While all species on our planet contribute to ecosystem services to some degree, some species are more valued than others for the services they provide. There has been a long, on-going debate regarding how to assign values to ecosystem services (Barton, 2016; Costanza et al., 2014, 1997; Fisher et al., 2009; Gómez-Baggethun et al., 2014). For the purpose of microplastic studies the schematic by Gómez-Baggethun et al. (2014) illustrating the hypothetical relationships between human values, Maslow’s pyramid of human needs and ecosystem services may aid as a good guide to identify valuable key species (Figure I. 3). While Figure I. 3 provides some insight into the nature of these relationships and their importance (arrow thickness) it is

necessary to recognise that these values vary with countries and regions based on economic situation as well as cultural and educational background. If a species can be classified as particularly valuable in accordance with this schematic, obtaining information in regard to their contamination by microplastics in nature will also be valuable. It is therefore essential for marine scientists to consult such schematics as they may be biased when selecting a study organism due to the nature of the profession. Nevertheless scientists should not fail to meet the data demands of decision-makers for e.g. economically important species and those of the public who want to safeguard species which are for instance, culturally important to them.

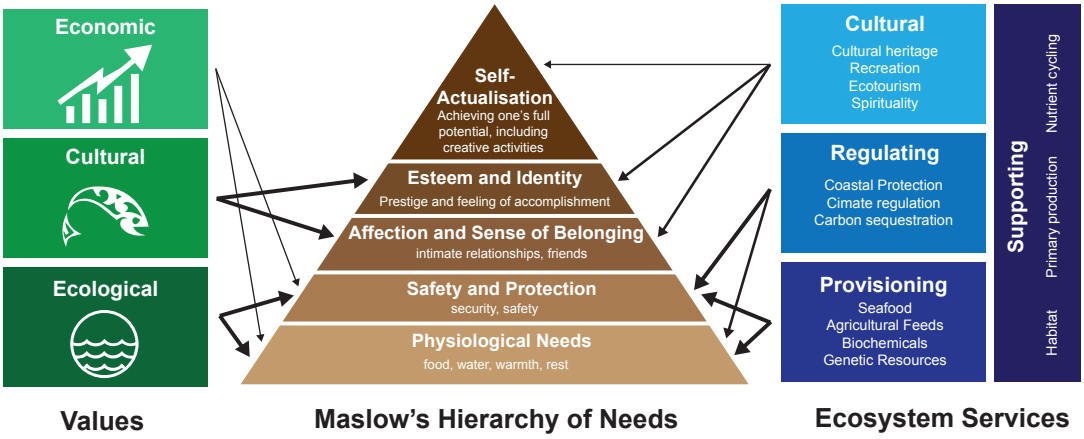



**Figure I. 3:** Adaptation of schematic by Gómez-Baggethun et al. (2014) illustrating the hypothetical relationships between human values, Maslow's pyramid of human needs and marine ecosystem services. ©Alina M Wieczorek

*Economically Important Species*

The most apparent example of key pelagic species of economic value are those exploited by fisheries. In 2016, first-sale value of global fisheries was estimated at 130 billion USD (FAO, 2018). Reports of ingestion of plastics by valuable pelagic species originate from all seas and occurrences and concentrations vary widely (Santillo et al., 2017). Unfortunately, so do extraction and analysis methodologies, which may explain some degree of variation amongst studies (Dehaut et al., 2016). With regard to pelagic finfish, 11 of the 20 most valuable species (by biomass) have been reported to ingest microplastics (Lusher et al., 2017). The most common type of microplastics identified from pelagic species appear to be fibers (Santillo et al., 2017). Yet, when comparing extracted microplastics from fish sampled from markets in Indonesia and the US, Rochman et al. (2015) found that samples from the US were heavily enriched in fibers whereas those from Indonesia did not contain any. They see a potential reason for this in the fact that washing machines are less common in Indonesia than the US and thus fibers, which among other sources are

thought to originate from washing machine wastewater, are also less common (De Falco, 2019). This study demonstrates that simple abundance studies can give valuable insights which need to be considered when trying to develop solutions. Despite there being a large number of studies on microplastic contamination in economically important species, there are still knowledge gaps. This becomes evident when considering that no microplastic contamination data exist for the three most important finfish species in terms of biomass. These are: 1. Alaska pollock - *Theragra chalcogramma*, 2. anchoveta - *Engraulis ringens* and 3. skipjack tuna - *Katsuwonus pelamis* (FAO, 2018). It is important to gather data for these and other economically valuable species, especially once there is more certainty about potential consequences for fish and human health (see “So what?”). In theory, assessment of microplastic contamination for these fish could in the future easily be accommodated alongside stock assessment procedures. Continuing investigations within individual studies applying differing analytical methods to a small number of species in a few regions will likely lead to patchy and skewed data which are of little use.

*Culturally Important Species*

The pelagic environment is home to numerous species of whales, dolphins, sharks and sea turtles, many of which have been assigned to our planets “charismatic megafauna”. Some of these species have been an integral part of long-rooted cultures such as the Māori (Native Polynesians) and Native Americans, while these days they are a big part of modern-day (eco-)tourism. The use of such species as a conservation tool has been acknowledged (and criticised) for quite some time now (Di Minin and Moilanen, 2014; McClenachan et al., 2012; Walpole and Leader-Williams, 1998). Interestingly, many of the earliest reports of plastic ingestion and entanglement were from species which we value as culturally important (e.g. Carr and Stancyk, 1975; Forrester et al., 1975; Walker and Coe, 1990). Over the past few years media coverage of ingestion of large plastic items by emblematic species has been the cause of global enragement (e.g. 40 kg of plastics found in Cuvier’s beaked whale in Philippines, March 2019). In terms of microplastics, several reports of microplastic ingestions exist but data is patchy and usually limited to opportunistic finds of stranded carcasses (Besseling et al., 2015; Lusher et al., 2015a). Despite this, it has been suggested that hard-shelled sea turtles be used as bio-indicators for microplastic pollution (Matiddi et al., 2017). Moreover, in a recent review the propriety of filter-feeding sharks and baleen whales as flagship species for microplastic pollution has been emphasised (Germanov et al., 2018). In order to

circumvent the problems of investigating these often protected and elusive species, researchers are starting to utilised the analysis of phthalates in the animals' tissue as an indication of plastic ingestion (Fossi et al., 2017; Savoca et al., 2018). There is also some promising on-going research utilising faecal matter collection by ecotourism operators to monitor microplastic ingestion by filter-feeding sharks (Wieczorek and Donati *ongoing research:* www.saveourseas.com/project/microplastics-a-macro-disaster/, accessed 26.09.2019).

*Ecologically Important Species*

The pelagic realm is highly diverse and many of its inhabitants are strongly interconnected while driving important chemical, physical and biological processes. In terms of microplastic studies of ecologically valuable species zooplankters have been a major focus of investigations. Zooplankton themselves, and the size of their prey, strongly overlap with the size range of microplastics. As a consequence, they may be particularly prone to ingest them. So far, most of the studies on zooplankton have been conducted in the laboratory (e.g. Cole et al., 2013; Coppock et al., 2019; Lee et al., 2013; Setälä et al., 2018) and very little data exists for *in situ* microplastic contamination. Among the few papers that currently exist are reports of microplastic contamination of copepods and euphausiids from the Northeast Pacific (1:34 and 1:17 microplastic to individual ratio, respectively) and fish larvae from the English Channel (2.9% with microplastics) (Desforges et al., 2015; Steer et al., 2017). A link between zooplankton trophic position and microplastic contamination has been suggested by Sun et al. (2017) who studied microplastic contamination of copepods, chaetognaths, jellyfish, shrimp and fish larvae in the South China Sea (microplastic/zooplankton encounter rates of 7%, 18%, 41%, 55% and 132%, respectively). Studies largely refer to microplastic contamination of these, and other species, as ingestion but one should consider that plastics may also get trapped by and attached to an organism as has been described for the mauve stinger (*Pelagia noctiluca*) (Macali et al., 2018). This is particularly true for cnidarians which possess stinging/adhesive cnidae. All these species are important sources of energy for pelagic predators and collectively they drive key ecological processes such as the biological pump (Turner, 2002). Furthermore, zooplankton larvae replenish global populations of echinoderms, crustaceans, polychaetes and nearly all fish, and gelatinous zooplankton have been recognised for making up a large fraction of biomass in deeper waters (Webb et al., 2010). Similarly, mesopelagic fish are thought to make up a major fraction of the biomass in the mesopelagic realm

(Kaartvedt et al., 2012). They have a wide-spread global distribution but few studies report on microplastic abundance in these fish: two studies from the North Pacific (Boerger et al., 2010; Davison and Asch, 2011) and one from the North Atlantic (Lusher et al., 2015b). They describe percentage occurrences of 9%, 35% and 11%, respectively. Mesopelagic fish link surface to deeper waters through their diurnal migrations and are a key energy source for large predators such as tuna and swordfish (Stillwell and Kohler, 1985; Varela et al., 2013). Of these large predators 4.2% of sampled albacore (*Thunnus alalunga*), Atlantic bluefin tuna (*Thunnus thynnus*) and swordfish (*Xiphias gladius*) have been found to have macro- and microplastics in their gut contents (Romeo et al., 2015). For other apex predators such as sharks and dolphins data are however, limited to entanglement with marine debris and opportunistic ingestion of larger plastics based on the extraction from carcasses (Colmenero et al., 2017; Denuncio et al., 2011; Laist, 1997; Parton et al., 2019; Sazima et al., 2002). In the deep pelagic it is hard to make definite conclusions about the ecological significance of some of these species. Nevertheless, can one safely assume that amphipods, due to the biomass they provide, and the fact that they are known to form symbiotic relationships, are likely key species of the abyssal and hadal pelagic (Gasca et al., 2007). Despite their remoteness from anthropogenic inputs, 72% of these deep-sea amphipods have, in a recent global study, been described to have been contaminated with microplastics (Jamieson et al., 2019).

## So What?

We now know that microplastics are present in some key economic, cultural and ecological species, but so what? One might argue that microplastics are relatively inert and thus if ingested, may simply be egested again. Analogous to this would be the in- and egestion of a piece of jewellery by a human; which made them favourable for smuggling in this way (www.livescience.com/34190-what-happens-when-you-swallow-a-diamond.html, accessed 25.09.2019). In fact, ingestion and subsequent egestion of microplastics with little to no effect on overall fitness has been described, for instance in sea urchin larvae (Kaposi et al., 2014). In contrast, ingestion of microplastics and associated chemicals has been shown to cause intestinal alterations and liver toxicity in some fish (Pedà et al., 2016; Rochman et al., 2013). This effect would be more comparable to the associated health risks of drug smuggling via body packing (Hartoko et al., 1988). What this illustrates is that

the nature of the substance of concern (inert piece of jewellery vs. hazardous drug packed in thin foil) determines its pathways and potential detrimental consequences.

In terms of microplastics in the marine pelagic environment this is determined by their initial composition (polymer mix, residual monomers and additives) as well as *in situ* associations with organic matter, bacteria and chemical pollutants (Galloway et al., 2017 and references therein). Furthermore, it needs to be considered that bioavailability and ecosystem pathways are governed by the dynamic changes a piece of plastic undergoes in the marine environment. These changes are driven by degradation, sheathing, colonisation, aggregation and ingestion.

Detrimental impacts of microplastics in marine pelagic organisms can manifest at different levels. For instance on an individual level microplastics have been demonstrated to have an effect on the fitness of copepods. For *Calanus helgolandicus* it was found that the ingestion of 20 µm fluorescent polystyrene beads affected feeding, leading to a 40% decrease in carbon biomass uptake and a reduction in reproductive output (Cole et al., 2015). In copepods, nanoplastics have been found to be particularly harmful. For *Tigriopus japonicus* it has been found that while plastics between 0.5 – 6 µm in size appeared to affect fecundity, nanoplastics (0.5 µm in size) also decreased survival of copepodites and nauplii and caused mortality in the second generation (Lee et al., 2013). A potential reason for this is the translocation of nanoplastics as is hypothesised by Jeong et al. (2017) who report an oxidative stress response mechanism by *Paracyclopina nana* in response to the exposure to nanoplastics of 0.05 µm size, but not by larger ones. These studies do provide some valuable first insights into potential pathways and effects of microplastics. Critical to consider, however, is that these are laboratory studies and the types of microplastics, as well as the concentrations to which the organisms were exposed, to do not reflect those present in the environment (Botterell et al., 2019; Lenz et al., 2016).

In the case of the sea urchin larva (*Tripneustes gratilla*) no effect was noted when they were exposed to environmentally relevant concentrations and plastics were simply in- and egested again (Kaposi et al., 2014). Antarctic krill (*Euphausia superba*) seem to respond in a similar manner (Dawson et al., 2018a) but have also been found to fragment microplastics during digestion (Dawson et al., 2018b). To get more realistic insights, researchers now aim to use microplastics which resemble those present in the environment (Cole, 2016). Using manufactured

microfibers, Coppock et al. (2019) found that exposure led to a behavioural change in food selectivity which resulted in a 6-8% overall reduction in feeding. Interestingly, when comparing uptake, egestion and effect of microplastics in freshwater amphipods (*Hyalella azteca*) it was found that fibers may be more harmful as they are not as readily egested as particles and hampered growth (Au et al., 2015). Very little insight exists for higher trophic levels such as fish but a recent study on glassfish (*Ambassis dussumieri*) that were exposed to environmentally relevant doses of microplastics showed a common trend of growth reduction (Naidoo and Glassom, 2019). Microplastics may actually accumulate at a faster rate in these higher level trophic organisms due to ingestion of microplastic contaminated prey. Trophic transfer of microplastics under lab conditions has already been demonstrated from smaller zooplankton prey to mysid shrimp (*Neomysis integer*) (Setälä et al., 2014). Transfer of microplastics from their mackerel prey (*Scomber scombrus*) may also have been the likely pathway of microplastics found inside grey seal (*Halichoerus grypus*) scat (Nelms et al., 2018). This is also of concern in terms of chemicals within (additives) and absorbed onto microplastics as these may then be absorbed into the animals tissue and bioaccumulate along food webs. Transfer of polybrominated diphenyl ether, a common flame retardant used in plastics, from microplastics into an organism's tissue has already been demonstrated in amphipods (*Allorchestes compressa*) and rainbow fish (*Melanotaenia fluviatilis*) (Chua et al., 2014; Wardrop et al., 2016). Exposure to absorbed pollutants has been shown to cause liver toxicity in medaka (*Oryzias latipes*) and in sea bass (*Dicentrarchus labrax*), where polluted microplastic exposure lead to increased intestinal alterations (compared to exposure to non-polluted microplastics), potentially causing blockage, and a decrease in nutrient absorption. However, the relevance and potential effects of pollutants absorbed onto microplastics is currently being debated (Gouin et al., 2011; Koelmans et al., 2016; Lohmann, 2017). Overall, studies on the impacts of microplastics on an organism's health, behaviour and potential trophic transfer, while supported by a few studies on freshwater/coastal species (Besseling et al., 2014; de Sá et al., 2015; Tosetto et al., 2016; Wang et al., 2019), are currently very limited.

In terms of large-scale ecological processes, some preliminary studies have been carried out to specifically investigate potential effects on particle sinking. In the pelagic environment, the downward transport of nutrients and organic material from the ocean surface, by the means of particle sinking and diurnally migrating species,

is a key supply of energy to species throughout the water column and the sea benthos (Kaiser et al., 2005). What is even more important, is the role of sinking particles for biogeochemical cycling and carbon sequestration, specifically within the biological pump (Turner, 2002). In a pioneering study Long et al. (2015) describe for the first time that microplastics may interact with sinking particles. They showed that 2 µm fluorescent polystyrene microspheres were readily incorporated into algae aggregates. This caused a decrease in sinking speed for aggregates produced by the diatom *Chaetoceros neogracile* and an increase for aggregates composed of microplastics and the cryptophyte algae *Rhodomonas salina* (Long et al., 2015). Apart from aggregates, faecal pellets of zooplankton contribute significantly to the downward flux of particulate organic matter (Turner, 2002). This has led researchers to investigate what impact microplastics incorporated into zooplankton faecal pellets may have on their sinking speed. For the copepod *C. helgolandicus* a preliminary study showed that sinking rates decreased significantly once microplastics were incorporated into the faecal pellets (Cole et al., 2016). While in this study the microplastics used were, again, not so environmentally relevant, a more recent study on *C. helgolandicus* utilised more environmentally relevant, fibrous microplastics. Here the authors observed a similar trend for microplastics of low densities (Coppock et al., 2019). Lastly, it is also important to consider findings on the larvacean *Bathochordaeus stygius* that, among other filter-feeders, is known to facilitate downward flux of organic matter by re-packaging captured prey into faecal pellets and through the sinking of their shed houses and carcasses (Conley et al., 2018; Turner, 2015, 2002). When exposed to high doses of varying sizes of fluorescent polystyrene particles, *B. stygius* was found to incorporate these into faecal pellets and shed houses, but through visual inspection the authors concluded that both faecal pellets and houses remained negatively buoyant (Katija et al., 2017). Filter-feeders may be particularly prone to the ingestion of microplastics as they display less selective feeding behaviour (e.g. Madin, 1974). Despite these findings, it is feasible to summarise that in order to make tangible conclusions about the ecological impact of microplastics, we are currently still lacking studies applying environmentally relevant types and concentrations of microplastics on key ecological species (Cunningham and Sigwart, 2019).

## Introduction

### What If?

What happens if? Any answer to a question beginning with these words will contain an element of uncertainty as the inclusion of the word “if” implies a hypothetical scenario. It is a common concept used in climate change studies and reports such as the special report “Global Warming of 1.5°C” by the intergovernmental panel on climate change which, among many other things, explores ecological consequences of 1.5$^{o}$C (versus 2$^{o}$C) warming (Hoegh-Guldberg et al., 2018). “If” scenarios aid in guiding key actors and policymakers in decision making (Krug et al., 2017). For plastic pollution one may remember the repeatedly referred-to scenario stating that if plastic input into our seas continues at predicted rates then by 2050 there will be more plastics than fish in the sea in terms of weight (MacArthur et al., 2016). While this statement definitely sparked the interest of the public, it is hard to guide decision making on the basis of these types of predictions as they give little insight into the environmental or human risk. More complex assessments are needed, but in order to conduct these, reliable standards and datasets are required. Koelmans et al. (2017) outline that for plastics it will be key to work with a range of concentrations in order to assess which are likely to cause adverse effects (i.e. effect and lethal concentrations). They also point out that coming up with ecologically relevant metrics for plastic pollution is rather challenging due to their multifaceted nature in terms of type, shape, additives and so forth (Koelmans et al., 2017). This gets further complicated by the fact that microplastics in the environment are highly changeable in their nature and distribution (Galloway et al., 2017). At present there are very few studies on the effects of microplastics and most studies explore effects using environmentally irrelevant microplastic types, in concentrations exceeding those recorded in the environment by many-fold (see “So what”) (Cunningham and Sigwart, 2019; Lenz et al., 2016). If these get incorporated into models for risk assessment they may underestimate concentrations causing adverse effects as no data for lower limits exist (Koelmans et al., 2017; Troost et al., 2018). To my knowledge, only two studies have thus far endeavoured to extrapolate the ecological risks of plastics based on currently available data. One study explored the effects of microplastics on pelagic ecosystem productivity in the Southern North Sea (Troost et al., 2018). Microplastic abundances within this study were extrapolated from current models and effects on primary production were assumed to be increased respiration, due to increased motility loss and damage repair (based on Bhattacharya et al., 2010), whereas effects on secondary production were based on reduction in *C. helgolandicus* feeding rates, as reported

by Cole et al. (2013). Interestingly, despite adverse impacts reported from exposure studies, in this study the authors predict no overall effect on production in the Southern North Sea. The second study assessing the ecological risks of microplastics was carried out by Everaert et al. (2018). The aim of this study was to predict the amount of microplastics likely to be found in the pelagic realm, the seafloor and beached on the coastline by 2100 and assess whether this amount is below concentrations causing adverse effects. For the pelagic environment, the authors predict that 9 – 49 microplastics will be present in one cubic meter of water, which they model to be below the safe concentration of 6,650 microplastics, above which they predict adverse effects based on currently available effect data (Everaert et al., 2018). Both studies emphasise to a great extent the uncertainties associated with their predictions and how these hamper efforts to make tangible conclusions about the ecological risk of microplastics. In order to address these uncertainties more well-designed studies are needed, estimating abundances and effects of microplastics. In particular studies need to consider:

1) The use of ecologically relevant metrics
2) The use of a range of concentrations from present and below to 50% effect and lethal concentration endpoints
3) Monitoring of long-term concentration response
4) Selection of key ecological species
5) Investigation of a wider range of organisms with different feeding strategies
6) Transport mechanisms between different environments

Only once these studies are available will it be possible to address the “What if?” question with an acceptable amount of certainty.

## Aims and Objectives

The research presented within this thesis investigates microplastic pathways in the marine pelagic environment along two gradients: from macro- to micro-scale organisms and eutrophic to oligotrophic waters (Figure I. 4). Three organisms of distinct trophic levels were selected, which each play important roles in key ecological processes in the pelagic realm, and the following objectives were addressed:

*Chapter 1 – Mesopelagic Fish*

Microplastic contamination in mesopelagic fish sampled from an eddy region in the Northwest Atlantic was assessed and it was investigated whether species, stomach fullness and the depth at which the fish were caught had a governing effect on the amount of microplastics present. Moreover, the type, shape, colour and size of microplastics found in the gut contents were examined and compared to those from the surface waters.

*Chapter 2 – Salps*

Salps were exposed to a range of concentrations (from current to arbitrary future levels) of environmentally relevant microplastics to investigate whether they do ingest microplastics and if so, whether they subsequently incorporate them into their faecal pellets. Following this, alterations in sinking speed of faecal pellets containing microplastics were assessed.

*Chapter 3 – Microzooplankton*

Utilising flow cytometry, it was assessed how microplankton communities of the South Pacific respond to nanoplastic exposure with a particular focus on potential ingestion by heterotrophic nanoflagellates and mixotrophs. These findings were then put into a theoretical concept of encounter rates between nanoplastics and the microplankton community.

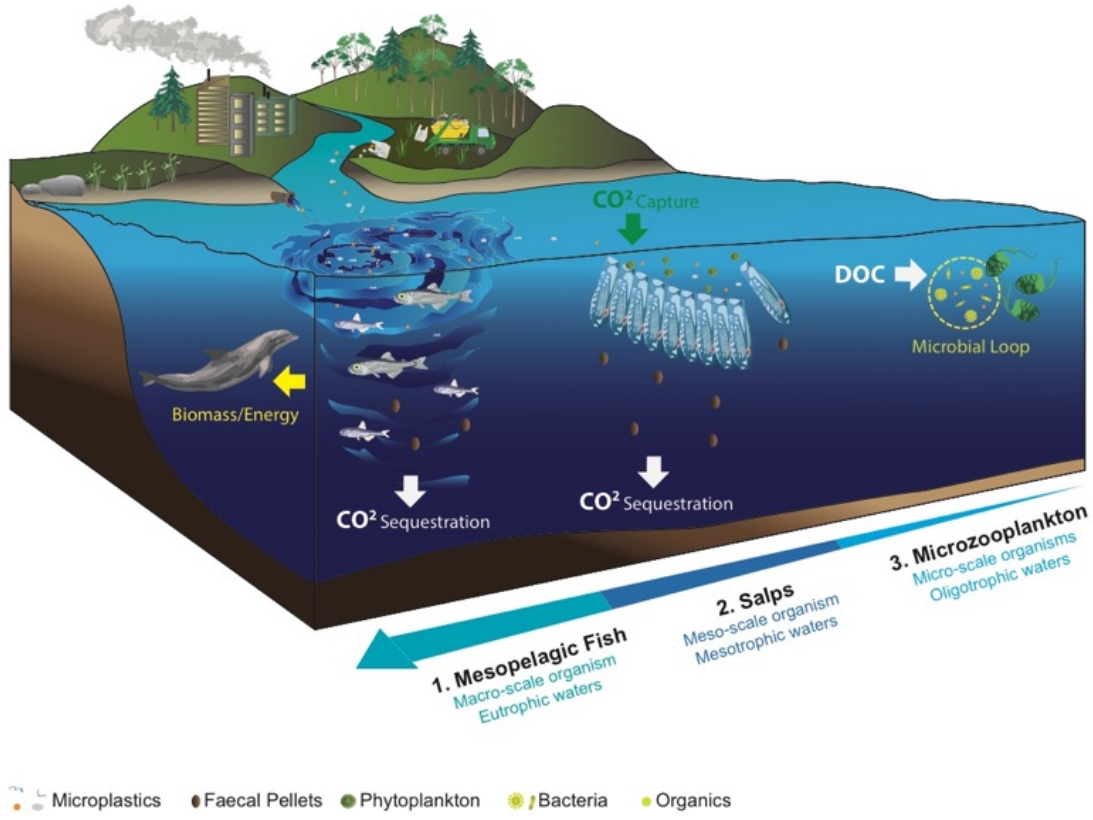



**Figure I. 4:** Graphical representation of the organisms investigated within the research chapters of this thesis and their ecological function in the pelagic realm. ©Alina M. Wieczorek

These objectives are in agreement with the above argued need for microplastic studies looking at key species, ecological effects, pathways and parameters needed to conduct ecological risk assessment. All three organisms are ecologically significant (Figure I. 2). Mesopelagic fish are important prey for large predators and undergo diurnal migrations moving organic matter within the water column (Radchenko, 2007). Salps facilitate an important downward flux mechanism for nutrients and carbon captured at the sea surface by photosynthetic organisms which they ingest and re-package into dense faecal pellets (Turner, 2002). Microzooplankton provide the pivotal link between the microbial loop and higher trophic organisms, particularly in oligotrophic waters (Fenchel, 1988). When considering plans to exploit mesopelagic fish and debates of carbon credits, mesopelagic fish and salps could also be considered of high economic importance in years to come (Irigoien et al., 2014). Knowledge about microplastic contamination and potential exposure pathways of any of the three species is thus scientifically valuable.

In terms of adverse ecological effects, the above objectives may allow for first insights of microplastics potentially transferred from mesopelagic fish to large predatory fish, from salp faecal pellets to coprophagous and benthic organisms and from microzooplankton to zooplankton. Moreover, for salps, effects on carbon sequestration within the biological pump, a key ecological process taking place in the pelagic environment, was enumerated in exposure studies using environmentally relevant types and concentrations of microplastics. These findings can directly feed into predictions of the ecological risks of microplastics. Lastly, the investigated organisms display three different feeding strategies and, as mentioned above, investigating organisms with varying feeding strategies is another criterion recently requested for future studies (Koelmans et al., 2017).

The overall aim and the specific research objectives of this thesis thus help address the question of whether microplastics are of environmental concern for the pelagic realm.

## Introduction

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# Chapter 1

# Frequency of Microplastics in Mesopelagic Fishes from the Northwest Atlantic

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# Chapter 1 – Mesopelagic Fish


## Abstract

Microplastics are a ubiquitous pollutant in our seas today and are known to have detrimental effects on a variety of organisms. Over the past decade, numerous studies have documented microplastic ingestion by marine species, with more recent investigations focussing on the secondary impacts of microplastic ingestion on ecosystem processes. However, few studies so far have examined microplastic ingestion by mesopelagic fish which are one of the most abundant pelagic groups in our oceans and, through their vertical migrations, are known to contribute significantly to the rapid transport of carbon and nutrients to the deep sea. Therefore, any ingestion of microplastics by mesopelagic fish may adversely affect this cycling and may aid in transport of microplastics from surface waters to the deep-sea benthos. In this study, microplastics were extracted from mesopelagic fish under forensic conditions and analysed for polymer type utilising micro-Fourier Transform Infrared Spectroscopy (micro-FTIR) analysis. Fish specimens were collected from depth (300 - 600 m) in a warm-core eddy located in the Northwest Atlantic, 1,200 km due east of Newfoundland during April and May 2015. In total, 233 fish gut contents from seven different species of mesopelagic fish were examined. An alkaline dissolution of organic materials from extracted stomach contents was performed and the solution filtered over a 0.7 µm borosilicate filter. Filters were examined for microplastics and a subsample originating from 35 fish was further analysed for polymer type through micro-FTIR analysis. 73% of all fish contained plastics in their gut contents with *Gonostoma denudatum* having the highest ingestion rate (100%), followed by *Serrivomer beanii* (93%) and *Lampanyctus macdonaldi* (75%). Overall, we found a much higher occurrence of microplastic fragments, mainly polyethylene fibres, in the gut contents of mesopelagic fish than previously reported. Stomach fullness, species and the depth at which fish were caught, were found to have no effect on the amount of microplastics found in the gut contents. However, these plastics were similar to those sampled from the surface water. Additionally, using forensic techniques we were able to highlight that fibres are a real concern rather than an artefact of airborne contamination.

## Chapter 1 – Mesopelagic Fish

### Introduction

As a consequence of decades of marine litter entering our seas (Ryan, 2015), microplastics have been found in coastal and pelagic environments around the globe with an ever-increasing distribution (Galgani et al., 2015; Barnes et al., 2009).

Considering the prevalence of microplastics, there is now a substantial research effort investigating their abundance in the gastrointestinal tracts of various organisms. For example, some early studies found that 83% of *Nephrops norvegicus* had microplastics in their tracts (Murray & Cowie, 2011) and Lusher et al. (2013) found microplastics in the tracts of 35% of the pelagic and demersal fish species examined. Indeed, Gall and Thompson (2015) have reported that over 690 marine species are impacted by marine litter. More recent studies have moved from quantifying which animals have ingested microplastics to examining the physical and health implications of microplastic ingestion (Pedá et al., 2016, Cole et al., 2014; Rochman et al., 2013). For example, Wright et al. (2013) demonstrated how the ingestion of microplastics by the polychaete *Arenicola marina* (lugworm), an important ecosystem engineer of Northern Europe's intertidal zones, caused inflammation and decreased feeding and ultimately depleted energy reserves. Such studies have prompted researchers to investigate the impact on ecosystem processes. Cole et al. (2016) found that microplastics alter the sinking rates of copepod faecal pellets if ingested and may consequently affect the downward flux of carbon to the ocean floor. With the increasing evidence that microplastics represent an ecosystem and environmental health concern, UNEP and the EU Commission have established bodies and efforts to guide in decision making and legislation (UNEP, 2016; Galgani et al., 2013). Furthermore, several governments have taken steps by introducing legislations to ban microbeads in cosmetics and detergents by 2020 (Sutherland et al., 2017).

Despite this substantial increase in studies investigating the ingestion of microplastics and their associated impacts, there are still important taxa playing key roles in ecosystem functioning that have not been well studied. Mesopelagic fish inhabiting the disphotic zone of the pelagic realm (200 - 1000 m depth) from the Arctic to the Antarctic (Gjøsaeter & Kawaguchi, 1980) are one of these understudied groups. Many species are known to undergo diurnal vertical migrations by residing at depth during the day before migrating to the surface at night to feed (Gjøsaeter & Kawaguchi, 1980). Smaller mesopelagic fish such as *Myctophum punctatum* and *Benthosema glaciale* feed by filtering zooplankton,

predominantly copepods, euphausiids, amphipods, eggs and larvae, over their gill rakers (Scotto di Carlo, 1982; Roe and Badcock, 1984). Larger mesopelagic fish such as *Stomias boa* and *Serrivomer beanii* also actively target decapods and fish using their anterior vertebrae and branchial apparatus to swallow larger prey (Roe and Badcock, 1984; Bauchot, 1986). Thus, mesopelagic fish are exposed to microplastics either through the direct consumption of a microplastic mistakenly identified as a prey item or indirectly, through the consumption of a prey item (e.g. copepod or euphausiid) that had already consumed microplastics.

As mesopelagic fish undergo large vertical migrations, they are known to play a key role in the cycling of carbon and nutrients to the deep ocean (Radchenko, 2007; Davison et al., 2013). For instance, Radchenko (2007) has shown that such species in the Bering Sea transport 15,000 tonnes of carbon daily to the deep ocean. Therefore, the ingestion of microplastics by mesopelagic fish may disrupt carbon cycling and aid in the transport of microplastics to deeper waters, as suggested by Lusher et al. (2016).

The importance of mesopelagic fish was recently further highlighted in studies by Kaartvedt et al. (2012) and Irigoien et al. (2014) who found that the mesopelagic fish biomass in the global oceans may have previously been underestimated by at least one order of magnitude due to avoidance behaviour and mesh extrusion. Because they make up such a large biomass in the pelagic realm, they provide an important food source for a variety of predatory fish and marine mammals which, through trophic transfer from their mesopelagic fish prey, may suffer from the impacts of microplastics and associated toxins (Lusher et al., 2016). Some of the species preying on mesopelagic fish such as tuna and swordfish (Scott and Tibbo, 1968; Varela et al., 2013) are commercially important food sources and thus toxins and microplastics transferred to these species may also pose a danger to human health. To date, mesopelagic fish have not been exploited as a human food source due to the high levels of wax esters in their tissue (Gjøsaeter & Kawaguchi, 1980). This may change in the near future as the demand for fish protein increases and new policies (e.g. Blue Growth Strategy by the European Union) encourage sustainable exploitation of potential resources (St. John et al., 2016). Furthermore, the food safety issues concerned with microplastics, and the associated toxin exposure through the consumption of commercially exploited fish, have recently been outlined in an extensive report by the Food and Agriculture Organization of the United Nations drawing attention to the potential threat of microplastics to human health (Lusher et al., 2017).

However, to date, only a few studies have investigated microplastic ingestion by mesopelagic fish: one in the North Atlantic (Lusher et al., 2016) and two in the North Pacific Gyre regions (Boerger et al., 2010; Davison & Ash, 2011). Since then, new and improved methodologies for microplastic extraction have been developed with an emphasis on ultra-clean techniques in order to prevent airborne contamination (Wesch et al., 2017).

This study set out to quantify microplastic ingestion by mesopelagic fish from an eddy region in the Northwest Atlantic known to be a hot spot for mesopelagic fish (Fennel & Rose, 2015; McKelvie, 1985) and potentially microplastics (Yu et al., 2018). Specifically, this study investigated whether: 1) species, stomach fullness and the depth at which fish were caught had an effect on the amount of microplastics found in the gut contents of mesopelagic fish, and 2) how the type, shape and size of microplastics found in the gut contents compared to those found in the surface waters. Importantly, we applied strict measures to prevent microplastic contamination during extraction and identified microplastic type using micro-FTIR spectroscopy.

## Methods

*Sample Collection*

Mesopelagic fish samples were collected during a Northern Atlantic crossing (*CE*15007) from Galway, Ireland to St. John's, Newfoundland aboard the RV *Celtic Explorer* between the 20$^{th}$ of April and the 5$^{th}$ of May 2015. In total, eight 30-minute pelagic trawls were carried out during daylight hours at a towing speed of 4 knots (Figure 1. 1). The opening of the net was fitted with a Scanmar depth sensor to enable three trawls to be conducted in the upper mesopelagic zone between 300 - 350 m (shallow) and five in the lower mesopelagic zone between 500 - 650 m (deep). Once hauled aboard, a random subsample of 35 intact mesopelagic fish was taken from each trawl.

Furthermore, surface water samples were taken during each trawl by utilising the ship's underway water pumping system with its intake located at 3 m depth. The intake water initially passed through a 1 mm mesh and was then pumped into the lab facilities, where the underway hose was positioned to allow water to pass through a 180 μm plankton sieve. Sieved particles were then washed down with 0.2 μm filtered ultrapure water into cylindrical aluminium containers (5 cm Ø) which

were then folded over at the top. The flow rate of water through the underway pump was measured at 10 L $min^{-1}$ and the volume of water filtered was estimated using the flow rate and duration of each trawl. Fish and water samples were stored in Ziploc® bags and immediately transferred into a -20 °C freezer and stored there until the vessel returned to Galway on the 15th of May 2015 when samples were transferred to a -20 °C freezer at the National University Ireland, Galway.

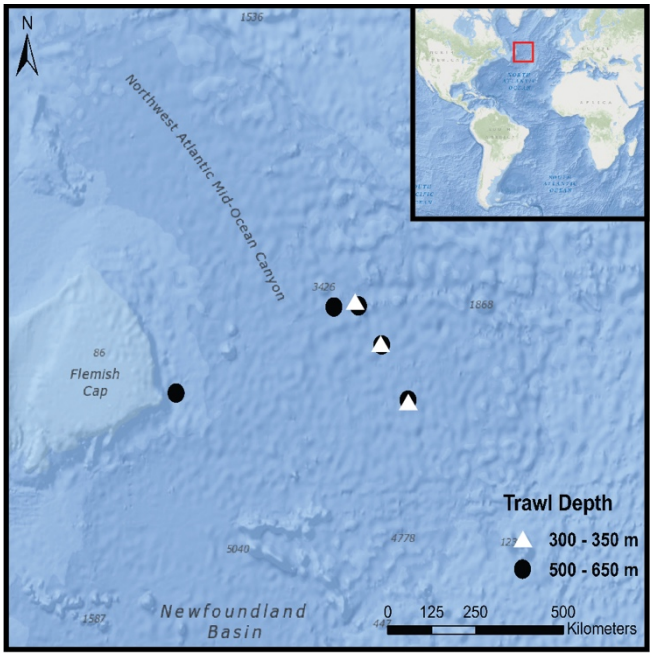


**Figure 1. 1:** Trawl locations during *CE*15007 survey aboard the RV *Celtic Explorer*; red rectangular box in the insert corresponds to outer figure margin.

*Ethics Statement*

Fish were taken dead from midwater trawls carried out to ground truth the backscatter from a Simrad EK60 scientific echo sounder investigating the deep scattering layer in the Northwest Atlantic and are thus exempt from ethical approval dealing with regulated animals; that is live vertebrates and higher invertebrates.

*Sample Processing and Polymer Analysis*

Samples were processed between September 2015 and June 2016. Fish samples were defrosted approximately 3 hours prior to processing and identified by counting number of dorsal, pectoral and anal fin rays and number of gill rakers as well as prominent features such as photophores and barbels (Marine Species Identification Portal, 2017). Fish which displayed visible physical damage to their digestive tract were excluded from analysis. The standard length (to the nearest millimetre) of each fish was recorded. Fish were rinsed with 0.2 μm filtered MilliQ™ water (18.2 MΩ $cm^{-1}$) (Millipore, Bedford, USA) and weighed (to the nearest 0.0001 g) before

being transferred into a borosilicate container located inside a laminar flow hood (AirClean600®: ISO class 5) where part of their alimentary tract - the oesophagus to the duodenum - was extracted. The extracted alimentary tract was then opened, and the gut contents emptied into 20 ml borosilicate scintillation vials and the alimentary tract lining thoroughly washed with 0.2 μm filtered MilliQ™. The removed alimentary tract and the dissected fish were then weighed (to the nearest 0.0001 g) to obtain gut contents weight. Vials containing gut contents were filled with MilliQ™ water and sodium hydroxide (Certified analytical reagent for analysis, Fisher Scientific, UK) to give a 1 M concentration and subsequently incubated at room temperature for 24 hours, following an effective and cost-efficient microplastic extraction protocol outlined by Cole et al. (2014). Water samples were processed in a similar fashion, whereby the frozen contents of the aluminium containers were emptied into glass scintillation vials and organic materials digested, also using a 1 M solution of sodium hydroxide solution over 24 hours.

After incubation, vial contents were filtered through borosilicate filters (42 mm Ø, 0.7 μm mesh) using a vacuum pump and Büchner flask; filters were then rinsed with 200 ml of 0.2 μm filtered MilliQ™ water to rinse sodium hydroxide from the filter and retained particles. Filters were kept in borosilicate glass petri dishes, covered with a lid and examined for microplastics using an Olympus SZX16 stereo microscope (Olympus, SZX16) with a digital camera attached (Olympus, DP17). Once all potential microplastics were identified on the filter, the glass lid was removed and potential plastics were examined and manually manipulated to confirm polymer characteristics (brittleness, softness, transparency). Plastic particle colours and sizes were recorded (to the nearest μm) using CellSense Standard software package (Olympus, version 1.2). Two microplastic fibres were gold coated (Emitech K550, Quorum Technologies Ltd, West Sussex, United Kingdom) and subjected to scanning electron microscopy (SEM) in secondary electron mode using a Hitachi model S-4700 (Hitachinaka, Japan). The analyses were performed at an acceleration voltage of 20 kv, an emission current (Ic) of 10 μA and a working distance of 12 mm (Morrison et al. 2009).

Five individuals of each species were randomly selected and microplastics originating from their gut contents, as well as those originating from one randomly selected surface water sample, were further analysed for polymer identification using micro-Fourier-transform infrared spectroscopy (micro-FTIR). The absorbance for each polymer was obtained using a Perkin Elmer Spotlight 200i FT-IR

Microscopy System (Perkin Elmer, USA) at 100 $\mu m^{-1}$ resolution with spectra collected over the wavenumber range of 7800 - 600 $cm^{-1}$.

*Contamination Prevention*

The extraction of microplastics and subsequent examination of the filters was performed in compliance with the most recent findings in microplastic contamination prevention methodologies (Woodall et al., 2015, Wesch et al., 2017). All equipment used was pre-rinsed with 0.2 μm filtered MilliQ™ water and all clothing worn during laboratory work was of non-polymer nature. Furthermore, samples and filters were not at any time air-exposed and always kept under a clean air laminar flow hood (HEPA filter, class ISO5) or maintained within covered borosilicate Petri dishes. During dissections and filtrations on each day a wet filter (blank) was kept in a borosilicate petri dish inside the laminar flow hood for control purposes. After filtration of all samples on each day the filter was then also assembled within the Büchner flask and 200 ml of 0.2 filtered MilliQ™ water were filtered through it and the filter was later assessed for microplastics for quality assurance purposes.

*Data Analysis*

A stomach fullness index (FI) was calculated for each fish by dividing the weight of the gut contents by the weight of the fish.

To test whether stomach fullness had any effect on microplastics being present or not in the alimentary tract of the fish, a Mann-Whitney-U test (as the distribution of FI was non-parametric) was carried out using R (R Development Core Team 2007), comparing the median stomach fullness value for fish that had microplastics with those that did not.

As the microplastic count data were non-parametric, a Kruskal-Wallis test (using R) was used to test whether there was any difference in the abundance of microplastics between the seven different species. A Mann-Whitney-U test (using R), was used to test whether more microplastics were present in fish found in shallow compared with those found in deep waters.

## Results

A total of 280 fish were captured, of which 233 were examined for the presence of microplastics in their gut contents. The most common species amongst the subsampled fish were the spotted lantern fish *Myctophum punctatum* (with 86 individuals, or 37% of catch), the glacier lantern fish *Benthosema glaciale* (69 indiv., 29%) and the white-spotted lantern fish *Diaphus rafinesquii* (34 indiv., 15%). The remaining species were the Rakery beaconlamp *Lampanyctus macdonaldi* (16 indiv., 7%), the stout sawpalate *Serrivomer beanii* (14 indiv., 6%), the scaly dragonfish *Stomias boa* (9 indiv., 4%) and *Gonostoma denudatum* (5 indiv., 2%). Where information on sexual maturity size exists (*Myctophum punctatum, Benthosema glaciale, Diaphus rafinesquii, Gonostoma denudatum*), every sampled fish was assessed as being sexually mature.

Overall, 73% of fish contained plastics in their stomachs with *Gonostoma denudatum* having the highest frequency of occurrence (100%), followed by *Serrivomer beanii* (93%) and *Lampanyctus macdonaldi* (75%) (Table 1. 1). In total, 452 microplastic fragments were extracted from the fish gut contents, with an average of 1.8 microplastic fragments per fish. The highest average number of microplastics in the gut contents was recorded in *Serrivomer beanii* (2.36), followed by *Myctophum punctatum* (2.28) and *Gonostoma denudatum* (2.2) (Table 1. 1).

**Table 1. 1:** Fish species, numbers and length examined for microplastic ingestion and associated microplastic abundances in gut contents.

| Species | No. Fish Dissected | Average Length (mm) (± SD) | No. of Fish with MPs | % of Fish with MPs | Average MPs in Fish |
|---|---|---|---|---|---|
| *Myctophum punctatum* | 86 | 67.86 ± 7.49 | 64 | 74.42 | 2.28 |
| *Benthosema glaciale* | 69 | 57.93 ± 5.80 | 47 | 68.12 | 1.46 |
| *Diaphus rafinesquii* | 34 | 75.15 ± 8.25 | 24 | 70.59 | 1.15 |
| *Lampanyctus macdonaldi* | 16 | 243.34 ± 221.15 | 12 | 75.00 | 1.75 |
| *Serrivomer beanii* | 14 | 496.76 ± 258.95 | 13 | 92.86 | 2.36 |
| *Stomias boa* | 9 | 70.31 ± 58.99 | 6 | 66.67 | 1.33 |
| *Gonostoma denudatum* | 5 | 17.84 ± 4.00 | 5 | 100.00 | 2.20 |
| ***Total*** | **233** | **-** | **171** | **73.39** | **1.80** |

There was no significant difference between the median stomach fullness indices of fishes that had microplastics in their stomachs and those that did not (W = 5253, P = 0.976). Furthermore, there was no significant difference in median microplastic

counts among the seven different species (H = 10.904, d.f. = 6, P = 0.091), nor between fish caught in shallower and deeper waters (U = 5877, P = 0.389).

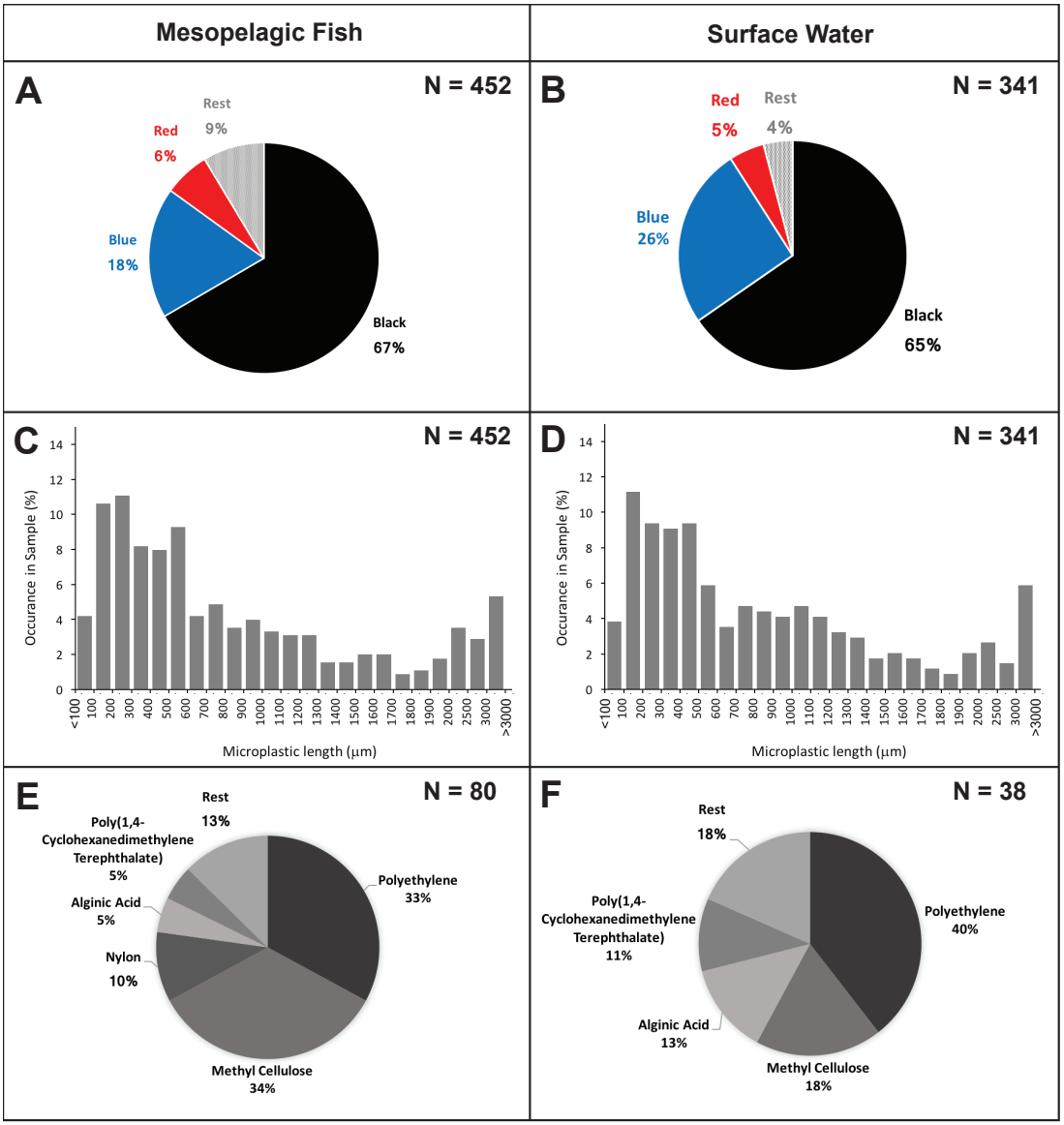


**Figure 1. 2:** Microplastic colours (A, B), length (C, D) and type (E, F) found in mesopelagic fish gut contents (left) and surface waters (right).

In total, 341 particles were found in the surface water samples (8 samples totalling 2,400 L of surface water) resulting in an estimated concentration of 14 microplastic fragments per 100 litres of water. Plastics identified from fish guts were very similar to those found in the surface waters (Figure 1. 2). 98% of microplastics identified from the fish and 99% of those identified from the water samples were classed as fibres, with the remainder being flattened fragments of plastics. Recorded microplastic colours included black, grey, blue, green, purple, red, yellow and white. Black and blue were by far the most common colours, followed by red, making up 67, 18 and 6% and 65, 26 and 5% of sampled plastics from fish guts and surface

waters, respectively. Polymers of other colours only made up a minor fraction of the particles in both cases (Figure 1. 2). Likewise, sizes of extracted microplastics were very similar between those found in fish (mean ± SD: 969 ± 1,048 μm) and in surface waters (mean ± SD: 985 ± 1,101 μm). The smallest recorded polymer fragment had a length of 42 μm and the largest a length of 8,150 μm.

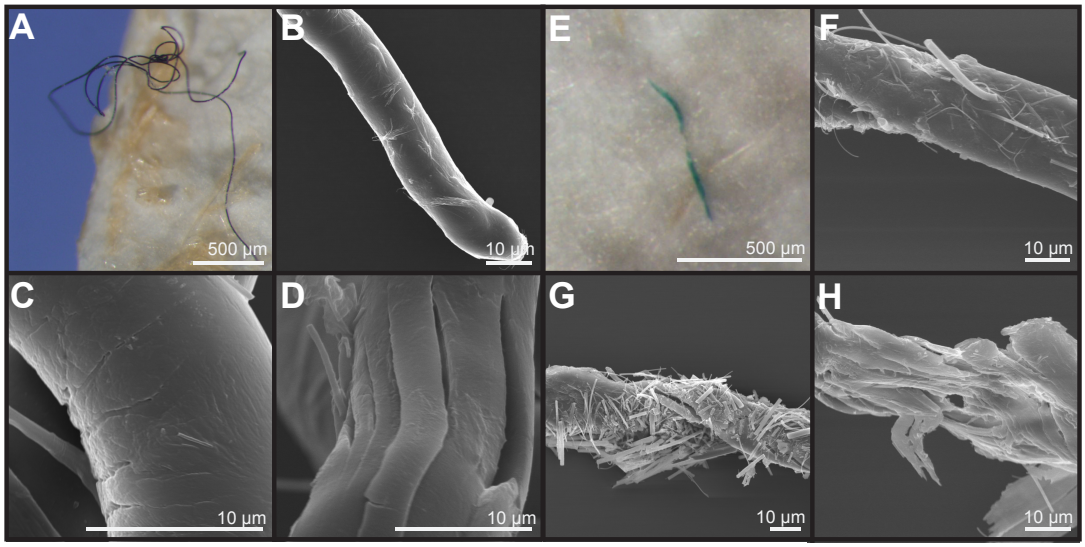


**Figure 1. 3:** Light microscopy and scanning electron microscopy images of a black (A-D) and a green (E-H) microplastic fibre recovered from gut contents of *Myctophum punctatum.*

Micro-FTIR analysis was successfully carried out for 118 of the 191 microplastic fragments originating from 35 fish and from one surface water sample. The 73 particles which could not be assessed for their polymer nature either fractured when pressure was applied by the diamond of the micro-FTIR machine or did not show a significant fit with any of the materials within the FTIR spectra library and thus were excluded from analysis. Polymers identified from fish and water samples were of similar polymer nature with the majority being polyethylene, followed by methyl cellulose and a relatively small proportion were identified as alginic acids. An exception was nylon, which comprised 10% of the particles found in the fish guts but was not identified amongst the particles extracted from the surface water (Figure 1. 2). Scanning electron microscopy images of two fibres extracted from fish gut contents had visible signs of polymer fracturing (Figure 1. 3).

No microplastics were found on the filters used as blanks to ensure no airborne contamination or any contamination from the filtration equipment and procedure.

## Discussion

Using forensic methods, this study assessed microplastic frequency of occurrence in mesopelagic fish gut contents from a warm-core eddy in the Northwest Atlantic. We detected a significantly higher occurrence rate of 73% in contrast to previous studies reporting occurrence rates of 11% in the North Atlantic and 9% and 35% in the North Pacific Gyre regions (Davison & Ash, 2011; Boerger et al., 2010). There are several reasons which may explain our much higher frequency of occurrence. Firstly, there are no standardised methods for the extraction of microplastics from gastrointestinal tracts of fish and so different research teams have used different protocols such as visual sorting of gut contents (Boerger et al., 2010), staining of organic materials (Davison & Ash, 2011) and extraction by the use of alkaline dissolution (Lusher et al., 2016). In the latter, the authors used a similar approach to this study but used a more concentrated caustic solution (1.8 M vs. 1 M) and incubated samples for a longer time period (14 days vs. 1 day). Cole et al. (2014) assessed many different extraction methodologies and found that the hydrolysis of organic compounds using a caustic solution was an efficient and viable method. However, they noted that extractions using higher concentrations and longer incubation times than recommended damaged and discoloured pH sensitive polymers such as nylon, uPVC and polyethylene and these may previously have been underestimated. Furthermore, we used fine-meshed borosilicate filters in contrast to Lusher et al. (2016) who used a 250 μm filter. As a result, our study detected much smaller microplastics (down to 42 μm) which made up 20% of all detected microplastics.

Another potential explanation for differences among reported microplastic occurrence rates is the abundances of microplastics found in the study areas. Samples for this study were collected around a warm core eddy ~1200 km off the Newfoundland coast which is known to be an area of enhanced biomass for all trophic levels (Dufois et al., 2016), including mesopelagic fish (Fennel & Rose, 2015; McKelvie, 1985), and may also potentially aggregate microplastics (Yu et al., 2018). Surface water samples collected within this study indeed showed ten times higher concentrations of microplastics than reported for other regions of the Atlantic (Lusher et al., 2014) where Lusher et al. (2016) collected their samples. The other two studies collected samples at the edge region of the North Pacific Gyre which, while potentially having slightly higher concentrations of plastics, were still not located close to the centre of the gyre which is known to be a hot spot for microplastics (Eriksen et al., 2014). At this point, it is also important to consider how

mesopelagic fish may be exposed to microplastics. All of the seven investigated species migrate to the surface at night to feed and therefore ingestion could happen either through the direct consumption of microplastics mistaken as prey items or through trophic transfer from their prey species. Indeed, the most common prey of mesopelagic fish are copepods, euphausiids, amphipods, larvae and decapods and all have been reported to ingest microplastics (Setälä et al., 2014; Desforges and Ross, 2015; Carpenter et al., 1972). Lusher et al. (2016) previously excluded trophic transfer as a likely route of exposure as the average size of microplastics they found in the gut contents was 1.9 mm. This was considered too large to be ingested by their prey species, but they also noted that their study only targeted plastic particles over 250 µm in size. In this study, the average size of identified microplastics from fish guts was 970 µm with 20% of all plastics being smaller than 250 µm and thus trophic transfer from planktivorous prey species may indeed be a likely route of exposure. In addition to size, it is also worth noting that the colour of any microplastics is unlikely to play an important role in the ingestion of microplastics by mesopelagic fish as the colours of the microplastics identified from the fish gut contents were similar to those identified from the surface waters.

Lastly, different microplastic abundances in the gut contents may be caused by some mesopelagic species being more selective or impacted than others. For example, it is well known that some bird species are more prone to microplastic and marine litter ingestion than others (e.g. petrels: Van Franeker & Bell, 1988). However, our study found no differences in microplastic occurrence rates between the seven mesopelagic fish species examined. Neither did depth seem to explain any variation in microplastic abundances amongst individuals caught at different depths. Therefore, we can conclude that the notably higher occurrence rates reported within this study are likely due to the differences in microplastic extraction methods as well as the fact that the present study was carried out in a hot spot for mesopelagic fish and microplastics alike. While this study reports one of the highest abundances of microplastics in the gut contents of fish, other studies have reported similar results for different species, particularly in polluted areas. For example, Tanaka and Takada (2016) reported a 77% encounter rate of microplastics in Japanese anchovies (*Engraulis japonicus*) sampled from Tokyo Bay, and Nadal et al. (2016) found microplastics to occur in 68% of seabream (*Boops boops*) sampled around the Balearic Islands. It is also noteworthy that while Lusher et al. (2016) and Davison & Ash (2011) reported a lower average microplastic count per individual

fish of 0.13 and 0.11 respectively, Boerger et al. (2010) found the average microplastic count per fish to be 2.1, higher than that observed by us (1.8).

Such high numbers of microplastics in the gut contents of mesopelagic fish is of great concern. Microplastics have previously been shown to adversely impact invertebrate species such as lugworms causing weight loss, reduced feeding activity and inflammation (Besseling et al., 2013; Wright et al., 2013). Detrimental effects on the intestinal functioning of seabass (*Dicentrarchus labrax*) have also been noticed (Pedá et al., 2016). Furthermore, there is growing concern about the effect of chemical pollutants sorbed to microplastics. For example, Mato et al. (2001) reported up to $10^6$ higher concentrations of PCBs on polypropylene pellets than in the surrounding sea water, and recently it has been shown that Japanese rice fish (*Oryzias latipes*) and rainbow fish (*Melanotaenia fluviatilis*) readily accumulate chemical pollutants from ingested microplastics (Rochman et al., 2013; Wardrop et al., 2016).

The ingestion of microplastics by mesopelagic fish may also have secondary implications for other species as well as the entire ecosystem. Mesopelagic fish are now known to make up a substantial biomass in the pelagic realm (Kaartvedt et al., 2012) and provide an important food source for many large predators such as dolphins, seals and tuna as well as sea birds (Spitz et al., 2010; Cherel et al., 2008; Varela et al., 2013; Danielsen et al., 2010). These taxa consume large amounts of mesopelagic fish and consequently ingest the microplastics within them. More importantly, due to trophic transfer, predators of mesopelagic fish may also bioaccumulate chemical pollutants absorbed from ingested microplastics. As some of the species preying on mesopelagic fish are commercially exploited fish the transfer of microplastics and bioaccumulated toxins in their tissues may also pose a threat to human health (Lusher et al., 2017).

Mesopelagic fish are also responsible for a significant amount of carbon and nutrient cycling (Radchenko et al., 2007). Organic material released as faeces or from dead and decaying organisms sink very slowly from the upper surface to the deep ocean. A large proportion of this organic material is recycled by other organisms and re-released before it can reach the ocean floor. Mesopelagic fish, however, undergo diurnal migrations, quickly travelling long distances from the epipelagic layer where they feed, to the deeper (below 300m) ocean where they deposit their faeces. Therefore, they play a key role in speeding up the downward flux of carbon and nutrients to greater depth and circumvent recycling by other

organisms (Irigoien et al., 2014). As discussed above, we are now aware that microplastic ingestion can have substantial effects on fish health and in particular digestive functions. Therefore, reported microplastic abundances in the fish gut contents may have implications for the cycling of carbon and nutrients by these species. Moreover, as suggested by Lusher et al. (2016), mesopelagic fish may aid in the downward transport of microplastics to the deep-sea benthos and cause potential harm to organisms in this habitat.

In terms of our methods, the applied protocols have resulted in the successful extraction of very small plastic particles. However, the micro-FTIR spectroscopy analysis identified a large proportion of the analysed microplastics as methyl cellulose and alginic acids. This, while seeming unusual at first, is very likely a consequence of insufficient cleaning of the microplastics after extraction. Methyl cellulose is produced synthetically by heating cellulose with a caustic solution. As we used a caustic solution to hydrolyse organic materials some of the sodium hydroxide may have remained on the plastic particles and potentially skewed the absorbance spectrum. Similarly, alginic acids are likely to be a reading of an outer biofilm coating of the microplastic particles which had not been removed during extraction. For future studies we strongly recommend a more thorough cleaning of plastic particles with filtered, ultrapure water. Furthermore, it is interesting to note that, despite taking a forensic approach during the extraction of microplastics (Wesch et al., 2017), we observed a large amount of fibres (98%) amongst the sampled microplastics. This is in agreement with other findings (Neves et al., 2015; Lusher et al., 2013; Lusher et al., 2016; Bellas et al., 2016; Rochman et al., 2015). However, fibres are often considered to be a contaminant of airborne nature and are sometimes excluded from analysis (Tanaka & Takada, 2016; Rummel et al., 2016; Foekema et al., 2013). As we did not observe any fibres on the filters used as blanks, we argue that fibres do indeed make up a large proportion of microplastics and are not of airborne nature. In support of this, Rochman et al. (2015) found high numbers of fibres in fish sampled from USA fish markets, but not in those sampled from Indonesian fish markets. The authors suggest that this is due to the large amount of wastewater effluents carrying synthetic fibres from washing machines as such machines are more common in developed areas. In fact, the microplastics we identified from the fish gut contents closely overlapped in colour, size, shape and type with those sampled from the surface water (Figure 1. 2) and we can thus assume that types of microplastics sampled from organisms are a reflection of those found in the environment they inhabit.

While large gyres have been a major focus of microplastic research, this study together with that of Yu et al. (2018) show that mesoscale features such as eddies may also be a hot spot for microplastics and should be further investigated. Furthermore, future studies quantifying microplastic ingestion by predatory fish species should also consider sampling their putative prey to investigate trophic transfer of microplastics.

In conclusion, this study reports the highest ingestion rates of microplastics in the gastrointestinal tracts of mesopelagic fish. This has important consequences for the health of pelagic ecosystems and biogeochemical cycling in general. Additionally, using forensic techniques, we provide more evidence that fibres are found throughout our oceans rather than being an artefact of airborne contamination.

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# Chapter 2

# Microplastic Ingestion by Gelatinous Zooplankton May Lower Efficiency of the Biological Pump

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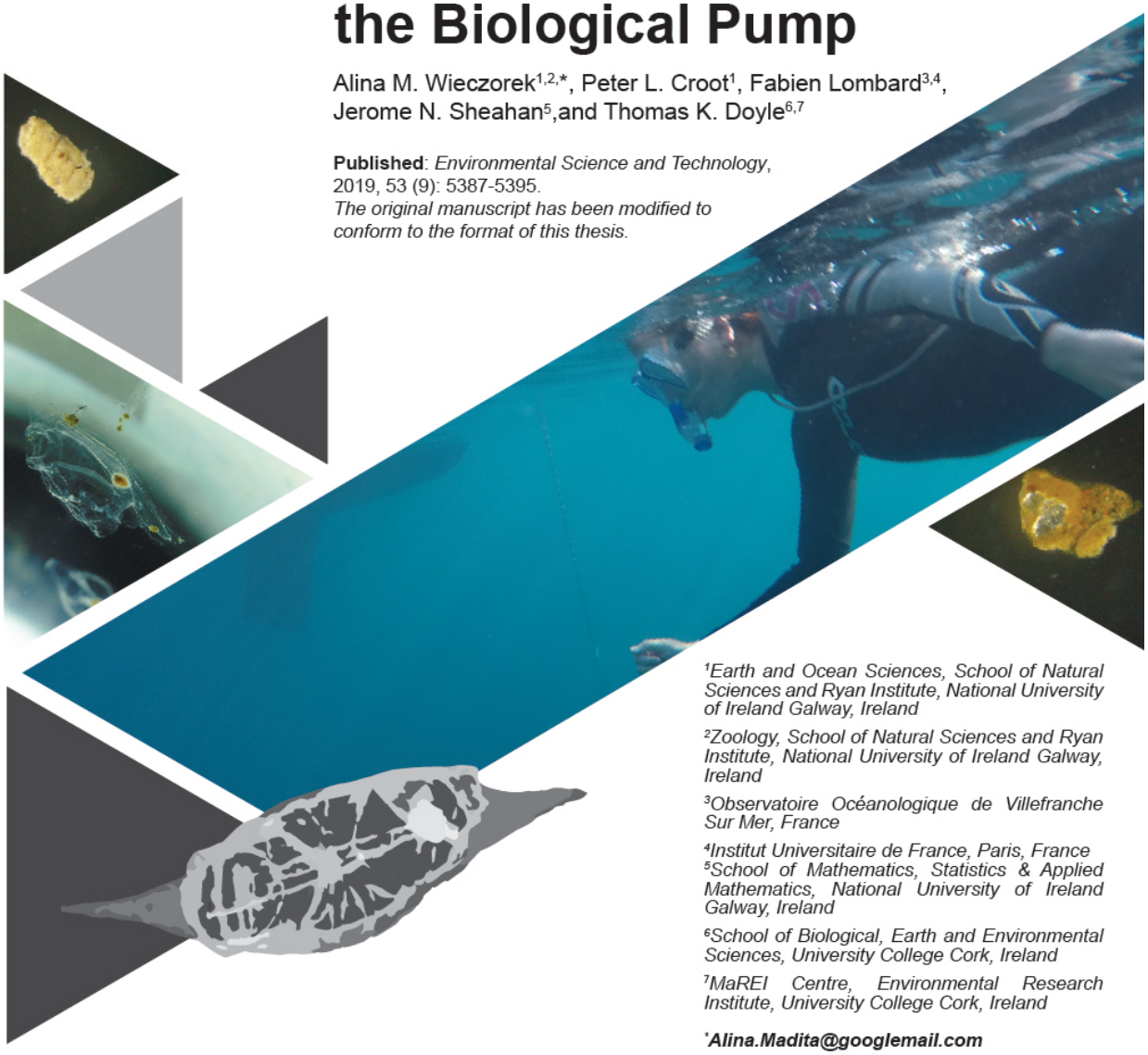
*The original manuscript has been modified to conform to the format of this thesis.*



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# Chapter 2 – Salps


## Abstract

The impacts of microplastics on some individual organisms have been well studied but it is less clear is what impacts microplastics may have on wider ecosystem processes. Using salps as model organisms, we studied the effect of microplastic ingestion on the downward flux of high-density particulate organic matter in the form of salp faecal pellets. While to date most microplastic studies used virgin microplastics at unrealistic environmental concentrations here we exposed *Salpa fusiformis* to fractured and UV exposed polyethylene and polystyrene microplastics possessing a biofilm. It was found that when exposed to environmentally relevant concentrations, reported for the Mediterranean and the South Pacific Gyre, only few faecal pellets had microplastics incorporated within them. Under potential future scenarios, however, up to 46% of faecal pellets contained microplastics. Incorporated microplastics significantly altered the size, density and sinking rates of salp faecal pellets (p-value < 0.05 in each instance). Sinking rates decreased by 1.35-fold (95% CI = 1.18, 1.56) for faecal pellets with polyethylene microplastics and 1.47-fold (95% CI = 1.34, 1.61) for polystyrene. These results suggest that microplastic ingestion by salps currently has minimal impact on the biological pump. However, under future microplastic concentrations (or in areas such as convergent zones) microplastics may have the potential to lower the efficiency of the biological pump.


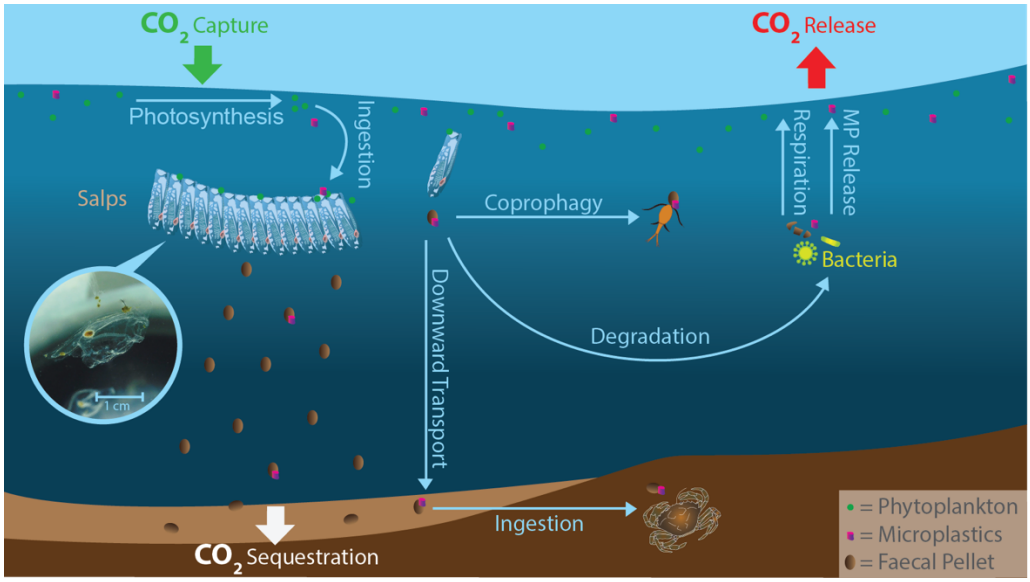


**Figure 2. 1:** Graphical abstract: potential pathways of microplastics incorporated into salp faecal pellets: Degradation by bacteria and re-release of microplastics and captured carbon in the form of $CO^2$; ingestion by coprophagous organisms; downward transport to seafloor where the faecal pellets may be ingested by the benthic community and microplastics transferred to these.

## Chapter 2 - Salps

### Introduction

It is well established that microplastics may pose a threat to the environment. They have been shown to be ingested by numerous marine organisms, causing physical harm and inflammation, and may act as a vector for hazardous chemicals (Watts et al., 2015; Pedà et al., 2016; Rochman et al., 2013). What is less clear, is what impact the ingestion of microplastics may have on populations and assemblages, or even on wider ecosystem processes (Sussarellu et al., 2016; Green, 2016; Galloway et al., 2017). This lack of evidence regarding the 'ecological harm' caused by microplastics is hampering efforts to develop effective legislation to reduce marine litter (Galloway and Lewis, 2016). A recent review identified three ecosystem processes which may be impacted by microplastics: (i) bioturbation, (ii) behavioural changes to predator–prey relationships and (iii) perturbations to carbon cycling (Galloway et al., 2017). For example, Wright et al. demonstrated that microplastics present in sediments decreased activity in the bioturbating marine worm *Arenicola marina,* and in a subsequent study it was found that this decrease in bioturbation had a secondary effect on the primary productivity of their habitats (Wright et al., 2013; Green et al., 2016).

In pelagic systems, some initial studies have investigated the potential impacts microplastic ingestion may have on the biological pump - the most important process driving sequestration of anthropogenic carbon in the ocean (Le Quéré et al., 2010; Passow and Carlson, 2012). It describes the uptake of atmospheric $CO_2$ by photosynthetic organisms in the euphotic zone and the sequestration of this carbon to the ocean floor through sinking particulate organic matter (POM) in the form of fish and zooplankton faecal pellets, aggregates, carcasses and phytodetritus. A potential effect of microplastics on the biological pump was demonstrated in copepods that were exposed to polystyrene microspheres (Cole et al., 2016). It was found that copepods readily incorporated polystyrene microspheres into their faecal pellets which decreased faecal pellet sinking rates by 2.25-fold. This reduction in sinking speed could lead to the degradation of faecal pellets in the upper ocean and cause captured carbon to be re-released to the atmosphere. Ultimately, this would reduce the amount of carbon, in the form of faecal pellets, that would be transported to the sea floor. The authors further demonstrated that faecal pellets containing microplastics are consumed by coprophagous copepods and thus microplastics could be transferred from the egests of one copepod to another. Similarly, polystyrene microspheres have been shown to be incorporated into aggregates of the diatom *Chaetoceros neogracile*

and decrease sinking rates by 2.87-fold.(Long et al., 2015) However, if incorporated into aggregates of the cryptophyte *Rhodomonas salina*, sinking speed increased by 1.64-fold, highlighting that sinking POM may also act as a downward vector for microplastics, as argued by Cózar et al. (2014; 2015) and Song et al. (2018). This is further supported by reported correlations between chlorophyll a content and microplastic numbers, indicating that aggregates may act as a transport mechanism for microplastics, as well as reports of microplastics being found in deep sea sediments (Bergmann et al., 2017; Van Cauwenberghe et al., 2013; Woodall et al., 2014).

While some studies indicate that microplastics may affect the efficiency of the biological pump, to date no study has investigated the impact of microplastics on salps which are one of the most significant contributors to the downward flux of carbon and other key elements (Turner, 2002). Salps are freely swimming pelagic tunicates occurring in long chains or in solitary form with a global distribution (Van Soest, 1998). They can transport a significant amount of carbon captured at the sea surface through phytoplankton photosynthesis, to depth via filter-feeding ingestion and defecation. The fine mesh of their mucous filters can efficiently capture particles ranging from 2 to 1000 µm in size (Madin, 1974). Daily filtration rates of some salp species can be as high as 458 L $day^{-1}$ $individual^{-1}$ (Sutherland and Madin, 2010) resulting in the transport of up to 576 mg C $m^{-2}$ $day^{-1}$.(Morris et al., 1994) Salps are so uniquely important in the cycling of carbon and key elements to the sea floor as their faecal pellets have unusually high sinking rates of up to 2700 m $day^{-1}$(Bruland and Silver, 1981). In contrast, copepod and larvacean sinking rates are 86 m $day^{-1}$ and 300 m $day^{-1}$, respectively (Cole et al., 2016; Katija et al., 2017). It has previously been argued that such sinking speeds are too low to significantly contribute to the downward flux of carbon to the seafloor. (Møller et al., 2010) A recent study conducted in oligotrophic waters found that POM reaching the deep sea, and thus contributing to the sequestration of carbon to the ocean floor, requires minimum sinking rates of 124 - 732 m $d^{-1}$(Agusti et al., 2015). These sinking rates are only met by a few zooplankton species, salps being one of them (Turner, 2015). The incorporation of low-density microplastics into high density salp faecal pellets may lead to a decrease in this sinking speed and cause it to be below the critical threshold required to reach the sea floor.

The potential consequences of microplastic ingestion by salps on the net flux of carbon and other key elements becomes evident as studies from the North Pacific have reported up to 90% of collected sinking matter to be composed of salp faecal

pellets (Dunbar and Berger, 1981; Coale and Bruland, 1985). Furthermore, it has been hypothesised that some salp species may experience favourable conditions under predicted climate change and thus could flourish in future years (Atkinson et al., 2004; Li et al., 2016). As with many other planktonic filter feeding species, filtration rates of salps have previously been determined by exposing them to small plastic beads (Kremer and Madin, 1992) which shows that they are indeed able to ingest microplastics. Similarly, in a more recent study ingestion of larger polystyrene beads (90-106 µm) has been demonstrated by some salp species in the Pacific (Chan and Witting, 2013).

To address the question of how microplastic ingestion may impact the biological pump, we exposed *Salpa fusiformis* to two abundant microplastic types – polystyrene and polyethylene of two size categories (150 – 250 µm and 250 – 500 µm), which underwent pre-treatment of ultraviolet (UV) exposure and biofilm formation. Microplastic concentrations for experiments were chosen to resemble those observed in the Mediterranean (MED) and the South Pacific Gyre (SPG), and two higher concentrations representing potential future, and yet unmeasured, concentrations of e.g. convergent zones (Clark et al., 2016). It was investigated whether salps do ingest microplastics at varying concentrations and what effect microplastics incorporated into faecal pellets had on their size, density and sinking speed. By including two different types of microplastics we further looked at how microplastic type may affect any of the above. This is the first study examining impacts of microplastics on the salp-driven downward flux of carbon and key elements and allows for coherent conclusions on potential consequences for the efficiency of the biological pump and element cycling to the deep-sea benthos.

## Materials and Methods

*Microplastic Preparation and Concentration*

Two plastic types – low density polyethylene (PTX131=LD/LLD polyethylene; density = 0.955 g $cm^{-3}$), from here on referred to as polyethylene, and general purpose polystyrene (PTX 300=GP polystyrene; density = 1.05 g $cm^{-3}$), from here on referred to as polystyrene, were purchased in two size categories (150 – 250 µm and 250 – 500 µm) from Carat GmbH. Carat GmbH produce microplastics from virgin pellets by grinding them with a cryogenic mill to prevent thermodegradation of the plastics, before they are size fractioned using a sieve shaker. Purchased microplastics were then exposed to UV radiation, equalling 30-day UV sea surface

exposure in the Mediterranean (Appendix 1.1). After being counted out in doses listed in Figure 2. 2: A, microplastics were transferred into glass scintillation vials which were then filled with 25 µm filtered seawater and left for 96 hours in a constant temperature room (17°C) with a natural light regime, to allow for the formation of a biofilm. This timeframe has been chosen based upon a study by Lee et al., who report formation of a biofilm over a 36-hour period (Lee et al., 2008).

Microplastic concentrations were chosen to resemble those of the Mediterranean (MED), the South Pacific Gyre (SPG), 10-fold that of the South Pacific Gyre (10 x SPG) and 100-fold that of the South Pacific Gyre (100 x SPG) (Appendix 1.2). Encounter rates were chosen, rather than concentrations of microplastics per litre of seawater, under consideration of the high filtration rates of salps and the duration of the experiments (see Appendix 1.2). The limitation of this is that salps may be more likely to come across microplastics as they are kept in a more confined space over this time period. The 10 x SPG and 100 x SPG concentrations were chosen as they are each one order of magnitude higher than those reported for the South Pacific Gyre, in reference to Jambeck et al. (2015) who predict the input of plastics into the oceans to increase by an order of magnitude by 2025. Thus, these concentrations represent potential future microplastic concentrations.

*Salp Collection*

*Salpa fusiformis* collection took place between the 8$^{th}$ and the 16$^{th}$ of March 2017 in the centre of Villefranche Bay in the Northwestern Mediterranean. Salps were collected in ziplock® bags from a kayak or via snorkelling, and immediately transferred into buckets filled with seawater using a ladle with rounded edges. Exposure experiments commenced within 3 hours of collection. The condition of salps was visually assessed and only active salps were selected for use in exposure experiments, with normal activity being assumed if salps were part of an aggregate chain or if actively pumping water. After completion of exposure experiments a subsample of 40 salps each day were measured using callipers. Measurements were taken from the anterior to the posterior process, to the nearest 0.1 mm.

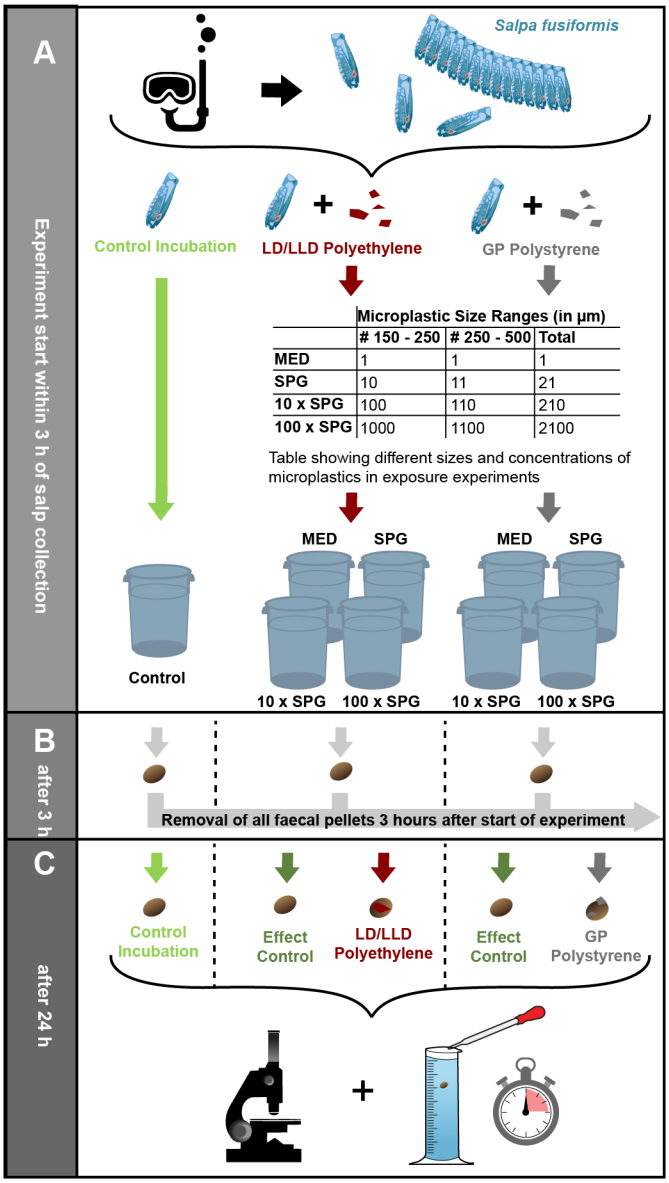


**Figure 2. 2:** Infographic of experimental procedures: (A) experiments commenced within 3 hours of salp collection; salps and microplastics in concentrations resembling those of the Mediterranean (MED), the South Pacific Gyre (SPG), 10 x that of the South Pacific Gyre (10 x SPG) and 100 x that of the South Pacific Gyre (100 x SPG) were added to the nine containers filled with 6 L of 25 µm filtered seawater. Each exposure experiment was repeated on five consecutive days (n=5). (B) 3 hours after start of exposure experiment all faecal pellets were cleared from the nine containers as these may still have contained microplastics ingested from the environment. (C) 24 hours after start of exposure experiment all faecal pellets from each of the nine containers were collected, microscopically inspected and a subsample of each group (control incubation, effect control, polyethylene and polystyrene) photographed, measured and their sinking speed timed.

*Exposure Experiment*

Nine containers were filled with 6 L of 25 µm filtered seawater (Crystal Filter®, SW-25-978-PP). Of the nine containers, four were spiked with polyethylene microplastics (from here on referred to as “polyethylene” group) and four were spiked with polystyrene microplastics (from here on referred to as “polystyrene” group). Two size ranges and four different concentrations of microplastics were used (listed in Figure 2. 2: A). The final container was used as a control (from here on referred to as “control

incubation") to which no microplastics were added. 180 salps were freshly collected each day and 20 randomly selected salps were added to each container. The containers were placed in a constant temperature room (17°C) with a natural light regime. To avoid airborne microplastic contamination each container was covered with a 25 µm nylon mesh, which was meticulously rinsed with filtered seawater beforehand to avoid shedding of microplastic filaments from the mesh itself. Each exposure experiment was carried out five times.

*Faecal Pellet Collection*

As outlined by Phillips et al. (2009), salps defecate approximately every 3 hours. Thus, faecal pellets were removed from the containers 3 hours after commencing the exposure experiment to ensure that sinking speed measurements were not influenced by microplastics potentially ingested in the field before being captured (Figure 2. 2: B). These faecal pellets were then microscopically inspected (under Zeiss STEMI SV 11 stereomicroscope equipped with an Olympus DP21 camera) for the presence of polyethylene and polystyrene microplastics. 24 hours after commencing the experiments, all (intact) faecal pellets were carefully collected from each of the nine containers using a glass pipette (outer ∅: 6.5 mm) (Figure 2. 2: C). Faecal pellets were immediately transferred into glass petri dishes and microscopically examined. All faecal pellets containing microplastics, as well as a subsample of faecal pellets from the control incubation and a subsample of microplastic free faecal pellets from the eight microplastic incubations (from here on referred to as "effect control" group), were photographed, and length and width measured (to the nearest 1 µm). Thickness of the faecal pellets was not obtained as faecal pellets started to fracture when manipulated. Therefore, to obtain the volume it was assumed that the faecal pellets have an ellipsoid shape with the thickness of the faecal pellets being roughly equal to the width: $V=4/3\times\pi\times a\times b^2$. The equivalent spherical diameter was then calculated as: $ESD=2\times(3V/4\pi)1/3$.

*Sinking Speed*

All photographed and measured faecal pellets were then assessed for sinking speed by allowing faecal pellets to settle out of a glass pipette into the centre of a 2 L graduated glass cylinder (520 mm height, 83 mm outer ∅) filled with 25 µm filtered seawater. Settling speed of a 300 mm distance was measured to the nearest 0.1 s. The top and bottom 100 mm were purposely excluded as at the top insertion of the glass pipette into the cylinder may cause small turbulences and at the bottom, as the convex shape at the bottom of the cylinder may hinder accurate

assessment of the time of settlement. Given the very low sphere to tube diameter ratio (mean ± SD = 0.013 ± 0.005), wall effects were assumed to be negligible with reference to Uhlherr & Chhabra (1995). For each faecal pellet, sinking speed was measured three times and an average of the three measurements was used in calculating the sinking speed in m $day^{-1}$. All experiments were carried out in a constant temperature room (17°C). The sinking speed was also used to calculate the density of each assessed faecal pellet after Lombard & Kiørboe (Appendix 1.3) (Lombard and Kiørboe, 2010).

*Data Analysis*

Following initial analysis, diagnostics were performed on the residuals to see if there was evidence of violation of assumptions needed to justify the analyses conducted, and transformations performed where appropriate.

It was tested whether mean ESD of the faecal pellets originating from the four different groups (“control incubation”, “effect control”, “polyethylene” and “polystyrene”) differed significantly from one another, using an ANOVA followed by post-hoc multiple comparison test (Tukey). These tests were run within the statistical software package R (R Team, 2017).

To assess differences in sinking speed for the four groups (control incubation, effect control, polyethylene and polystyrene), statistical analyses applying general linear models (GLMs) were carried out in Minitab (Minitab 17 Statistical Software, 2010). Firstly, evidence for a difference in sinking speed between the control groups was tested by running a GLM with sinking speed as the response variable and with input variables being Type (factor at two levels: “control incubation” and “effect control”) and ESD as a covariate, including a possible interaction term between Type and ESD. The reason for inclusion of the interaction of ESD with Type, along with the possible main effects of one or both of ESD and Type, is not only to test if the interaction is present but if it is, to be careful not to misinterpret any main effects that appear to exist. To compare the effect of microplastics incorporated in the faecal pellets on sinking speed, similarly, a GLM was performed with sinking speed as the response variable and input variables being Type (factor at three levels: “control”, “polyethylene” and “polystyrene”) and ESD as a covariate.

While initially ESD, Type and their interaction were included in the model it was decided to drop the interaction because it was not significant ($F_{1,126} = 0.65$, p-value = 0.423), and it was decided to measure the effect of each of ESD and Type

without the (non-significant) contribution of the interaction. A post-hoc Tukey pairwise comparison test was performed to see which pairs among the three levels differed significantly. For each pair, the difference between means in that pair was used as an estimate of the mean difference in sinking speed among the three groups.

To obtain faecal pellet volume it was assumed that faecal pellets are ellipsoidal in shape. In an attempt to compensate for this assumption non-parametric tests were applied when comparing faecal pellet densities, as these rely on the ranking of the derived faecal pellet density and not the density values themselves. Again, the control faecal pellets from the separate incubations ("control incubation") were first compared to the control group from the microplastic incubations ("effect control") using a Mann-Whitney-U test. To investigate the effect of the two different incorporated microplastic types on the density of the faecal pellets, the control group ("control" = "control incubation" + "effect control") was compared to the faecal pellets which had polyethylene ("polyethylene") fragments incorporated within them and those which had incorporated GP polystyrene ("polystyrene") fragments by applying a Kruskal-Wallis test. Post-hoc Mann-Whitney-U tests were then run to compare each pair of faecal pellet group with one another. These tests were run within the statistical software package R (R Team, 2017).

## Results

On five separate days 180 salps were captured successfully. Salps ranged in size from 16.7 to 58.3 mm (mean ± SD: 34.8 ± 7.4 mm). Out of the 900 salps used for the experiments, all but five individuals were assessed to be alive by the end of the experiment (i.e. actively pumping water or being part of an aggregate).

*Microplastics Abundances in Faecal Pellets*

No polyethylene or polystyrene microplastics were detected in faecal pellets retrieved within 3 hours of commencing the experiments. Also, all faecal pellets collected from the "control incubation" were found to be free of microplastics after microscopic inspection. From the incubations containing microplastics in concentrations equivalent to those of the Mediterranean Sea, only one faecal pellet had microplastics incorporated within it. Similarly, for faecal pellets removed from incubations containing microplastic concentrations equivalent to those of the South Pacific Gyre (SPG), only one faecal pellet contained microplastics (Table 2. 1). From the incubations mimicking 10 x SPG between 3 – 15 (2 – 10%) of the faecal

pellets contained microplastics and from those mimicking 100 x SPG between 20 – 82 (16 – 46%) of faecal pellets contained microplastics (Table 2. 1). Overall, salps were more susceptible to ingestion of polystyrene compared to polyethylene microplastics. The percentage of faecal pellets that contained polystyrene was over 3 times higher than those which contained polyethylene (Table 2. 1).

**Table 2. 1:** Count of faecal pellets which had no microplastics within them (# without MPs) and those which did (# with MPs) for the eight different exposure experiments and the percentage of faecal pellets containing microplastics for each of those incubations.

| | Polyethylene | | | Polystyrene | | |
|---|---|---|---|---|---|---|
| | # without MPs | # with MPs | % with MPs | # without MPs | # with MPs | % with MPs |
| **MED** | 125 | 1 | 0.8 | 94 | 0 | 0.0 |
| **SPG** | 141 | 0 | 0.0 | 125 | 1 | 0.8 |
| **10 x SPG** | 128 | 3 | 2.3 | 133 | 15 | 10.1 |
| **100 x SPG** | 107 | 20 | 15.8 | 95 | 82 | 46.3 |

*Microplastic Effect on Faecal Pellet Equivalent Spherical Diameter (ESD)*

A comparison of ESDs using an ANOVA indicated that the mean ESDs of faecal pellets were not all equal across the four different groups, being “control incubation”, “effect control”, “polyethylene” and “polystyrene” ($F = 22.31$, d.f. = 3 and 247, p-value < 0.05). The mean ESDs (± SD) of each group is listed in Table 2. 2. No significant difference of mean ESD was noted between the control group and the polyethylene group (Tukey’s test, p-value = 0.932) or between the polyethylene and polystyrene group (Tukey’s test, p-value = 0.069). All other groups showed a significant difference in mean ESD to each other (Tukey’s test, p-value < 0.05 in all four cases).

**Table 2. 2:** ESD, sinking speed and faecal pellet density measured for control incubation and effect control group and control (control incubation and effect control combined), polyethylene and polystyrene group.

| Faecal pellet group | n | ESD (mean ± SD in μm) | Sinking speed (mean ± SD in m $d^{-1}$) | Density (median ± IQR in g $cm^{-3}$) |
|---|---|---|---|---|
| **Control incubation** | 66 | 1141 ± 411 | 1116 ± 844 | 1.063 ± 0.051 |
| **Effect control** | 64 | 1384 ± 517 | 1142 ± 554 | 1.059 ± 0.029 |
| **Control** | 130 | 1261 ± 480 | 1128 ± 714 | 1.060 ± 0.037 |
| **Polyethylene** | 24 | 1093 ± 362 | 509 ± 278 | 1.041 ± 0.020 |
| **Polystyrene** | 97 | 909 ± 237 | 380 ± 275 | 1.040 ± 0.014 |

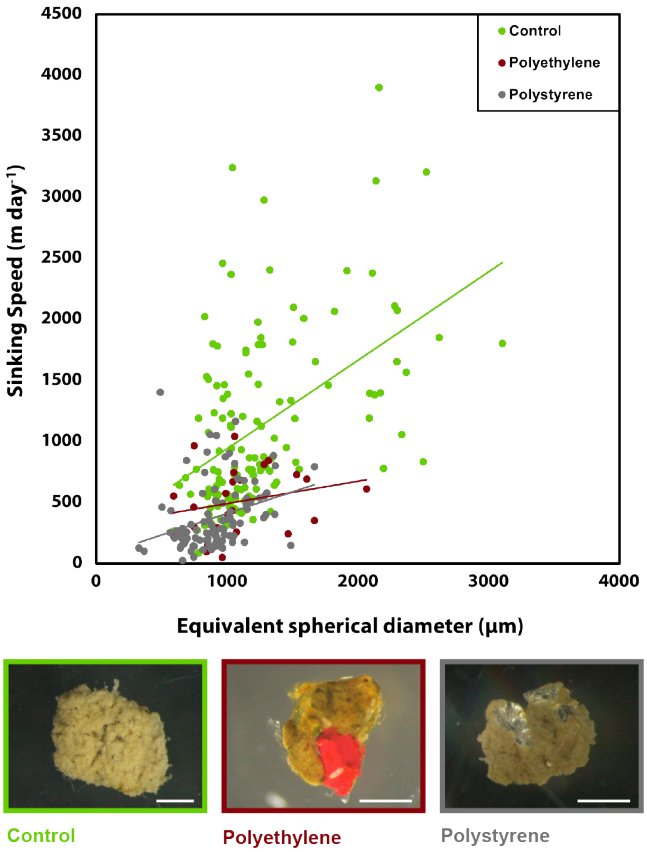


**Figure 2. 3:** Sinking speed (m day$^{-1}$) as a function of equivalent spherical diameter (µm) for the faecal pellets from control, polyethylene and polystyrene incubations, together with sample images of microplastic free faecal pellets (control) and faecal pellets containing polyethylene and polystyrene; scalebar = 500 µm.

*Microplastic Effect on Sinking Velocity*

Faecal pellets originating from the two control groups had an average sinking speed of 1128 m day$^{-1}$ (SD ± 714 m day$^{-1}$), while faecal pellets containing polyethylene and polystyrene had lower average sinking rates of 509 m day$^{-1}$ (SD ± 278 m day$^{-1}$) and 380 m day$^{-1}$ (SD ± 275 m day$^{-1}$), respectively (Table 2. 2, Figure 2. 3). The results from the general linear model (GLM) with Sinking Speed as the response variable and input variables of ESD and Type (with Type at two levels: "control incubation" and "effect control") indicated that the covariate ESD had a significant effect on Sinking Speed (F1,126 = 38.9, p-value < 0.05), but that there was no evidence that the type of control had any effect (F1,126 = 0.52, p-value = 0.472). Incidentally, there was no evidence for an interaction between ESD and Type either ($F_{1,126}$ = 0.65, p-value = 0.423). As a result, the two types of controls were amalgamated (and are referred to as "control") for the purposes of comparison with the "polyethylene" and "polystyrene" groups. A GLM test for an effect of ingested microplastics on sinking speed, incorporating ESD as a covariate, showed that there was statistical evidence that both ESD and Type had an effect on the

response ($F_{1,247}$ = 51.85, p-value < 0.05 and $F_{2,247}$ = 52.22, p-value < 0.05 respectively). The quality of the model fit for the two GLMs is given in Appendix 1.4. The post-hoc Tukey pairwise comparison test showed that the sinking speed for polyethylene and polystyrene containing faecal pellets differed significantly to the control (p-values < 0.05) and that there was no significant difference between the sinking speed of pellets which contained either of the two different plastic types (p-value = 0.351). By taking the inverse log of the difference between the means of each pair it was estimated that, on average, the sinking speed of the control faecal pellets was 1.35 (95% CI = 1.18, 1.56) times faster than faecal pellets containing polyethylene and 1.47 (95% CI = 1.34, 1.61) times faster compared to faecal pellets containing polystyrene.

*Microplastic Effect on Density*

There was no significant difference (U = 3344, p-value > 0.05) between the sample median density of faecal pellets from the “incubation control” (median ± IQR: 1.063 ± 0.051 g $cm^{-3}$) and the “effect control” (1.059 ± 0.029 g $cm^{-3}$) and both groups were amalgamated into “control”. When comparing the sample median density of the faecal pellets from the “control” group and the “polyethylene” and “polystyrene” there was significant evidence that not all three groups were equal in terms of sample median densities (H = 85.18, df = 2, p-value < 0.05). Faecal pellets from the “control” group were more dense (median ± IQR: 1.060 ± 0.037 g $cm^{-3}$) than those containing polyethylene (1.041 ± 0.020 g $cm^{-3}$) or polystyrene (1.040 ± 0.014 g $cm^{-3}$) (Table 2. 2, Figure 2. 4).

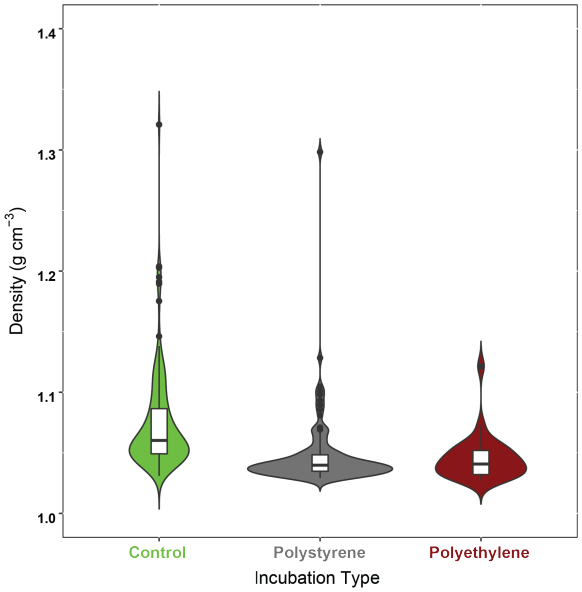


**Figure 2. 4:** Violin plot showing spread, interquartile range and median of faecal pellet densities of microplastic free faecal pellets (control) and those containing polyethylene or polystyrene.

Here we provide evidence of a potential ecosystem impact from the ingestion and subsequent egestion of microplastics by salps. Using environmentally relevant microplastics (size range, UV treated, possessing biofilm) we demonstrate that salps readily ingest microplastics, which significantly decreased their faecal pellet sinking speed by a factor of 1.35 in the case of polyethylene and 1.47 in the case of polystyrene. These results have implications for the efficiency of the biological pump and the cycling of key elements to the deep-sea benthos. The efficiency of the biological pump is largely determined by the rate of transport of POM to the ocean floor, which is in turn governed by POM sinking rates, degradation and grazing activities in the upper ocean (Turner, 2015). If sinking rates of POM are sufficiently lowered, POM may be degraded and broken down in the upper ocean by bacterial activity and the carbon captured within the POM will be re-released in form of $CO_2$ instead of being sequestered to the seafloor. Initial studies investigating effects of microplastic incorporation into POM on the biological pump have focussed on species which produce relatively slow sinking POM (Cole et al., 2016; Long et al., 2015). While this POM may somewhat contribute to the downward flux of carbon, recent findings underline that POM reaching the ocean floor would require higher sinking rates (Agusti et al., 2015). As salp faecal pellet sinking rates surpass sinking rates of any other POM, salps are known to significantly contribute to the flux of POM to the benthos (Turner, 2002; Madin et al., 2006; Henschke et al., 2016). Indeed, Coale & Bruland (1985) found that over 90% of sinking matter at a site within the California Current was made up by salp faecal pellets. Where salps and

microplastics overlap in high concentrations, the downward flux of faecal pellets is likely to be decreased due to longer residence times in surface waters where they are available for ingestion and bacterial breakdown. It is likely that faecal pellets produced by smaller salp species will be more affected by this as their faecal pellets are estimated to remineralise at lower depths (Henschke et al., 2016). Faecal pellets produced by larger salps may possess sinking rates sufficiently high to reach the seafloor despite containing microplastics. Nevertheless, we demonstrate an important concept here which not only holds true for small to medium sized salps, but also for other mucus-web filter feeders (Conley et al., 2018), especially those possessing faecal pellet sinking rates close to the critical threshold necessary to reach the sea floor.

Changes in remineralisation depths of salp faecal pellets may have overarching consequences not only for the cycling of carbon, but also that of other key elements. It has previously been highlighted that elemental transport through salp faecal pellets could, in places, control the distribution of elements within the water column (Krishnaswami et al., 1985). Transport of iron, strontium, thorium, calcium and manganese in particular seem to be facilitated by salp faecal pellets (Krishnaswami et al., 1985; Cabanes et al., 2017). Consequences of salp faecal pellet sinking speed alterations due to microplastic incorporation may thus also affect the cycling of other elements and could deprive the deep-sea benthos not only of carbon but also of other key elements.

Microplastics-enriched faecal pellets which reside at the surface for a prolonged period are also more readily available to coprophagous organisms resulting in microplastic transfer, as previously demonstrated by Cole et al. (2016). Furthermore, the presence of microplastics in deep sea sediments (Van Cauwenberghe et al., 2013; Woodall et al., 2014; Bergmann et al., 2017) and benthic deep sea organisms (Courtene-Jones et al., 2017; Taylor et al., 2016) indicate that, despite a potential impact on faecal pellet sinking speeds, an unknown proportion of microplastics are likely being transported to the seabed where they become available to benthos communities. This may be particularly true for smaller microplastics of high density and/or microplastics which are fibrous in shape. Fibers have a lower overall volume and as a consequence are likely to impact less on the density of sinking matter. Recent findings of fibers in deep sea waters and sediments, as well as deep sea organisms, further support this view (Courtene-Jones et al., 2017; Wieczorek et al., 2018; Peng et al., 2018).

Previous research on the impact of microplastics on the sinking speed of POM in the form of copepod faecal pellets and *Chaetoceros neogracile* aggregates showed similar results in terms of sinking speed reduction. However, an increase in sinking speed has been reported for *Rhodomonas salina* aggregates and no effect (based on observations) has been noted in a study investigating the uptake of microplastics by the larvacean *Bathochordaeus stygius* through *in situ* exposure to varying sizes of polyethylene (Long et al., 2015; Katija et al., 2017). One of the reasons which might explain these disparate results, is that each study used different microplastic types with different specific densities. For example, Cole et al. (2016) and Long et al. (2015) used polystyrene microspheres which have densities of 1.050 g $cm^{-3}$ (denser than sea water), and Katija et al. (2017) used polyethylene microspheres which have densities similar to that of seawater (about 1.027 g $cm^{-3}$). This study used two microplastic types; polyethylene having a density of 0.955 g $cm^{-3}$ and the higher density polystyrene (1.05 g $cm^{-3}$). One would expect to see a greater effect on sinking speed when lower density microplastics were used but the opposite is the case. Indeed, comparing the two microplastic types used within this study a similar trend can be observed with the higher density polystyrene microplastics having a greater effect on density and sinking speed of the faecal pellets than the polyethylene microplastics. This might be explained by three possible, but not necessarily mutually exclusive reasons. Firstly, one type of microplastic may be more readily incorporated within sinking particles than another, which would result in a higher ratio of low-density microplastics to high-density organic matter. Our findings support this idea as salps were more susceptible to ingestion of polystyrene microplastics compared to polyethylene microplastics (Table 1). One reason for this could be the surface properties of the plastic particles which may affect their retention in the mucous strand. Another reason could be the location of microplastics within the water column; polyethylene has a low density and is likely only available to the salps at the very surface, whereas polystyrene, having a higher density, would be more homogenously mixed within the seawater. This would be particularly significant for vertically migrating species such as *Salpa fusiformis.* At this point, it is however, also important to mention that UV and biofilm pre-treatment of microplastics within this study may have caused some changes to the overall density of the microplastics to which the salps were exposed to as previously argued by Kowalski et al. (2016) and Cozar et al. (2014). Lastly, we need to consider that Cole et al. (2016) found that faecal pellet sizes decreased when microplastics were incorporated into faecal pellets. An effect on size was also noted within this study as faecal pellets containing polystyrene were found to have a

significantly smaller ESD than microplastic free faecal pellets and those containing polyethylene. This suggests that certain types of microplastics (in these cases polystyrene) seem to not only decrease faecal pellet density, but also affect their structure and size. Smaller faecal pellets are known to sink and remineralise at a slower pace (Turner, 2002, Henschke et al., 2016) and thus the effect on size has further implications for the efficiency of the biological pump.

As is the case for many microplastic exposure experiments, studies investigating microplastic impacts on sinking particles have mostly used very high concentrations of small-sized virgin microplastics, to date unobserved in any marine environment. Lenz et al. (2016) rightfully point out that much of the microplastic exposure research conducted thus far has used concentrations two to seven orders of magnitude higher than those observed in the environment. In the microcosm experiments carried out within this study, microplastics concentrations were chosen to reflect those observed in the environment, in addition to two higher concentrations, to represent areas of exceptionally high microplastic abundance or future scenarios. Furthermore, microplastics were pre-treated through UV exposure and seawater incubations to allow for biofilm formation, whereas other studies commonly use small-sized virgin microplastics or microspheres (Paul-Pont et al., 2018; Botterell et al., 2018). Importantly, our study shows that salps only occasionally incorporate microplastics into their faecal pellets when exposed to environmentally relevant concentrations. Thus, despite microplastics being so abundant in our oceans (Cozar et al., 2014; Eriksen et al., 2014), under realistic microplastic concentrations very few salps are likely to ingest enough microplastics to significantly impact on the downward flux of carbon or other nutrients. However, when exposed to higher concentrations (100 x SPG), 16% (polyethylene) and 46% (polystyrene) of salp faecal pellets contained microplastics. Such impacts may not be too far into the future, as one of the higher concentrations chosen for this study (10 x SPG = 5,000,000 MPs $km^2$) is close to the current reported maximum abundance of microplastics (3,500,000 MPs $km^2$) (Yamashita and Tanimura, 2007). Additionally, negative interactions may already occur where surface abundances of salps co-occur with microplastics in areas where they are both advected by currents (Graham et al., 2001). Further, Jambeck et al. (2015) predict that even with severe reductions in our every-day use of plastics their abundance in the marine environment is likely to increase.

We conclude that where salps and microplastics overlap in high concentrations, microplastics are likely to be incorporated into faecal pellets and decrease the downward flux of these to the sea floor. This effect will increase with increasing inputs of marine litter into our oceans. We selected salps as a model organism because of their great importance in biogeochemical cycling and carbon sequestration (Caron et al., 1989; Henschke et al., 2016), but other mucus-web filter feeders such as pteropods, dolliolids and appendicularians may be similarly affected (Conley et al., 2018). Future studies on the impact of microplastic ingestion by key ecological players within the biological pump may be able to quantify the disruptive effect of microplastics on the biological pump by applying our microplastics-altered sinking rates to biogeochemical models such as PISCES-v2 (Aumont et al., 2015). Furthermore, it will be of great interest to investigate abundances of microplastics within faecal pellets collected from various environments across the globe as this will give a true indication of the prevalence of microplastic ingestion by salps.

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# Chapter 3

# Nanoplastic Interactions with Microplankton Communities in the Subtropical South Pacific

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# Chapter 3 – Microzooplankton


## Abstract

Nanoplastics are the under-studied size fraction of plastic pollution in our oceans. Their size resembles that of bacteria who build the basis of food webs in oligotrophic waters such as the subtropical South Pacific. Here, heterotrophic nanoflagellates (HNFs) and mixotrophs act as the pivotal link between the microbial loop at the basis of these food webs to higher trophic organisms. Previous applications of nanoplastics in grazing rate experiments demonstrate that there is a potential for HNFs and mixotrophs to ingest nanoplastics. Here we exposed microplankton communities, sampled from the subtropical South Pacific to nanoplastics in the form of fluorescently labelled beads (FLBds). Exposure experiments were performed within a dilution series set-up, run over 48 hours. Microplankton community responses and potential ingestion by HNFs and/or mixotrophs were monitored every 24 hours via flow cytometry. It was found that no noticeable amount of either HNFs or mixotrophs ingested FLBds. However, FLBds seemed to be incorporated into heteroaggregates, likely made up of FLBds and colloidal matter. These findings are supported when put into the context of mathematical encounter rate models. Aggregation processes, rather than ingestion by microzooplankton may therefore, be the dominant pathway distributing nanoplastics within oceanic, oligotrophic environments.


## Introduction

Over the past decade, marine science journals have been inundated with articles reporting on the abundance and detrimental impacts of microplastics in all seas and environments (Burns and Boxall, 2018). Despite this rapid increase in studies, there is still concern about how hazardous microplastics really are to the biosphere (Burns and Boxall, 2018; SAPEA, 2019). Of particular concern is the lack of information on the fate, effect and risks posed by microplastics smaller than 1 μm in size (SAPEA, 2019). These plastics, commonly referred to as “nanoplastics”, have been defined by Gigault et al. (2018) as a “plastic particle within a size ranging from 1 to 1000 nm resulting from the degradation of industrial plastic objects and that can exhibit a colloidal behaviour”.

Thus far, to our knowledge, only one study from the North Atlantic Subtropical Gyre reports on the abundance of this size fraction of microplastics in the marine environment (Ter Halle et al., 2017). Despite the lack of knowledge of their abundance in the marine environment, nanoplastics and microplastics of the smallest sizes, in the form of fluorescently labelled beads (FLBds), have been used in numerous microplastic exposure studies (e.g. Bhattacharya et al., 2010; Cole and Galloway, 2015; Paul-Pont et al., 2016). Furthermore, in the past, nano-sized FLBds have often been used to trace ingestion rates of marine filter-feeders such as appendicularians, choanoflagellates and rotifers (Bedo et al., 1993; Fernández et al., 2004; Marchant and Scott, 1993; Ronneberger, 1998).

Other organisms often studied in this way are heterotrophic nanoflagellates (HNFs) and mixotrophic organisms (e.g. Safi and Hall, 1999). HNFs are unicellular, eukaryotic organisms that possess at least one flagellum which aids in drawing their prey towards them to make them available for ingestion via phagocytosis (Fenchel, 1988). Mixotrophs on the other hand, are protists who are able to obtain energy from photosynthesis as well as through phagocytosis of prey cells (Stoecker, 1998). Both are known to heavily prey on bacteria to the degree that they can control their abundances (Fenchel, 1988; Sherr and Sherr, 1994). In oligotrophic waters, such as experienced in the subtropical South Pacific, bacterial communities form the basis of a long-chained, stable, low energy food web which is heavily reliant on the principle of nutrient recycling (Sarmiento and Gruber, 2006). Here HNFs and mixotrophs play a key role as the intermediate of the bacterial loop to higher trophic levels (Fenchel, 1988; Stoecker, 1998).

The subtropical oligotrophic region of the South Pacific has also been highlighted as a microplastic hotspot (Eriksen et al., 2013; Martinez et al., 2009; Van Sebille et al., 2015). Additionally, the meteorological conditions of high ultraviolet (UV) radiation coupled with current action may enhance the breakdown of larger plastics into microplastics and of microplastics into nanoplastics in this region. Resembling the size of bacteria, nanoplastics may be confused as prey and engulfed by HNFs and mixotrophs, which would provide a pathway for nanoplastics into the oligotrophic food web.

As mentioned above, a number of studies have previously looked at nanoplastic engulfment by HNFs and mixotrophs. While they do seem to ingest FLBds, the majority of studies report lower FLBd ingestion rates compared to fluorescently stained bacteria with HNFs, in particular, discriminating against FLBds (Gonzalez

and Suttle, 1993; Jürgens and DeMott, 1995; Nygaard et al., 1988; Safi and Hall, 1999; Vaque et al., 1994). Selection against FLBds was usually observed to be stronger under eutrophic conditions when there was a saturation of natural prey (Jürgens and DeMott, 1995; Nygaard et al., 1988). In general, ingestion rates of potential prey particles seem to be higher with higher temperatures (up to 30$^{o}$C), higher particle surface charge and higher prey swimming speed (Matz et al., 2002; Vaque et al., 1994). Furthermore, grazing rates appeared to be higher in culture-based experiments compared to grazing rates observed in the field (Vaque et al., 1994). Ingestion may, however, not be the only way HNFs interact with nanoplastics. Fukuda & Koike (2000) describe that HNFs associated to aggregates only rarely ingested FLBds but rather, by beating their flagella, draw in sub-micron particles, such as FLBds, towards the aggregate and caused their attachment to it.

With this study we aimed to gain insights into how microplankton communities of the subtropical South Pacific respond to nanoplastic exposure and in particular, investigate ingestion by HNFs and mixotrophs. For this, we sampled microplankton communities at three stations during the SO245 “UltraPack” cruise crossing the subtropical South Pacific. At each station, we carried out incubation experiments, during which microplankton communities were exposed to nanoplastics within a dilution series setup (Landry and Hassett, 1982), and monitored their responses via flow cytometry. These responses were then put into the context of theoretical encounter rates and revealed a potential pathway of nanoplastics in oceanic, oligotrophic environments.

Importantly, in this study, the responses of organisms were observed within their natural community rather than investigating cultures in a lab setting. Further, concentrations of FLBds used, while still much higher than those estimated to be found in the environment, were several orders of magnitudes lower than those commonly used in the context of grazing rate experiments (Bratvold et al., 2000; Fukuda and Koike, 2000; Gonzalez and Suttle, 1993) and some microplastic exposure experiments (e.g. Bhattacharya et al., 2010; Lee et al., 2013).

## Material and Methods

*Sampling Area and Sample collection*

Samples were obtained during the SO245 “UltraPac” cruise aboard the RV *Sonne* which departed from Antofagasta, Chile on the 17.12.2015 and arrived in Wellington, New Zealand on the 28.01.2016. This expedition was focused on the oligotrophic South Pacific Gyre, with a total of 15 stations sampled for biogeochemical and bio-optical properties along the transect. In the Southwest subtropical Pacific, at station 12, 14 and 15, depth profiles of chlorophyll *a* and nanoplanktonic communities were obtained from discrete depths samples and water samples for dilution-series incubations with nanoplastics were taken as indicated in Table 3. 1 and Figure 3. 1. Seawater for incubations was sampled from 60 m depth, just below the first chlorophyll *a* peak, at station 12 and 14 and from 10 m depth at station 15 using a Seabird sbe911+ CTD (Seabird Scientific, WA, USA), attached to a SBE32 water rosette sampler containing 24x12-litre Niskin bottles (Figure 3. 1, Table 3. 1, sample dates and times are listed in Appendix 2.1). To obtain depth profiles at each station seawater was sampled by filling it from Niskin bottles, fired at various depths, into triple-rinsed brown Nalgene® bottles. For the incubations, 5 l of seawater were filled from the relevant Niskin bottles into triple-rinsed carboy containers (Figure 3. 2: A).

**Table 3. 1:** Location of stations, sampling depth and associated temperature and salinity obtained from and available on Pangaea (www.pangaea.de; Zielinski et al., 2018).

| | Latitude (Degrees Minutes) | Longitude (Degrees Minutes) | Depth (m) | Temperature (°C) | Salinity (PSU) |
|---|---|---|---|---|---|
| **Station 12** | 39°18.613’S | 139°48.621’W | 60 | 14.36 | 34.46 |
| **Station 14** | 39°00.014’S | 160°00.050’W | 60 | 12.83 | 34.79 |
| **Station 15** | 39°00.005’S | 170°00.019’W | 10 | 17.66 | 35.06 |
| **Average** | **n/a** | **n/a** | **n/a** | **14.95** | **34.77** |

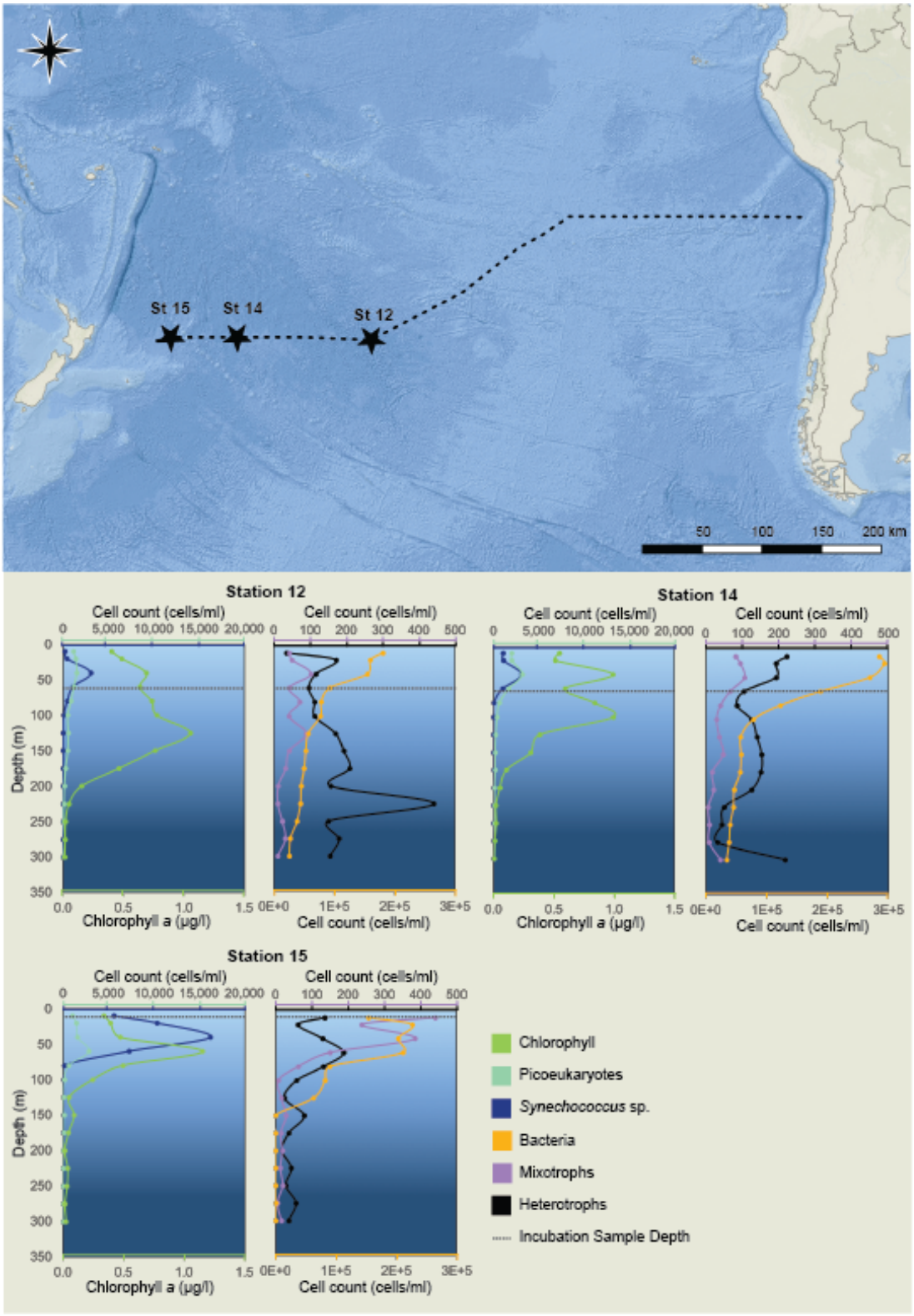


**Figure 3. 1:** SO245 cruise track with locations of station 12,14 and 15 together with depth profiles for each station of chlorophyll a content (µg/l) and counts (cells/ml) of picoeukaryotes, *Synechococcus*, bacteria, mixo- and heterotrophs; incubation sample depth is marked on each depth profile as black dashed line. ©Alina M. Wieczorek

*Flow Cytometry*

For identification of picoeukaryotes, *Synechococcus* and *Prochlorococcus,* the untreated seawater was immediately run on a BD Accuri C6 Flow Cytometer for 5 minutes with a fast flow rate (66 µl/min) and detector settings of 800-FSC and 800-FL3. After Rose et al. (2004), heterotrophic nanoflagellates' food vacuoles were stained by the use of LysoTracker Green® which also proved to be effective for phagocytosing mixotrophs, as described by Carvalho and Granéli (2006). For this, LysoTracker Green® (Molecular Probes®, S-7526, 1 mM in DMSO) was diluted in 5.5 ml MilliQ™ water and added to 2 ml of sample to give a final concentration of 75 nM LysoTracker Green®. The samples were then transferred to the refrigerator and left there for 20-30 minutes before being processed on the flow cytometer which was set to run for 5 minutes with a fast flow rate (66 µl/min) and detector settings of 800-FSC and 8,000-FL1. For the identification of bacteria, 20 µl of glutaraldehyde (25%) were immediately added to 2 ml of the water sample which was then left in the refrigerator for at least 20 minutes (Marie et al., 1997). Afterwards, SybrGreen® (Molecular Probes®, S-7585, 10000 concentrate in DMSO) was added to the samples to give a final concentration of 1:10000. The samples were again transferred to the refrigerator and left there for a minimum of 20 minutes. Samples were then run on the flow cytometer for 5 minutes with a fast flow rate (66 µl/min) and detector settings of 800-FL1 and 800-FSC (Figure 3. 2: D and E).

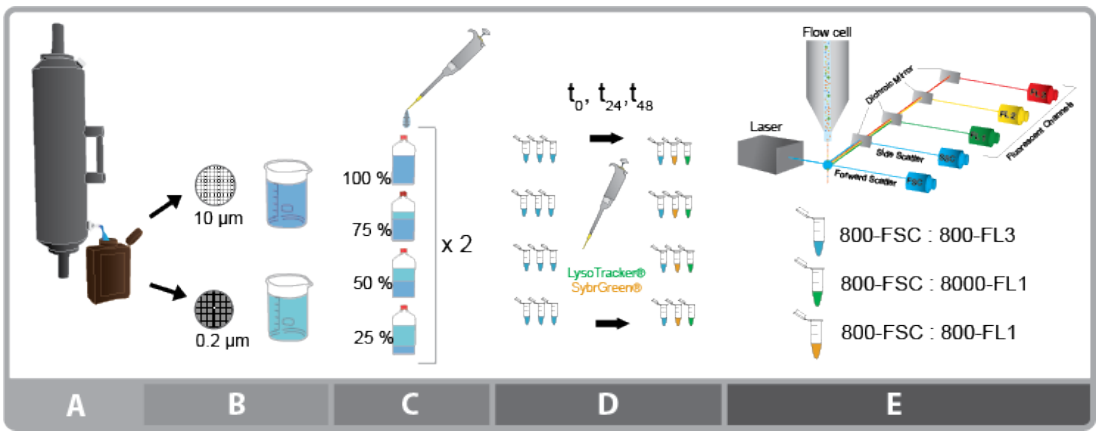


**Figure 3. 2:** (A) 5 litre of the surface seawater were sampled from Niskin bottle; (B) one half was filtered through a 10 µm filter the other through 0.2 µm filter; (C) eight 200 ml incubation flaks were made up with two each containing 100%, 75%, 50% and 25% of 10 µm filtered water and the remainder 0.2 µm filtered water, to each of the flask 40 µl FLBds were added; (D) at $t_0$, $t_{24}$ and $t_{48}$ of the incubation 3 x 2ml Eppendorf were filled from each incubation bottle and either left untreated (blue) or treated with Lysotracker Green® (green) or with glutaraldehyde and SybrGreen® (orange); (E) treated samples were analysed on BD Accuri C6 Flow Cytometer. ©Alina M. Wieczorek

*Chlorophyll a content*

Seawater (1 L) was collected from discrete depths by the CTD sampling rosette and filtered onto 25 mm GF/F filters which were stored in 10 ml polyethylene tubes at -20 $^{o}$C until they were analysed. Analysis for chlorophyll *a* content was carried out according to Arar and Collins (1997) using a Tuner fluorometer (Trilogy Laboratory Fluorometer). The fluorometer was calibrated with chlorophyll *a* standard and the fluorescent readings of the sample were obtained from the regression line of the calibration curve.

*Incubation/ - Dilution Series*

Half of the incubation sample water was filtered through a 10 µm PTFE filter (Omnipore™) and the other half through a 0.2 µm GF/F filter (Whatman®) (Figure 3. 2: B). A total of eight 40 ml Cellstar® culture flasks with Advanced TC™ surface were then filled with a 40 ml seawater mix with two bottles each containing 100%, 75%, 50% and 25% of the 10 µm filtered seawater with the remaining volume being comprised of the 0.2 µm filtered seawater. Then 40 µl of yellow-green fluorescent beads (Bangs Laboratories, Inc.: 488 nm excitation, green emission) were added to each incubation bottle (Figure 3. 2: C). At station 12 and 14, fluorescent beads of mean diameter 0.76 µm were added and at station 15, fluorescent beads of mean diameter 0.5 µm were added. In regard to the FLBds, it is important to note that these are commonly provided in a 2 mM sodium azide solution. Sodium azide has previously been used as a motility inhibitor for microscopic, planktonic organisms but has been clearly demonstrated not to have an efficient effect at concentrations below 50 mM (Cabrol et al., 2017; Winter et al., 2012; Sretenovic et al., 2017). The final concentration of sodium azide experienced within the present incubations was 0.002 mM and thus the potential effect of sodium azide was deemed negligible. Once the FLBds were added, the flasks were inverted several times and transferred into a Heratherm IMC™ incubator set at 23$^{o}$C which was equipped with 12V LED lights (5 W 400 LM) connected to a timer set to natural light regimes. At $t_0$, $t_{24}$ and $t_{48}$ three 2 ml samples were pipetted from each of the incubation flasks into 2 ml Eppendorfs and analysed via flow cytometry as described above (Figure 3. 2: D and E).

Nutrients (phosphate, nitrite and nitrate) and regenerated nutrients (dissolved organic nitrogen (DON)) were measured from seawater sampled at the same depth as the seawater used in the incubations. This allowed for the assessment of incubations to determine whether they became nutrient-limited over time. Phosphate ($PO_4^{3-}$), nitrite ($NO_3^-$) and nitrate ($NO_2^-$) concentrations were determined

on board, in accordance with Strickland and Parsons (Strickland and Parsons, 1969), using a QuAAtro autoanalyzer (Seal Analytical Ltd, England). Data for dissolved organic nitrogen were obtained from Osterholz et al. (*unpublished data, in preparation for publication*). For each station, it was then calculated how many bacterial and *Synechococcus* cells could theoretically be produced by the nutrients available within each incubation. Elemental cell compositions were assumed to be as described by White et al. (2019) for bacterial cells (SAR11: average of HTCC1062 P replete and P limited) and Heldal et al. (2003) for *Synechococcus* (average of WH8103 and WH7803 strain grown on artificial seawater).

*Gating*

Flow cytometry data were further analysed using FlowJo software (Treestar, Inc., San Carlos, CA). Picoeukaryotes and *Synechococcus* were identified by plotting red fluorescence (FL3) versus orange fluorescence (FL2). *Prochlorococcus* were identified in some samples but were hard to resolve from bacteria and thus excluded from further analysis. SybrGreen® stained bacteria were gated by plotting green fluorescence (FL1) versus red fluorescence (FL3). Identification of HNFs was done after Rose et al. (2004) and mixotrophs were distinguished from heterotrophs by the amount of red fluorescence (FL3) displayed by the two populations. Lastly, FLBds were identified by plotting forward scatter (FSC) against green fluorescence (FL1) and green fluorescence (Fl1) against orange fluorescence (FL2). This was done for the untreated samples but also for the Lysotracker treated samples as thresholds for green florescence were set higher when the latter were run. This gating was also used when investigating for any hetero- or mixotrophs that may have ingested beads. As FLBds have the tendency to stick together and form aggregates of multiple beads this was corrected for by adding up the cumulative green fluorescence (FL1) of all beads and dividing that by the mean fluorescence of the singlet peak. This corrected count is referred to as “FLBds derived” from here on.

*Growth and Grazing Rates*

Using the counts of bacteria, *Synechococcus*, picoeukaryotes, hetero- and mixotrophs of the different dilutions at timepoints $t_0$ ($P_0$), $t_{24}$ and $t_{48}$ ($P_t$) one can estimate growth and grazing rates for each (Landry and Hassett, 1982). After Landry et al. (1995), firstly, the net rate of change ($k_n$ $d^{-1}$) was calculated as

$$k_n = \frac{1}{t} ln \frac{P_t}{P_0}$$

Where *t* is the duration of the experiment - in this case one day as growth and grazing was calculated separately for $t_0$-$t_{24}$ and $t_{24}$-$t_{48}$. Secondly, the net rate of change was plotted against the dilution factor. The regression equation for each group was then used to get proxies for growth (y-intercept) and grazing (regression slope) rates for the $t_0$–$t_{24}$ and $t_{24}$–$t_{48}$ timeframe. It is important to note that these estimates underly the assumption that there is a constant proportion of microzooplankton (HNFs and mixotrophs) and prey (bacteria, picoeukaryotes) and a constant rate of prey growth during the experiment (Calbet and Saiz, 2018).

*Calculation Nanoplastic Abundances*

To obtain an estimate of how many nanoplastics one may encounter in the South Pacific it was calculated how many nanoplastics would result from the breakdown of all of the smallest microplastics (0.355-0.499 mm) estimated to be present in this region (Eriksen et al., 2013). Current abundance estimates are commonly given in the two-dimension unit g $km^{-2}$. As it was desirable to acquire an abundance estimate for 1 ml of seawater it was assumed that all microplastics are found in the top 1 cm of water. Nanoplastic abundance *NP* (count $ml^{-1}$) can then be calculated as:

$$NP = \frac{MP_{SP}\ g\ km^{-2}\ /\ \frac{4}{3}\ \pi\ {r_{NP}}^3\ d_{pol}}{1\ x\ 10^{10}\ ml}\ x\ 1\ ml$$

Where $MP_{SP}$ is equal to 0.2 g $km^{-2}$, the abundance estimates for the smallest microplastics in the South Pacific as given by Eriksen et al. (2013); $r_{NP}$ is equal to the radius of nanoplastics. Here the radius was chosen to be 0.38 µm-and 0.25 µm in accordance with nanoplastics used within the incubation experiment. Plastics possess different densities, given as $d_{pol}$, and to account for this, calculations were carried out for low (LD/LLD polyethylene = 0.955 g $cm^{-3}$) and high density (GP polystyrene = 1.05 g $cm^{-3}$) polymers.

*Encounter Rate Calculation*

Encounter rates were calculated for HNFs and mixotrophs with living cells (throughout referring to: bacteria, *Synechococcus* and picoeukaryotes) and FLBds under incubation conditions (lower turbulent shear) for each station. Encounter rates of FLBds with living cells and other FLBds were also calculated under these conditions. To get insight into encounter rates in the environment it was also looked at how many living cells and how many nanoplastics HNFs and mixotrophs would encounter under environmental conditions (higher turbulent shear). Lastly,

encounter rates for nanoplastics with living cells and other nanoplastic were also calculated under environmental conditions.

Abundances of living cells were estimated from the counts of the undiluted incubation samples for each station. For HNF and mixotroph abundances the counts were taken from the depth profiles as the similar fluorescence of the LysoTracker® Green and the FLBds may have caused inaccuracies in the counts of the incubation samples. FLBds counts used were the derived FLBd counts as previously stated. Encounter rates were determined according to the coagulation theory (Baird and Emsley, 1999):

$$Encounter\ rate = \phi_{j,k} P_j$$

Where $\phi_{j,k}$ is the encounter rate coefficient ($m^3\ s^{-1}$) of prey *j* and predator *k* and $P_j$ the concentration of prey. The encounter rate coefficient is given as:

$$\phi_{j,k} = \phi_{j,k,diffusion} + \phi_{j,k,relative\ velocity} + \phi_{j,k,turbulent\ shear}$$

For the purpose of this study, a curvilinear encounter rate coefficient was calculated which is commonly applied to simple heterotrophic organisms and assumes that smaller organisms follow streamlines around larger organisms (Baird and Emsley, 1999). For a curvilinear encounter rate coefficient diffusion, relative velocity and turbulent shear are given as:

$$\phi_{j,k,diffusion} = \frac{2K_B T}{3\eta}\left(\frac{1}{r_1} + \frac{1}{r_2}\right)(r_1 + r_2)$$

$$\phi_{j,k,relative\ velocity} = 0.5\pi r_1^2 U_{1,2}$$

$$\phi_{j,k,turbulent\ shear} = 9.8\frac{p^2}{(1+2p)^2}\left(\frac{\in}{v}\right)^{0.5}(r_1 + r_2)^3$$

Where *T* is the temperature (K), $K_B$ the Boltzmann constant (1.38066 x $10^{-23}$ $m^2$ $kg/(s^2 \cdot K)$), $r_1$ the radius of the prey and $r_2$ the radius of the predator (in m) and *p* the smaller divided by the larger radius. $\in$ refers to the mean rate of dissipation of turbulent kinetic energy which was set to log(-8) $m^2\ s^{-2}$ after Fernández-Castro et al. (2014) for environmental encounters in South Pacific and to log(-10) $m^2\ s^{-2}$ for incubation conditions, as here one would expect low turbulent kinetic energy in a

rectangular bottle placed in an incubator. Further, in the above equations, $\eta$ and $v$ represent the dynamic and kinematic viscosity (for average properties of stations as listed in Table. 1) calculated to be 1.213 x $10^{-3}$ kg $m^{-1}$ $s^{-1}$ and 1.183 x $10^{-6}$ m $s^{-1}$, respectively and $U$ is the relative velocity of the predator and prey species. To obtain $U$ one first needs to acquire estimates of the swimming motilities and sinking velocities of the predator and prey species.

Swimming motilities of bacteria and heterotrophic nanoflagellates were taken from the literature (Appendix 2. 2), and swimming speed was assumed to be 0 µm $s^{-1}$ for the FLBds. Sinking velocities ($V$) were calculated according to Kiørboe (1993) citing Jackson (1989) who accounts for the decline in cell density with cell size due to their empirical carbon content:

$$V = 2.48r^{1.17}$$

To obtain sinking velocities for the beads Stokes law was applied:

$$V = 0.222g\eta^{-1}r^2(p_p - p_f)$$

Where $g$ is the gravitational acceleration (9.82 m $s^{-2}$), $r$ is the radius of the sinking beads (0.38 µm and 0.25 µm), $p_p$ is the density of the beads (1050 kg $m^{-3}$) and $p_f$ the density of seawater (1025 kg $m^{-3}$) with properties as listed in Table 1.

Assuming that the swimming direction of prey and predator is random, the relative velocity can be calculated as:

$$U_{swim,j,k} = \frac{U_{slow}^2 + 3U_{fast}^2}{3U_{fast}}$$

Where $U_{slow} = min[U_{swim,j}, U_{swim,k}]$ and $U_{fast} = max[U_{swim,j}, U_{swim,k}]$.

## Results

*Microplankton and Nanoplastic Abundances*

Depth profiles at station 12 and 14 resembled those expected for oligotrophic waters with deep chlorophyll *a* peaks, relatively high bacterial abundances and lower abundances of picoeukaryotes and *Synechococcus* (Figure 3. 1). At station 15, which was located closest to the shelf edge, the depth profile showed one distinct chlorophyll *a* peak at around 60 m as well as lower bacteria and higher picoeukaryote and *Synechococcus* abundances. Here, phagocytosing mixotrophs also appeared to be more abundant than at the other stations (Figure 3. 1).

Samples taken for the incubations run at $t_0$ with no dilution (but with added FLBds) generally showed counts coherent with those taken from the same depth to construct the depth profiles (Table 3. 2). There were two exceptions to this, one being the bacterial counts at station 15 which, in the sample taken for incubations, appeared to be higher than in the depth profiles. The other exception to this was that mixotrophs seemed to be more abundant in the 10 m sample taken to construct the depth profiles for station 15 than that taken for the incubation experiments (Table 3. 2). Lastly, it is important to mention that the use of LysoTracker® Green in combination with yellow-green FLBds made it difficult to distinguish aggregates of multiple beads from potential HNFs. Thus, HNF abundances given for incubation experiments throughout are associated with uncertainties.

In regard to nanoplastic abundances, it is noteworthy that the amount of FLBds which should theoretically be in each of the incubation bottles is on average considerably higher than those which have been measured in each of the incubation bottles (11 and 4 times for simple and derived FLBds counts respectively; Table 3. 2). Further, the amount of nanoplastics which would result from the breakdown of all of the smallest microplastics estimated to be present in the South Pacific would result in much fewer nanoplastics than the amounts to which the organisms have been exposed to within the incubation experiments (on average 148 times less compared to the derived FLBd counts; Table 3. 2).

**Table 3. 2:** Abundances (in # $ml^{-1}$) of: **Environment**: amount of nanoplastics if all of the smallest microplastics from South Pacific were to break down into nanoplastics; **FLBds Theoretical**: FLBDs that should theoretically be in each incubation bottle; **FLBds Count**: FLBD count without correcting for multiple bead aggregates; **FLBds Derived**: FLBD count corrected for multiple bead aggregates; and abundances measured for bacteria, *Synechococcus*, picoeukaryotes, mixo-and heterotrophs for each station.

| | | Station 12 | | Station 14 | | Station 15 | |
|---|---|---|---|---|---|---|---|
| | | Inc. 1 | Inc. 2 | Inc. 1 | Inc. 2 | Inc. 1 | Inc. 2 |
| **Nanoplastics** | Environment | 88 | | 88 | | 306 | |
| | FBLd Theoretical | 100,000 | | 100,000 | | 100,000 | |
| | FLBd Count | 5,218 | 5,352 | 6,470 | 8,661 | 12,730 | 14,476 |
| | FLBd Derived | 12,503 | 12,376 | 15,058 | 19,997 | 41,076 | 41,542 |
| **Bacteria** | | 482,715 | 470,606 | 587,761 | 574,173 | 797,015 | 760,712 |
| ***Synechococcus*** | | 4,188 | 4,224 | 2,815 | 2,697 | 21,248 | 15,697 |
| **Picoeukaryotes** | | 2,576 | 2,621 | 5,567 | 5,318 | 5,294 | 3,848 |
| **Mixotrophs** | | 148 | 203 | 221 | 270 | 300 | 15 |
| **Heterotrophs** | | 288 | 315 | 339 | 330 | 282 | 70 |

*Growth and Grazing Rates*

At $t_0$, bacteria, picoeukaryotes, *Synechococcus* and mixotrophs showed counts expected according to the relevant dilution factor (Appendix 2. 3). This was not the case for heterotrophs, which indicates that the similarly fluorescing FLBds prevented accurate estimates of HNFs, and they were consequently excluded from the growth and grazing analysis. At station 12 at $t_{48}$, there was a problem with the bacteria counts which also were excluded from the analysis.

Within the incubations, neither the investigated nutrients nor regenerated nutrients were likely to be a limiting factor for cell growth (Appendix 2. 4, Table 3. 2 and 3. 3, Figure 3. 3). Under these conditions, bacteria displayed fast growth rates during the first day, but their growth appeared substantially reduced on the second day. Overall, there seemed to be very little grazing on bacteria during both days at all stations (Figure 3. 3; Table 3. 3). Similarly, *Synechococcus* did not seemed to be grazed upon over the incubation timeframe at any of the stations. Growth patterns of *Synechococcus* showed no growth during the first day for all stations and no growth during the second day at station 15 but appeared to have grown during the second day at station 12 and 14 (Figure 3. 3; Table 3. 3). In the same manner, picoeukaryotes were also not grazed upon but showed some growth during the second day at station 12 and 14 but not during any other timeframe at any other station (Figure 3. 3; Table 3. 3). Mixotrophs generally did not follow as clear of a trend as any of the other groups with generally much lower $R^2$ values (Table 3. 3).

Overall, mixotrophs did not appear to be grazed upon during any timeframe at any of the stations but did grow during the first day at stations 12 and 14 and during the second day at all stations (Figure 3. 3; Table 3. 3).

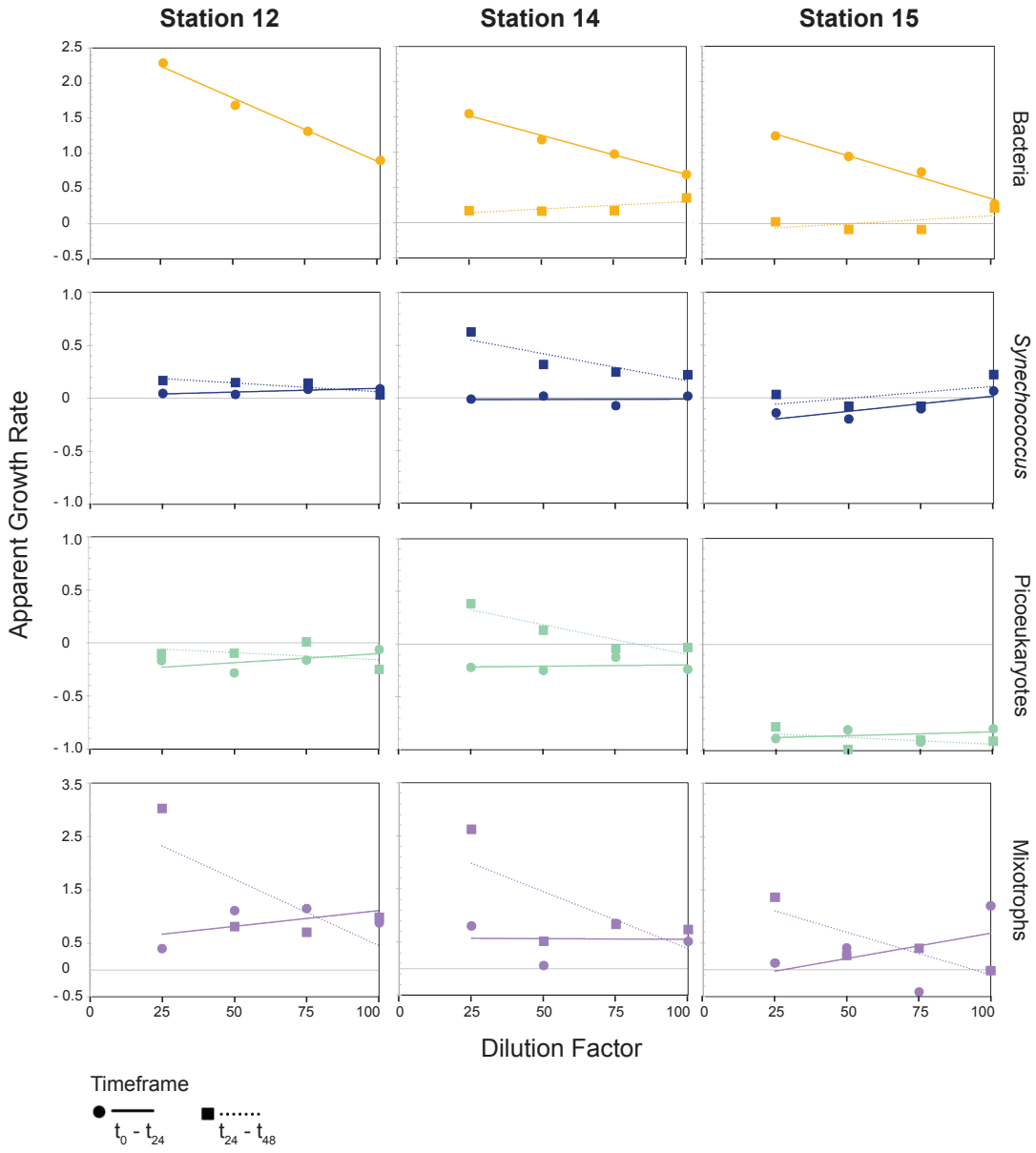


**Figure 3. 3:** Results of the dilution incubations carried out at each station for bacteria (orange), *Synechococcus* (dark blue), Picoeukaryotes (light green) and mixotrophs (lilac). The first day of the experiment is marked with circles and a solid line and the second day with squares and a dashed line.

**Table 3. 3:** Growth and grazing rates of bacteria, *Synechococcus*, picoeukaryotes and mixotrophs estimated from Landry and Hassett (1982) method as well as $R^2$ values associated with each trendline.

| | | Station 12 | | Station 14 | | Station 15 | |
|---|---|---|---|---|---|---|---|
| | | 0-24 | 24-48 | 0-24 | 24-48 | 0-24 | 24-48 |
| **Bacteria** | Grazing | -0.018 | - | -0.011 | 0.002 | -0.012 | 0.002 |
| | Growth | 2.682 | - | 1.796 | 0.087 | 1.576 | -0.119 |
| | $R^2$ | 0.987 | - | 0.989 | 0.591 | 0.977 | 0.270 |
| ***Synechococcus*** | Grazing | 0.001 | -0.002 | 0.000 | -0.005 | 0.003 | 0.002 |
| | Growth | 0.024 | 0.227 | -0.009 | 0.675 | -0.271 | -0.132 |
| | $R^2$ | 0.751 | 0.747 | 0.000 | 0.786 | 0.674 | 0.971 |
| **Picoeukaryotes** | Grazing | 0.002 | -0.001 | 0.000 | -0.006 | 0.001 | -0.001 |
| | Growth | -0.275 | -0.022 | -0.220 | 0.467 | -0.894 | -0.813 |
| | $R^2$ | 0.385 | 0.177 | 0.015 | 0.852 | 0.128 | 0.214 |
| **Mixotrophs** | Grazing | 0.000 | -0.021 | 0.006 | -0.025 | 0.010 | -0.016 |
| | Growth | 0.583 | 2.512 | 0.519 | 2.939 | -0.257 | 1.512 |
| | $R^2$ | 0.001 | 0.502 | 0.311 | 0.530 | 0.209 | 0.744 |

*FLBd Ingestion by HNF and Mixotrophs*

As mentioned above, due to the similarity in fluorescence of the LysoTracker® Green and the FLBd, it was not possible to clearly distinguish HNFs from bead accumulations. Likewise, it was not possible to say with certainty that HNFs and mixotrophs stained with LysoTracker® Green did not have any beads inside them. However, when looking at the untreated samples none of the particles displayed red and green fluorescence which one would expect for a mixotroph with engulfed FLBds. Further, only a marginal number of cells met the size and the (slightly lower than the FLBd) green fluorescence criteria in the untreated samples as one would expect from a HNF or mixotroph containing a FLBd. Thus, it appears that during all of the incubations we did not observe any FLBds ingested by HNFs or by phagocytosing mixotrophs.

Yet, at station 15, one accumulation of particles which appeared to increase over the period of the two days clearly stood out. These particles were slightly larger in size than the beads, but still smaller than any HNF, with a green fluorescence slightly weaker than that of the FLBds and low red fluorescence (blue dots Figure 3. 4). Their size made them too small to be cells which had engulfed FLBds and they possessed too little green fluorescence to be a simple FLBd aggregate. Consequently, these particles can be classified as organic aggregates with incorporated FLBds (Figure 3. 4).

In Figure 3. 5: A, the abundance change over time of the organic aggregates containing FLBds is shown for each dilution. For all dilutions, the abundances of the FLBd aggregates increased over time, but no trend in the amount of increase in FLBd aggregates was observed for incubations of different dilutions (Figure 3. 5: B).

At stations 12 and 14 the amount of FLBds in the incubations remained relatively unchanged, with marginally higher FLBd counts over time, for all but one incubation (Figure 3. 6: B). There were also no trends observed among the different dilution series of the amount of FLBds over the 48-hour period (Figure 3. 6: C). It is noteworthy that at $t_0$ of the station 12 and 14 incubations the FLBds appeared to be more aggregated with each other than at the later timepoints, for which the single bead green fluorescence peak was higher (Figure 3. 6: A). For station 15 the opposite was the case, with the beads aggregating with a lower single bead green fluorescence peak at $t_{24}$ and $t_{48}$ as well as higher multiple bead aggregate peaks (Figure 3. 6: A). At station 15 there was a small decrease in the amount of FLBds over time for the 50, 75 and 100 dilutions (Figure 3. 6: B) which followed a trend of the lower dilution incubations having a higher decrease in FLBds (Figure 3. 6: C).

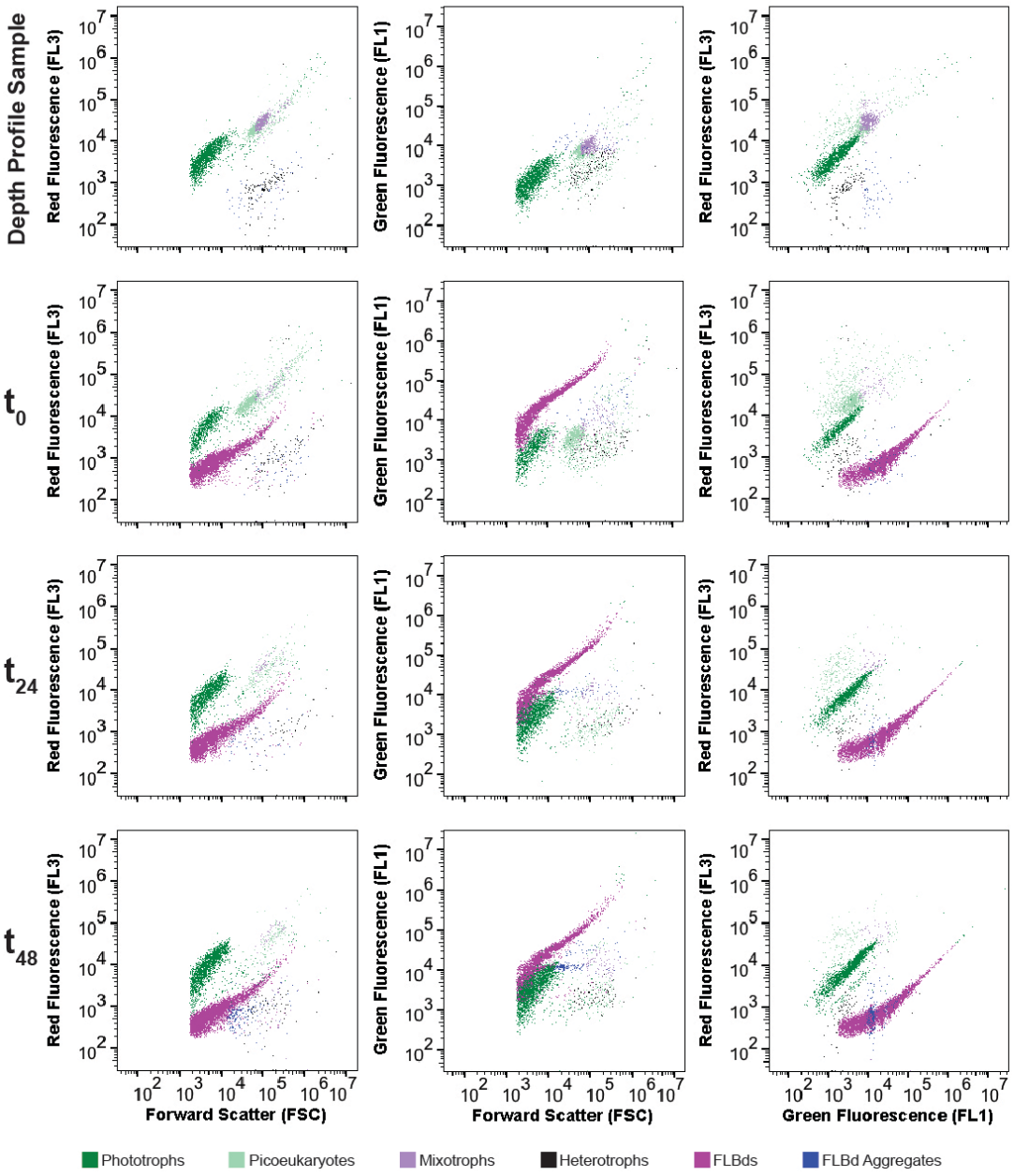


**Figure 3. 4:** Cytometric analysis of LysoTracker® Green treated samples from station 15 taken for the depth profile and the undiluted incubation sample at $t_0$, $t_{24}$ and $t_{48}$. The plots illustrate how the different groups of cells/particles were distinguished from each other using green (FL1) and red (FL3) fluorescence together as well as forward scatter (FSC) and show the increase in organic aggregates with FLBds inside them (blue: FLBd Aggregates) over time.

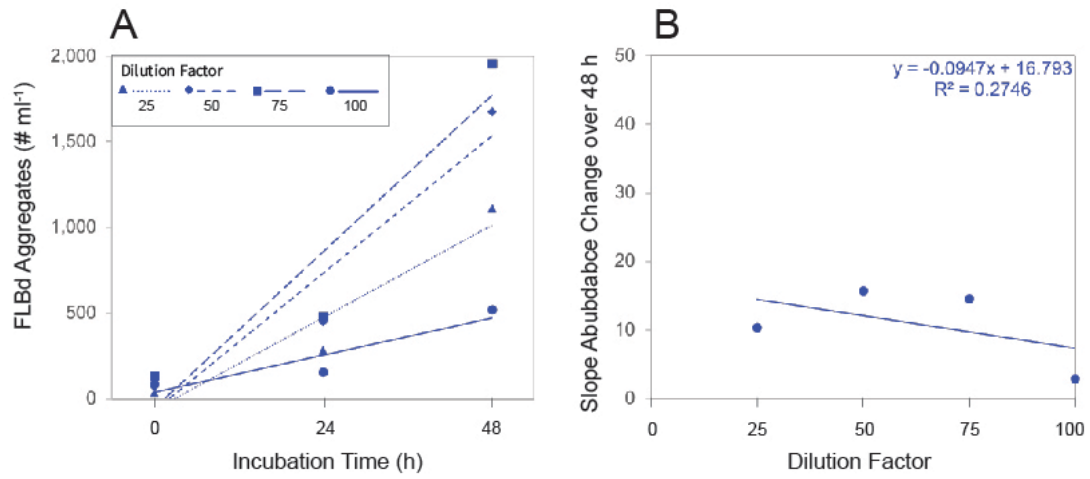


**Figure 3. 5:** A: Biplot of FLBd aggregate numbers (per ml) for the average of each dilution series at $t_0$, $t_{24}$ and $t_{48}$; B: Slope of abundance change of FLBd aggregates over a 48-hour period for the average of each dilution series.

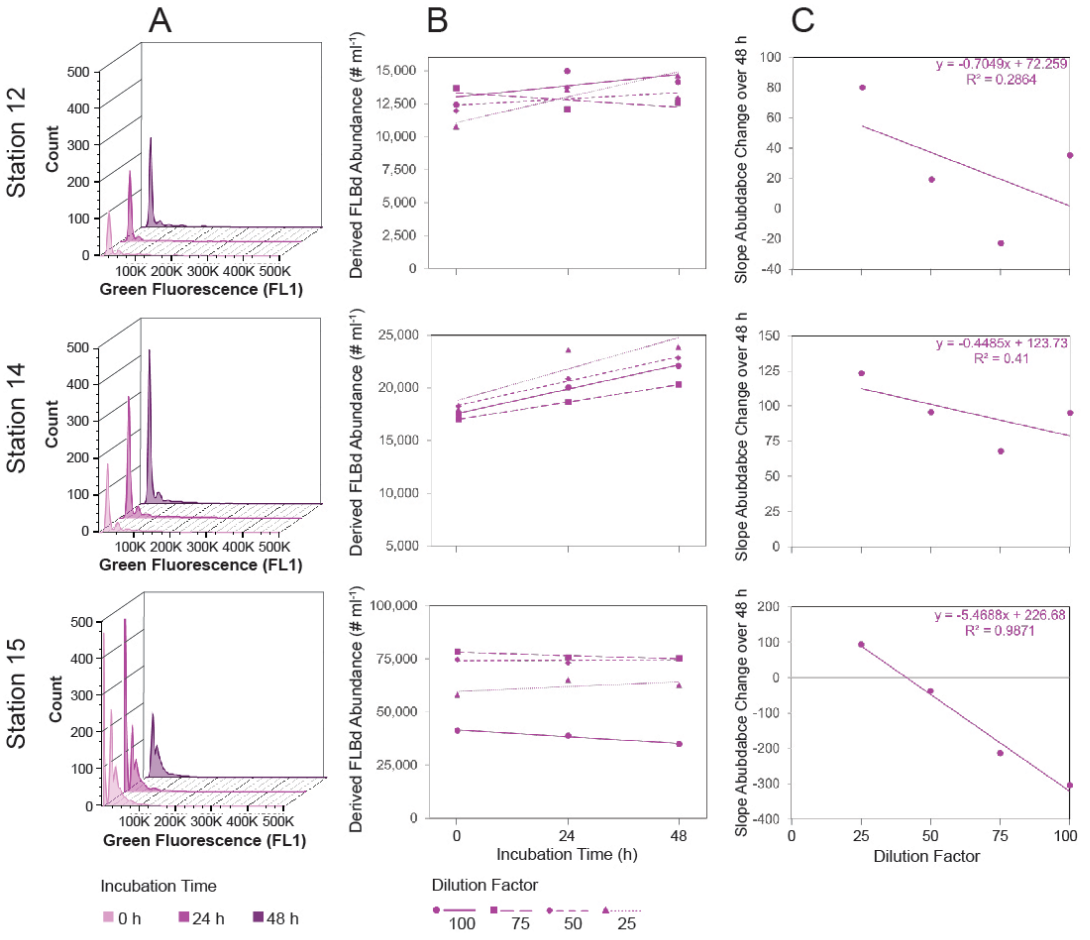


**Figure 3. 6:** A: Staggered biplot of green fluorescence (FL 1) of FLBds over time for one of the undiluted incubations from each station; B: Biplot of derived FLBd numbers (per ml) for the average of each dilution series at $t_0$, $t_{24}$ and $t_{48}$; C: Slope of abundance change of FLBds over a 48-hour period for the average of each dilution series.

*Encounter Rates*

When inspecting the encounter rates listed in Table 3. 4 it is evident that overall encounter rates of HNF and mixotrophs with FLB and nanoplastics are low. On average, in the incubation setting, HNF and mixotrophs are 26 times more likely to encounter a living prey cell than a FLB. For encounter rates calculated for the environment this trend is even more prominent with HNF and mixotrophs being 3897 and 3421 times less likely to encounter a nanoplastic rather than a living prey cell.

The encounter rate of the inert FLBs with a living cell was on average 2117 times higher than encounter rates of a HNF or mixotrophs with a FLB in the incubations. Similarly, nanoplastics in the environment were 2122 times more likely to encounter a living cell rather than being encountered by a HNF or mixotroph (Table 3. 4).

**Table 3. 1:** Encounter rates (in number $ml^{-1}$ $d^{-1}$) of HNF, Mixotrophs and FLB (in incubation) and nanoplastics (in wild) with living cells (bacteria, *Synechococcus* and picoeukaryote) and each other under incubation conditions and in the wild.

| | | Station 12 | Station 14 | Station 15 | Average |
|---|---|---|---|---|---|
| **In Incubation** | | | | | |
| **HNF** | Living cell | 731,364 | 965,535 | 1,718,542 | 1,102,778 |
| | FLB | 18,818 | 28,724 | 88,528 | 41,931 |
| **Mixotroph** | Living cell | 320,079 | 612,743 | 5,572,625 | 1,801,861 |
| | FLB | 8,236 | 18,229 | 287,162 | 68,528 |
| **FLB** | Living cell | 94,722,065 | 162,729,718 | 521,715,179 | 233,853,513 |
| | FLB | 2,438,445 | 4,841,161 | 26,865,189 | 8,892,272 |
| **In Environment** | | | | | |
| **HNF** | Living cell | 22,981,554 | 30,222,040 | 53,983,858 | 34,605,307 |
| | Nanoplastic | 4,206 | 4,556 | 20,718 | 8,940 |
| **Mixotroph** | Living cell | 10,108,869 | 19,358,196 | 176,054,134 | 56,925,598 |
| | Nanoplastic | 1,840 | 2,891 | 67,181 | 14,607 |
| **Nanoplastic** | Living cell | 21,179,906 | 25,810,928 | 122,160,012 | 49,977,551 |
| | Nanoplastic | 122 | 122 | 1,474 | 406 |

## Discussion

Despite the use of FLBds in HNF and mixotroph grazing rate experiments here we report no noticeable ingestion of FLBds by either group. We did, however, find that sub-micron sized plastic particles may coagulate with colloidal material and form micron-sized aggregates. This may provide a pathway for nanoplastics to enter oceanic, oligotrophic food webs and be distributed throughout the water column.

*Microplankton Community Composition and Nanoplastic Abundances*

Overall, abundances of the different microplankton groups resembled those one would expect to find in oligotrophic waters (Figure 3. 1). Although at station 15, waters were more productive with a greater amount of picoeukaryotes, *Synechococcus* and mixotrophs present. At station 15 we also found some

discrepancies in the counts of bacteria and mixotrophs between the samples taken to construct depth profiles and those used for incubations (Figure 3. 1, Table 3. 2). A possible reason for this could be diurnal variations in the microplankton communities as the two samples were taken more than 10 hours apart (Appendix 2. 1). Lastly, for the second incubation at station 15, HNF and mixotroph numbers were markedly lower, which could have been caused by errors during pipetting, resulting in the addition of too little LysoTracker® Green stain.

While there is a lack of studies describing microplankton communities in the central part of the subtropical South Pacific (station 12 and 14) some comparison can be made between the data from station 15 and a study by Safi & Hall (1999). In their study they sampled the waters approximately 800 km Southwest from station 15 around the same time of the year. They describe, slightly higher, but similar, numbers of HNFs and mixotrophs, and bacterial abundance levels four times lower. What is of interest is that by exposing HNFs and mixotrophic nanoflagellates (MNFs) to dead fluorescently-labelled bacteria and counting their decline over time they found that 55% of grazing on the bacterial standing stock was attributed to mixotrophs when accounting for both grazing rates and abundance (Safi and Hall, 1999).

*Growth and Grazing of Microplankton Exposed to FLBds*

Throughout all stations, bacteria grew at relatively high rates during the first day (Figure 3. 3). To allow for these growth rates, which levelled during the second day, the water must have been saturated in dissolved organic matter (DOM). Enrichment in DOM could have happened during filtration as cells may have lysed while passing over the filter (Ayukai, 1996). Interestingly, our observations of an initial increase in bacterial growth in response to nanoplastic exposure are in coherence with those of Sun et al. (2018). In their study, they describe how *Halomonas alkaliphila* showed an initial increase in growth rate when exposed to 55 nm polystyrene beads which they attributed to a hormetic response mechanism. However, in that study bacteria were exposed to nearly 30 times the amount of FLBds used in our exposure experiments (Sun et al., 2018).

*Synechococcus* and picoeukaryotes showed low growth rates during the first day which, for most incubations, picked up slightly during the second day (Figure 3. 3). These similar response patterns of photosynthetic organisms within the incubations may indicate that phototrophs struggled with the change from natural to artificial light. Another potential reason could be shading and airflow blockage by FLBds, as

previously hypothesised by Besseling et al. (2014) and Bhattacharya et al. (2010). Lastly, it is also important to consider that *Synechococcus* growth and grazing rates have previously been found to be inaccurate if extrapolated from dilution series experiments (Anderson and Harvey, 2019; Ayukai, 1996). Potential reasons for this are sensitivity to metals as well as lack of regenerated nutrients (Ayukai, 1996; Brand et al., 1989).

*FLBd Ingestion by HNFs and Mixotrophs*

Our results show no noticeable ingestion of FLBds by HNFs or mixotrophs (Figure 3. 6). Interestingly, several studies on the grazing rates of HNFs and mixotrophs did describe ingestion of FLBds (Vaque et al., 1994). In these studies concentrations of FLBds commonly used were, however, one to three orders of magnitude higher which made them more available to potential predators. Furthermore, most of these studies describe that FLBds were discriminated against when offered together with fluorescently labelled bacteria or natural prey (Bratvold et al., 2000; Gonzalez and Suttle, 1993; Jürgens and DeMott, 1995). In some cases, this led to selectivity of 15 – 20 for fluorescently labelled bacteria over FLBds (Landry et al., 1991; Safi and Hall, 1999). Despite describing such a selectivity by HNFs and MNFs sampled off the coast of New Zealand, Safi and Hall (1999) demonstrated that some HNFs and MNFs ingested FLBds when exposed to similar concentrations as used within our study. This may be explained by two reasons; firstly, they covered the FLBds in bovine serum prior to exposure which may have enhanced uptake by HNFs and MNFs, and secondly that the bacteria to FLBd ratio was higher in their study (Table 3. 2) (Safi and Hall, 1999). Nevertheless, HNFs and mixotrophs within this and many other studies show strong selectivity against FLBds and, when taking into account that under environmental concentrations encounter rates may be even lower than calculated for our incubation experiments, it appears unlikely that ingestion by HNFs and mixotrophs presents a pathway for microplastics into oligotrophic pelagic foodwebs (Table 3. 4).

Non-selective, filter-feeding mucous grazers, on the other hand, may provide a more likely route for nanoplastics into marine food webs. In addition to being less selective, some filter-feeders draw in vast amounts of water, which considerably increases encounter rates (Turner, 2002). Likely candidates of filter-feeding ingestion are larvaceans and salps (Conley et al., 2018). Both have been observed to ingest and re-package microplastics into shed filters and faecal pellets (Katija et al., 2017; Wieczorek et al., 2019). Lastly, while direct ingestion by HNFs and MNFs seems unlikely, they may facilitate nanoplastic incorporation into large marine

aggregates to which they are attached to, by beating their flagella, thereby creating currents and drawing such particles towards the aggregates (Fukuda and Koike, 2000).

*Formation of FLBd Aggregates*

Flow cytometric analysis of incubations at station 15 revealed the formation of FLBd heteroaggregates (Figure 3. 4) which increased in number with time for each dilution (Figure 3. 5: A). These heteroaggregates may be a result of coagulation of FLBds with colloids. Colloids were likely to be abundant in the incubations as the water used to dilute the samples within the dilution series was filtered through a 0.2 µm filter which allows passage of much of the colloidal fraction. This would also explain why there was no difference noted in the amount of aggregates formed over time within each of the dilutions (Figure 3. 5: B).

Assuming a colloid abundance of $1 \times 10^7$ colloids $ml^{-1}$ (Isao et al., 1990) and no swimming nor sinking of the colloids it is possible to get an estimate of colloid – FLBd/nanoplastic encounter rates using the equations applied within this study. According to this, both FLBds in the incubations and nanoplastics in the environment would be 16 times more likely to encounter a colloid than a living cell and roughly 89,000 times more likely to encounter a colloid compared to a HNF or mixotroph. However, encounter rates alone do not determine whether submicron particles form heteroaggregates. Surface properties of the nanoplastic, pH of medium and other factors have been shown to determine whether colloids coagulate with nanoparticles (Alimi et al., 2018). Here we used fluorescently labelled polystyrene beads with encapsulated dyes which are not particularly representative of nanoplastics present in the environment and this should be improved in future studies.

What is of interest is that heteroaggregates have only been observed at station 15. This may be explained when recalling that incubation samples were retrieved from 60 m at stations 12 and 14 and from 10 m at station 15. Isao et al. (1990) highlight that colloids are most abundant in surface waters with 95% of colloids being found in the top 50 m. This, of course, depends on the mixed layer depth, productivity and other environmental factors but their predominance in the surface are in support of our findings. It is also noteworthy that at station 15 we observed an increase in FLBd homoaggregates with time, which was not the case at stations 12 and 14 where FLBds had the tendency to disaggregate over time (Figure 3. 6: A). While fluorescence and size of the FLBd homoaggregates at station 15 suggest no major

contribution of organics within these aggregates (hence classified as homoaggregates), it may be possible that sugars and proteins present in the water did contribute to this aggregation process.

Overall, the findings presented here are in support of what Fillela (2015) has previously emphasised in her review paper where she stresses that, as with natural suspended particles, interactions with natural colloids, such as compact inorganics, humic-like substances and biopolymers, need to be given adequate consideration when investigating nanoplastics (Filella, 2015). This not only determines their dispersal in the water column but also governs their bioavailability. In fact, findings by Ward & Kach (2009) have previously described how the incorporation of nanoplastics into aggregates made them available for ingestion by suspension-feeding bivalves. Aggregates are an important food source for many zooplankters (Lampitt et al., 1993). In this context, findings of copepod mortality associated with the exposure to nanoplastics are of concern (Lee et al., 2013).

*Study Conditions and Recommendations for Future Studies*

The use of yellow-green FLBds in combination with LysoTracker® Green, which overlap in their fluorescence, caused complications within this study and made the distinction of HNFs from aggregates of two or more FLBds a difficult task. This generated uncertainties in the estimated abundance of HNFs within the incubation samples. More importantly, it also caused complications when investigating HNFs and mixotrophs who may have ingested FLBds. Only by considering the green fluorescence within the samples which were run untreated was it possible to confirm that no notable number of particles resembled HNFs or mixotrophs with FLBds inside them. Despite this, and in coherence with previous studies (Long et al., 2017), the use of FLBds in combination with flow cytometry proved to be a useful tool to get a first idea of micro- and nanoplastic pathways within marine systems. In future studies, the use of a flow cytometer possessing more channels and detectors may allow for an easier distinction between different organisms and FLBd aggregates.

Another advantage of utilising flow cytometry is that actual concentrations of nano- and microplastics available (i.e. those suspended in incubation) to exposed organisms can be determined. This is important as microplastics may sink or stick to incubation container walls which is an artefact of studying organisms in the laboratory and should be given consideration (Long et al., 2017; Sussarellu et al., 2016). In fact, this may have also happened within this study as the amount of

FLBds which should have theoretically been present in the experiments was much lower than those observed within each incubation (Table 3. 2).

In regard to the use of FLBds it is important to highlight that concentrations and type of FLBds used within this study do not resemble nanoplastics one would expect to find in the environment. While the application of FLBds is useful in initial investigations into the fate of micro- and nanoplastics in marine environments (e.g. Cole et al., 2016; Katija et al., 2017) it is important that plastic particle type and concentrations used should more closely resemble those one would expect to see in the environment (Botterell et al., 2018). Moreover, plastic particles should undergo some form of pre-treatment such as done by Pace & Bailiff (1987) who covered FLBds with proteins to make them resemble natural prey more closely. Lastly, while the main aim of this study was to investigate nanoplastic uptake by HNFs and mixotrophs which does not necessarily require running a control dilution series (without FLBds), doing so would have helped to investigate potential effects of nanoplastics on bacteria, *Synechococcus* and picoeukaryote growth rates.

Here we provide some first insights into coagulation processes of nanoplastics and small microplastics with colloidal material. This is a pathway worthy of investigation, but future studies should take methodological considerations mentioned above into account. In the next instance, it will then be important to evaluate how re-packaging into micron-sized aggregates may affect their dispersal and bioavailability as this may have far-reaching implications for their bioavailability for species of higher trophic levels.

## Conclusion

The nano-sized fraction of plastics within our oceans is eminently understudied with only a handful of studies investigating their abundances and interactions with the marine biota (SAPEA, 2019). While not much is known about their abundances, we can assume that with an increase in alternatives to traditional plastic polymers, such as oxo-biodegradables, we may face a substantial increase of this size fraction in years to come (Kubowicz and Booth, 2017). Furthermore, the breakdown of microplastics to nanoplastics may actually be promoted through ingestion of microplastics by marine life (Dawson et al., 2018). Only a few organisms target this size class as a food source - HNFs and mixotrophs are thought to be the major ones (Fenchel, 1988). However, our findings together with encounter rate considerations show that they are unlikely to ingest nanoplastics in oligotrophic

waters. Here, we found that coagulation with colloids and formation of heteroaggregates may be more likely. Incorporation of nanoplastics into heteroaggregates could distribute them in the water column and make them available for ingestion by other organisms. Lastly, in “super“-oligotrophic waters concentrations of colloidal matter may be too low which may cause nanoplastics to accumulate in such environments. Here filter-feeding mucous grazers such as salps and larvaceans could provide the only means of advection (Conley et al., 2018).

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# DISCUSSION

- Summary of Findings
- Microplastic Interactions with Individual Organisms and Potential Impacts
- Adverse Ecological Effects
- Understanding microplastic pathways in the marine pelagic environment
- Outlook
- Perspective

# Discussion

Microplastics were first described in the pelagic environment in 1972 in a paper by Carpenter and Smith, but only in 2004 was the term “microplastic” introduced to the literature (Thompson et al., 2004). Since then microplastics have emerged as a global research topic and several high-profile studies have also sparked great concern among the public (e.g. Bergmann et al., 2019; Lebreton et al., 2018; Wieczorek et al., 2018). This also brought the issue to the attention of policy makers (e.g. Galgani et al., 2013). The majority of published microplastic studies either quantified microplastic contamination in selected environments and species or investigated effects of microplastics on individual organisms through laboratory-based experiments. This great body of work has given some first insights and allowed for general conclusions, but there are still some important questions that need to be addressed. Here, I have taken a holistic approach to investigate whether microplastics may affect the normal functioning of marine pelagic systems. This was achieved by firstly studying the interactions of microplastics with three distinct groups of organisms of ecological and economic significance along two gradients (macro- to microorganisms and eutrophic to oligotrophic waters) and secondly, by evaluating potential far-reaching adverse effects of these interactions. By doing so, important insights into microplastic pathways in the marine pelagic system were gained. A greater understanding of these pathways will significantly improve efforts assessing which organisms are at risk (“key species”), what potential risks microplastics pose (“so what?”) and help evaluate potential ecological knock-on effects (“what if?”). Ultimately, these insights will help to address the question of whether microplastics are of environmental concern.

## Summary of Findings

*Chapter 1 – Mesopelagic Fish*

Of the 233 mesopelagic fish which were sampled from the Northwest Atlantic and assessed for microplastic presence, 73% had microplastics within their gut contents. This is one of the highest abundances reported for marine fish thus far and exceeds previous findings of microplastic contamination in mesopelagic fish. While varying methods of microplastic extraction and analysis may explain this variation, the high abundances reported within this study could be a result of microplastics accumulating within the eddy feature from which the fish were sampled. In fact, microplastics identified from the sea-surface waters of the eddy

and those from the gut contents of the fish were similar in type, shape, colour and size. Whether microplastics are ingested directly from the water, or transferred by microplastic-contaminated prey, is uncertain. Yet, this study is the first to report abundances of microplastics in mesopelagic fish in the size range of those which could have been ingested by their planktonic prey. The number of microplastics did not vary significantly with stomach fullness, species nor the depth from which the fish were sampled (Figure D. 1). Overall, the high abundances of microplastics within these fish are of concern, especially when considering that they make up a large proportion of the biomass in the pelagic environment. They are a vital resource for large predatory fish and play an important role in the cycling of organic matter by undertaking large diurnal migrations. Lastly, as microplastics have been proposed as a potential vector for toxins to enter an organism's tissue, this potential hazard should be considered in terms of food safety when exploiting mesopelagic fish in the future, and during current exploitation of species preying on mesopelagic fish (e.g.: tuna, swordfish).

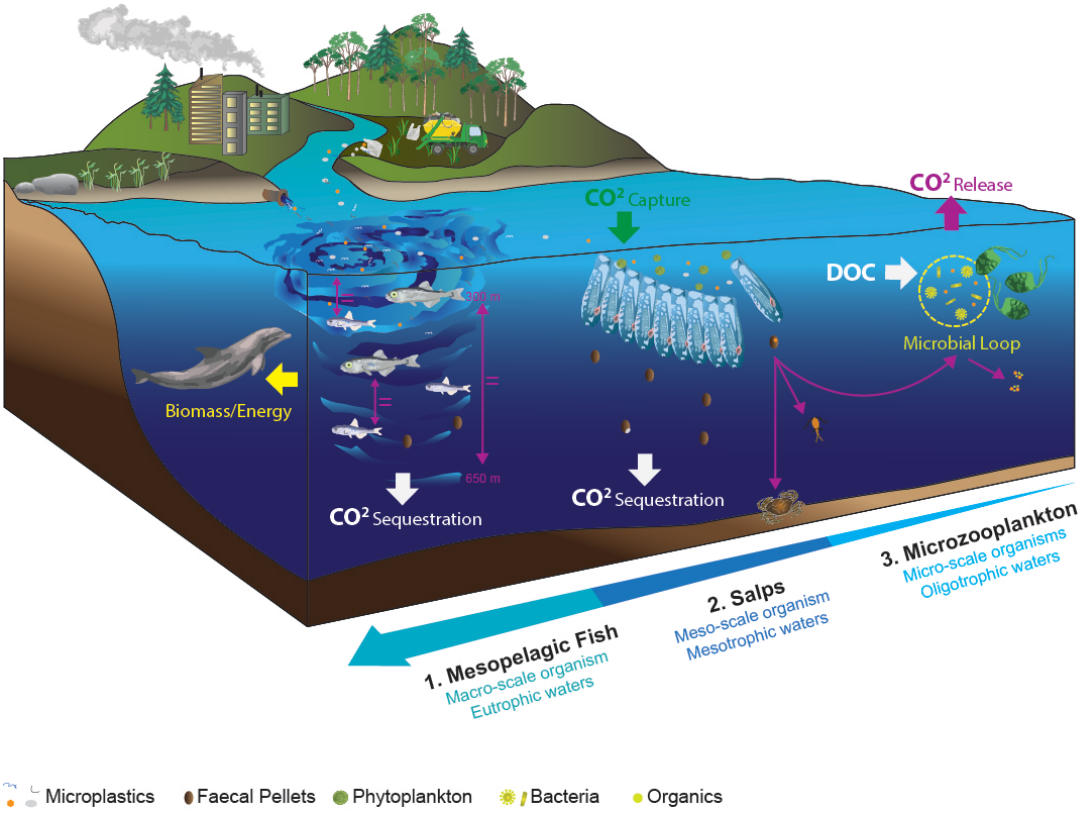


**Figure D. 1:** Graphical summary of the most important findings of the three research chapters indicated in purple. **Mesopelagic fish** had high contamination of microplastics which were similar to those sampled from the sea surface; no significant difference was observed between different species and fish caught at different depths. **Salps** were shown to ingest microplastics and incorporate the into their faecal pellets which decreased the faecal pellet sinking speed. **Microzooplankton** were shown to have low encounter rates with nanoplastics and it was found that nanoplastics more likely form aggregates with colloidal matter. ©Alina M. Wieczorek

## Discussion

*Chapter 2 – Salps*

*Salpa fusiformis* which were exposed to environmentally relevant (size range, UV treated, possessing biofilm) polyethylene and polystyrene microplastics were found to readily ingest these and incorporate them into their faecal pellets. Few faecal pellets contained microplastics when subjected to current environmental concentrations, yet when subjected to concentrations 1-2 orders of magnitude higher than reported for the South Pacific Gyre up to 46% of all pellets contained microplastics. Once microplastics were incorporated, sinking speed decreased by an average of 1.35 times for polyethylene-containing pellets and 1.47 times for polystyrene-containing pellets. Interestingly, of the two tested plastics, the higher density polystyrene was more frequently in- and subsequently egested and had a greater effect on sinking speed compared to the lower density polyethylene. Potential reasons for more frequent ingestion may be that higher density plastics are more evenly distributed in the water column or that plastics are retained differently by the mucous strand due to varying surface properties. This may also lead to higher loads of one type of microplastic over another which would explain the differing sinking rates. Lower sinking rates could further be explained by potential alterations in the faecal pellet structure due to properties such as the shape of the microplastic fragment. In fact, an effect on faecal pellet size was also noted for polystyrene within this study, which supports this hypothesis. In the pelagic environment, the decrease in salp faecal pellet sinking velocities may cause them to remain at the sea surface for a prolonged period. This makes them available for ingestion by coprophagous organisms and breakdown by bacteria. Bacterial breakdown would cause the carbon, which is initially captured by photosynthetic algae and tightly packaged within the faecal pellet, to be re-released to the atmosphere as $CO_2$. Further, a decreased flux of faecal pellets to the ocean floor may also deprive deep sea species of energy inputs from the sea surface. Alternatively, salp faecal pellets may act as a vector for microplastics to deeper waters and the seafloor where they are then available for ingestion by other organisms. The findings presented within this study give important insights into adverse ecological effects by using environmentally relevant microplastics in concentrations ranging from current estimates to those at which adverse ecological effects were noted, thereby contributing to the much-needed knowledge of critical doses while also providing altered sinking speed data. This data could be incorporated into current models of biogeochemical cycling to predict potential overarching consequences of microplastics in the future.

## Discussion

*Chapter 3 – Microzooplankton*

Heterotrophic and mixotrophic microplankton sampled from three stations, ranging from the central to the western subtropical South Pacific, showed no notable ingestion of nanoplastics in the form of fluorescently labelled beads to which they were exposed to within dilution series experiments. These findings were supported when abundances of phagocytosing organisms and potential environmental nanoplastic concentrations were put in the context of encounter rates. Calculated encounter rates revealed low encounter rates of the selectively feeding, phagocytosing organisms with nanoplastics. Rather than being ingested, nanoplastics appeared to form heteroaggregates with organic matter or colloidal material within the surface waters of this oligotrophic environment. Incorporation of nanoplastics into micron-sized aggregates may allow for their dispersal and sinking in the water column and enable them to enter the food web. Non-selective filter feeders such as larvaceans and salps may provide a more likely pathway for larger nanoplastics and those incorporated within described heteroaggregates. Other noteworthy findings of this study include an initial increase in bacterial abundance after the addition of nanoplastics, which has previously been described and may be attributed to a hormetic response mechanism. Further, phototrophs within this study showed an initial decline when exposed to nanoplastics. While this could have been a response to changing light regimes within the incubation setting, it may also have been attributed to shading and airflow blockage by nanoplastics. Overall, this study gives important insights into the, as of yet, understudied size fraction of plastics in our seas and their potential pathways as well as their interactions with microplankton communities and colloidal matter in oligotrophic waters.

### Microplastic Interactions with Individual Organisms and Potential Impacts

In the three papers presented in this thesis, I show that each of the three studied groups displays a very different response to microplastic exposure. Mesopelagic fish seem to be particularly prone to microplastic contamination (Chapter 1). This may be of concern as some initial studies have reported adverse effects of microplastics on fish health (Naidoo and Glassom, 2019; Pedà et al., 2016). Furthermore, microplastics have been proposed as a potential vector for toxins to enter an organism's tissue (Batel et al., 2016; Wardrop et al., 2016). This vector mechanism is, however, currently subject to debate (Gouin et al., 2011; Koelmans et al., 2016; Lohmann, 2017). Nonetheless, microplastics acting as a vector for toxins should be considered as a potential hazard to the fish themselves and to the

health of their predators. This has further implications for food safety in terms of future exploitation of mesopelagic fish, and the current exploitation of species preying upon them (e.g.: tuna, swordfish) (Lusher et al., 2017).

It remains unclear how mesopelagic fish may be exposed to microplastics, but their planktonic prey may provide a likely pathway of exposure. In fact, by applying forensic methods, coupled with the use of small-meshed microplastic retention filters, I was able to identify microplastics in the size range of those which may be ingested by their planktonic prey. Transfer of microplastics from smaller to larger zooplanktonic species has already been demonstrated under lab conditions (Setälä et al., 2014). Additionally, some zooplankton species sampled from the environment have been reported to be contaminated with microplastics (Desforges et al., 2015; Steer et al., 2017; Sun et al., 2017). This may have consequences not only for mesopelagic fish, but also for the many other species which rely on planktonic prey as a source of energy. For large filter feeders, such as baleen whales and whale sharks, microplastic contamination may be amplified due to the vast amount of planktonic prey they ingest - as indicated by recent findings (Besseling et al., 2015; Fossi et al., 2017, 2012; Wieczorek and Donati, *ongoing research*).

What is of further interest regarding this, is that the different species assessed within Chapter 1 showed no marked difference in the number of microplastics in their gut contents (Figure D. 1). This is of significance as some of the species feed rather non-selectively by filtering zooplankton over their gill-rakers, whereas others display more active predatory behaviour. Selective feeding by planktivorous fish has been described in amberstripe scad (*Decapterus muroadsi*) that ingested blue polyethylene microplastics resembling their blue copepod prey (Ory et al., 2017). In a subsequent study on palm ruff (*Seriolella violacea),* selective ingestion of microplastics-resembling prey (food pellets in this case of tank-reared fish) was confirmed and it was further found that microplastics were commonly expelled if they were not mixed with food in the fish's mouth (Ory et al., 2018). Findings presented in Chapter 1, however, suggest non-selective feeding by mesopelagic fish. This is further supported by the fact that plastics extracted from the fish strongly resembled those which were present in the seawater (Figure D. 1), which would indicate direct, non-discriminative ingestion from the water. Lastly, it was found that stomach fullness did not relate to the number of microplastics within their gut contents. This may be a sign that microplastics could, in fact be retained within the gut rather than being ingested and subsequently egested as had been shown for palm ruff by Ory et al. (2018). Ingestion and egestion with little effect on the

individual organism also appeared to be the pathway of microplastics presented to the non-selective filter-feeding salps studied within Chapter 2, as they incorporated microplastics into their faecal pellets. This has previously been described for other zooplankton species such as larvaceans, copepods, sea urchin larvae and krill (Cole et al., 2016; Coppock et al., 2019; Dawson et al., 2018; Kaposi et al., 2014; Katija et al., 2017) and thus seems to be a common phenomenon. What is of interest is that in the process of ingestion and egestion by krill, microplastics were fragmented into nanoplastics (Dawson et al., 2018).

During exposure experiments carried out within Chapter 2, salps were only exposed to a certain size fraction (150 – 500 µm) of microplastics of shapes and types which, at the time when the study was conducted, were reported to be prevalent in the environment. Since then, researchers have become more concerned about the understudied nanosized fraction of plastics in our oceans (SAPEA, 2019). Given that salps and other gelatinous filter-feeders possessing mucous structures are able to retain particles down to the submicron scale (Conley et al., 2018), they may indeed be prone to interact with these particles. This is of particular significance in the context of Chapter 3. In this chapter, experimental and theoretical evidence is presented which shows that microzooplankton are unlikely to actively interact with nanoplastics, particularly under extrapolated environmental concentrations. The reason for this lies in insufficient encounter rates. Some mucous-mesh grazers, on the other hand, are able to retain nanosized particles and actively filter large amounts of water over their mucous strands, leading to higher encounter rates (Conley et al., 2018). Such interactions may be particularly relevant in “super”-oligotrophic waters, low in colloidal and particulate matter, which would otherwise allow for coagulation of nanoplastics resulting in aggregate formation and dispersal as well as increased bioavailability (see “Understanding microplastic pathways in the marine pelagic environment”). As a consequence, microplastics may accumulate in these waters and mucous grazers provide the only means of advection. Concerning encounter rates, findings presented in Chapters 2 and 3 lead to question the significance of results presented within studies over-exposing organisms to microplastics in the lab. Indeed, in the environment, encounter rates may actually be so low that within the timeframes chosen for such experiments an organism would only encounter a single microplastic by chance.

Lastly, in Chapter 3, I give some first insights that, while not directly interacting with microplastics themselves, bacteria and photosynthetic microplankton present in oligotrophic waters may be affected by the exposure to nanoplastics. In the case of

bacteria, an initial increase followed by a decrease may be ascribed to a hormetic response mechanism as argued by Sun et al. (2018). For photosynthetic organisms, a lack of growth may be the consequence of shading and airflow blockage (Bhattacharya et al., 2010). However, none of these effects have been specifically investigated and it is therefore hard to draw any conclusions about any potential adverse effects at this stage.

Overall, I show that interactions and impacts on individual organisms strongly depend on the trophic position and feeding mechanism of an organism as well as the saturation of nutrients in the seawater (see also “Understanding microplastic pathways in the marine pelagic environment”).

### Adverse Ecological Effects

In the marine pelagic environment, the cycling of particulate organic matter (POM) presents a key ecological process. Specifically, the downward flux of POM into deeper waters and to the ocean floor provides a source of energy to deep-water and benthic species (Kaiser et al., 2005). Moreover, on a global scale, this downward flux accounts for the sequestration of $CO_2$ initially captured by photosynthetic organisms at the sea surface (Le Quéré et al., 2010; Passow and Carlson, 2012). Microplastics have the potential to affect this downward flux by causing structural and density changes to particles, which will in turn affect their sinking velocities.

Effects on sinking velocities have been described for copepod faecal pellets (Cole et al., 2016; Coppock et al., 2019) and algae aggregates (Long et al., 2015). Those studies did, however, overexpose organisms to microplastics and are thus less representative of processes which may occur in the environment. By using environmentally relevant microplastics in concentrations aligned with those described for the environment, I show that only when exposed to high concentrations a significant proportion of salp faecal pellets contained microplastics. However, once microplastics were incorporated, salp faecal pellet sinking speeds were significantly reduced (Chapter 2). A decrease in sinking velocities of faecal pellets causes them to remain at the sea surface for a prolonged period which makes them available for ingestion by coprophagous organisms and breakdown by bacteria (Figure D. 1). This breakdown would cause captured carbon to be respired and re-released to the atmosphere as $CO_2$ (Figure D. 1). Salps are thought to be of major importance in the downward flux of particulate organic matter (Turner, 2002).

Under natural conditions, their faecal pellets sink particularly fast causing them to bypass breakdown in the surface waters. Another way this breakdown is bypassed is through diurnal vertical migrations as undertaken by mesopelagic fish which feed at the surface at night and reside at depth during the day. These diurnal migrations are thought to facilitate the downward advection of large amounts of POM in the form of faecal matter and through the fallout of carcasses (Radchenko, 2007). This sinking POM may then either continue to sink, be recycled or be re-packaged by grazers in the mesopelagic. Interestingly, re-packaging by mesopelagic grazers has recently been argued to potentially increase downward advection of such POM (Stukel et al., 2019). In Chapter 1 I describe exceptionally high microplastic abundances found in the gut contents of mesopelagic fish. If these microplastics were expelled with their faeces, they potentially impact on faecal matter sinking rates as described for salps or remain in the gut, which could have long-term consequences for the fish's health. The latter may have knock-on effects on their normal functioning and in turn also affect the downward flux of POM.
A decreased downward flux of POM to deeper waters may also deprive deep-sea species of energy inputs from the sea surface which has recently been highlighted to be of key importance (Kelly et al., 2019). Alternatively, POM containing microplastics may act as a vector for microplastics to deeper waters and the seafloor where they are then available for ingestion by other organisms (Figure D. 1). Katija et al. (2017) propose that this is the case for sinking larvacean houses and faecal pellets which they describe as remaining negatively buoyant despite containing microplastics (Katija et al., 2017). Similarly, in Chapter 3 I show that nanoplastics may coagulate with colloidal materials and form aggregates (Figure D. 1). This could also result in their dispersal throughout the water column and make them bioavailable to a wider range of organisms (Filella, 2015). Bioavailability may be increased not only due to dispersal but also through interactions with organic matter causing them to resemble naturally occurring suspended particles. In fact, mussels have been shown to more readily ingest nanoplastics once re-packaged (Ward and Kach, 2009).

In summary, findings presented here and in currently available literature demonstrate that, under current environmental microplastic concentrations, the impact of microplastics on the sequestration of carbon within the biological pump appears to be negligible. Nevertheless, with increasing concentrations of microplastics there is potential for microplastics to cause disruptions to the sequestration of carbon to the seafloor in the future. How far into the future we can

expect notable impacts can be revealed through biogeochemical models incorporating data such as those presented within Chapter 2. At present it is, however, likely that incorporation into POM and colloidal aggregates causes microplastics to become distributed in the water column and makes them more bioavailable, which is supported by reports of microplastic abundances in several planktonic and deep-sea species (e.g. Courtene-Jones et al., 2017; Sun et al., 2017).

### Understanding Microplastic Pathways in the Marine Pelagic Environment

The multiple pathways of plastics within the pelagic environment are depicted in Figure D. 2. Pathways revealed within this thesis are: 1) ingestion of microplastics by mesopelagic fish; 2) ingestion and subsequent egestion of microplastics possessing a biofilm by salps; and 3) micron-sized aggregate formation of nanoplastics with colloidal matter. These pathways are highlighted with a white contour in Figure D. 2. Which pathway a piece of plastic will follow once it enters the pelagic environment, and what harm it may cause, very much depends on its intrinsic properties as well as physical, chemical and biological factors acting upon it in the environment (Andrady, 2017; Galloway et al., 2017). The diverse nature of plastics makes it particularly difficult to study their fate in this highly dynamic system. Depending on the desired application, plastics are moulded into varying shapes, given different crystalline formations and are enriched with different additives (Andrady, 2017). Responses to stressors such as ultraviolet (UV) radiation resulting in photooxidation, physical fragmentation resulting in cracking or ablation and biological breakdown by micro- and macro-organisms depends on the makeup of the plastics and what stressors are prevalent in the environment (Andrady, 2017; Dawson et al., 2018; Khatmullina and Chubarenko, 2019; Zettler et al., 2013). This results in a very diverse pool of macro-, micro- and nanoplastics possessing different characteristics (Figure D. 2: black pathways).

Key characteristics determining their distribution and fate are thought to be the size, density and shape of the plastics (Andrady, 2017; Chubarenko et al., 2016; Filella, 2015; Khatmullina and Chubarenko, 2019). For instance, microplastics of different shapes have been described as displaying different settling velocities and patterns of movement through the water column (Khatmullina and Isachenko, 2017; Kowalski et al., 2016). Further, when modelling movements of fibers compared to spheres in the Baltic, Chubarenko et al. (2016) described shorter surface residence

times for fibers. This is very interesting given that, in another study investigating microplastics in sediments, fibers were found to be less common than other shapes (Dai et al., 2018). A potential explanation for this disparity is that organisms present within the water column ingest these fibers. This theory is supported by the many studies reporting fibers to be the most abundant shape of microplastics found among the gut contents of several species (e.g. Bellas et al., 2016; Peters and Bratton, 2016). This indicates that fibers may be more bioavailable to some organisms. Results presented within the first chapter of this thesis are coherent with such findings since fibers made up the majority (98%) of microplastics extracted from mesopelagic fish. However, fibers were also the most abundant microplastic shape in the seawater samples. Nevertheless, the shape of a microplastic has implications for its bioavailability which in turn dictates the fate of these microplastics. They may either be egested with faeces, transferred to predators or encapsulated within sinking carcasses (Figure D. 2: blue pathways on the right-hand side).

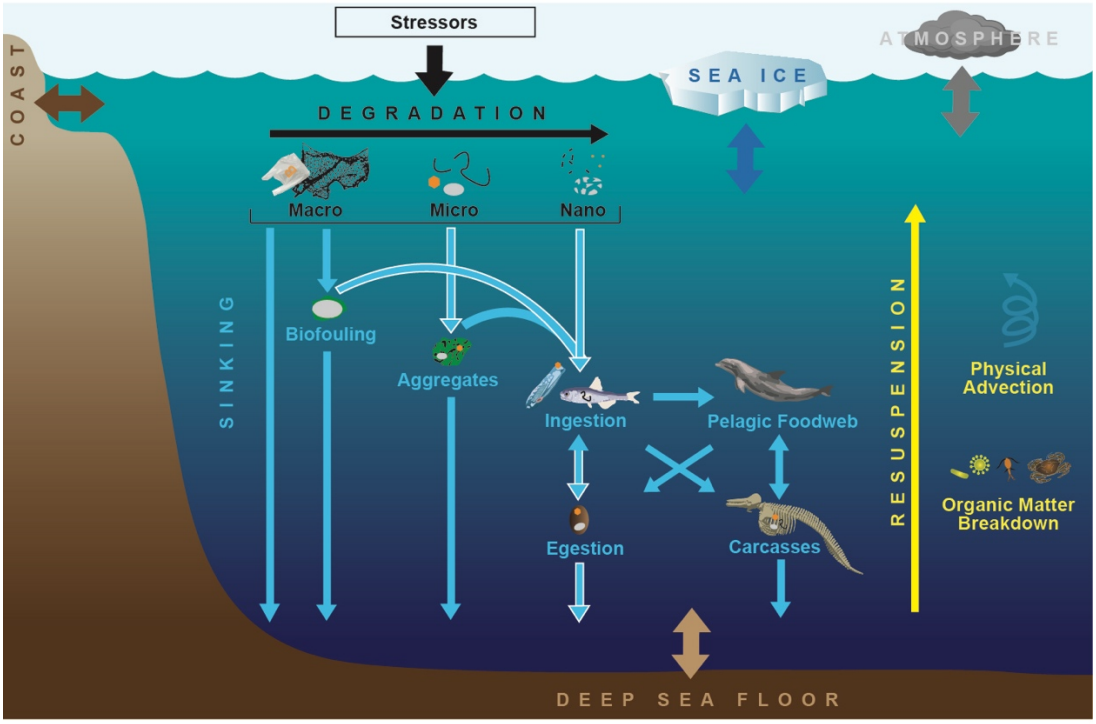


**Figure D. 2:** Microplastic pathways in the marine pelagic environment. Black arrows indicate the degradation process within the pelagic; light blue arrows pathways which facilitate the downward flux and bioavailability of microplastics and yellow arrows those which facilitate resuspension. The double-sided thick arrows represent interconnectivity to other environments. White contours mark which pathways have been revealed within this thesis. ©Alina M. Wieczorek

## Discussion

To what degree shape influences the transport of microplastics depends on the size of the microplastic fragment. It has for instance been demonstrated that in terms of settling velocities shape is more important for larger microplastics (Khatmullina and Isachenko, 2017). Smaller microplastics have been described to be more abundant at depth (Dai et al., 2018; Reisser et al., 2015) and thus may be more prone to vertical transport. This can be explained in a number of ways: One of them is that smaller microplastics have lower rise velocities (Reisser et al., 2015). Another reason is that small microplastics have a larger surface to area : volume ratio which makes them more prone to develop a biofilm sufficient to cause them to sink (Dai et al., 2018). Combined, these and other such factors may lead to a size-selective distribution of microplastics. Interestingly, Filella (2015) proposed that microplastics may possess a particle size distribution which fits the power-law relationship of natural suspended particles. She also argued that, in theory, small particles will coagulate, medium ones remain longer in the system and larger ones will sink. In fact, coagulation and interactions with compact inorganic colloids, large rigid biopolymers and humic-like substances have in the past been widely understudied (Filella, 2015). Results presented within the third chapter of this thesis indicate, however, that coagulation may indeed be an important mechanism determining the fate of the smallest plastic size fraction. Finally, concerning polymer size, it is worth considering that there is a current trend in the industry to produce rapidly degrading plastics which are often marketed as a biodegradable solution to combat plastic pollution. However, some of these polymers types, such as oxo-biodegradables, merely break down at a faster rate than standard polymers and this may lead to an increase of the smallest size fraction of microplastics in the ocean (Kubowicz and Booth, 2017).

The last key characteristic important to consider when investigating pathways of microplastics in the pelagic marine environment is density. Density is a robust first denominator of microplastic position within the water column and can determine whether it is buoyant and remains at the sea surface or whether it is likely to sink. Evidence of this is given by Chubarenko et al. 2016 who modelled movements of different types of particles in the Baltic and found that low-density foam microplastics have the potential to move across the surface at a speed four times that of surface currents. This would explain why positively buoyant particles have such a wide distribution. Slightly buoyant particles, on the other hand, may be more submerged and be subjected to biofouling which may increase their density and initiate vertical transport (Chubarenko et al., 2016). And yet even more dense

particles may start to settle by themselves. Density may also play an important role in the preferential ingestion of one microplastic type over another as indicated in Chapter 2. Here, the more dense microplastics were preferentially ingested by salps. This could be explained by the fact that the denser plastic may have been more mixed within the water and thus be more available for ingestion. Despite being denser they also caused a greater decrease in sinking speed. However, as the microplastics were also pre-exposed to allow for biofilm formation it may be that one type of microplastic more readily accumulated a biofilm compared to the other. In fact, evidence exists suggesting that different types of microplastics accumulate different biofilms and that amount and composition differ with the environment (Kaiser et al., 2017; Oberbeckmann et al., 2015). It is thus important to emphasise that not one single characteristic will determine a microplastic's pathway, but rather a combination of many, as well as environmental factors.

In terms of environmental factors, organic and inorganic matter content of the seawater are very important. For coagulation to occur, sufficient matter needs to be suspended in the seawater. This is supported by findings presented in Chapter 3 as coagulation of micron sized particles seems to have occurred at only one station. At this station, water was sampled closer to the surface where colloids are more abundant. The importance of organic matter content is further emphasised in a study by Kooi et al. (2017) who modelled the vertical movement of different sizes of microplastics in response to biofilm formation. Their models reveal oscillation patterns of initial sinking followed by re-surfacing, with oscillation frequency being dependant on size. They attribute these oscillation patterns to algae collision rates, growth, mortality and respiration which are all dependent on the position in the water column (Kooi et al., 2017). Likewise, physical factors play an important role. Despite the common understanding of gyres being accumulation zones of microplastics, further evidence is given in Chapter 1 that small-scale current features such as eddies may also aggregate microplastics. Khatmullina and Chubarenko (2019) and Filella (2015) rightfully point out that much can be learned about microplastic pathways when considering present knowledge of particle movements within our oceans (Khatmullina and Chubarenko, 2019). For instance, it is known that turbidity can strongly influence particle behaviour. In calmer waters, small microplastics, in particular, may readily sink, whereas in turbid waters they may remain in suspension (Filella, 2015). Turbidity, generally caused by mixing water masses, also dictates collision rates of particles. Indeed, when these were considered within encounter-rate calculations carried out in the context of Chapter

3, it became evident that rather than ingestion by microzooplankton, coagulation with natural submicron particles was a much more likely pathway for nanoplastics.

Two very interesting studies to consider in terms of physical factors acting upon particles in this context are those of MacIntyre et al. (1995) and Croot et al. (2007) who describe that marine snow and surfactants accumulate at density discontinuities in the water column. These areas may, therefore, present a hot spot for particles and organic exudates, both of which are likely to interact with microplastics (Galloway et al., 2017). MacIntyre et al. (1995) see a potential reason for this in the turbidity caused by the mixing of the water masses. However, they further note that timescales of these events are short and thus hypothesise that accumulated particles may have lower densities than previously considered and that large flocs may have disaggregated due to turbulence and resulted in smaller and slower-sinking particles. In the context of these findings, results presented within Chapter 2 may have far-reaching implications for microplastic pathways in the pelagic environment and the cycling of organic matter in general. The presented results show that once microplastics were incorporated into salp faecal pellets they caused a significant reduction in density and sinking speed. Such reductions could cause microplastic enriched particulate organic matter and microplastics possessing a biofilm to accumulate in these density discontinuities and the organic matter content to fragment there. This indicates that microplastic pathways in the pelagic are unlikely to be unidirectional within the water column. It has often been considered that processes acting upon microplastics mostly facilitate their downward transport (Figure D. 2: blue pathways) and that the seafloor is the ultimate sink of microplastics (Andrady, 2011; Woodall et al., 2014). However, it is important to also consider processes facilitating the accumulation and upward transport through resuspension (Figure D. 2: yellow pathways). Advection by internal waves, for instance, may cause such resuspension (Ballent et al., 2013). Another process facilitating resuspension is the breakdown of organic matter surrounding the microplastic. This can happen when bacteria colonise microplastic-enriched particles or those which possess a biofilm, and feed upon the organic matter. This is a common phenomenon observed in natural sinking particles (Turner, 2002) but has also been reported in the environment for microplastics (Ye and Andrady, 1991). Alternatively, organic material could also be broken down by removal or through ingestion and egestion by organisms throughout the water column and at the benthos (Rummel et al., 2017).

**Table D. 1:** Intrinsic properties, physical and biological factors acting upon microplastics in the pelagic environment.

| | *Which* | *Effect on* |
|---|---|---|
| ***Intrinsic Properties*** | Chemical makeup (additives, amount of monomers) | Photooxidation<br>Thermooxidation<br>Physical fragmentation (cracking, ablation)<br>Biological breakdown (hydrolysis by bacteria)<br>Absorbance of pollutants from the environment |
| | Shape | Settling velocities<br>Bioavailability<br>Horizontal movement |
| | Size | Settling velocities<br>Bioavailability<br>Biofilm formation |
| | Density | Settling velocities |
| | Colour | Ingestion by predators |
| ***Physical Factors*** | Gyres | Accumulations |
| | Eddies | Accumulations<br>Vertical transport |
| | Internal waves | Resuspension |
| | Upwelling | Resuspension<br>Vertical transport |
| | Density discontinuities | Accumulation |
| | Wind forcing | Vertical and horizontal transport |
| | Turbidity | Coagulation<br>Fragmentation |
| ***Biological factors*** | Organic and inorganic matter in seawater | Coagulation<br>Aggregation |
| | Bacterial communities | Associated organic matter breakdown |

It remains to highlight that many environmental factors act upon microplastics of all sizes and compositions (Table D. 1) which are cycled through our environments in a quite dynamic fashion rather than having ultimate sinks. While out of the scope of this discussion, one should also consider that factors governing microplastic pathways in the pelagic environment also affect its interconnectivity to other environments; these being the coast, sea ice, the atmosphere and the seafloor (Galgani et al. 2019; Bergmann et al., 2019; Dris et al., 2016; Jambeck et al., 2015; Obbard et al., 2014; Peeken et al., 2018) (Figure 2: thick double-sided arrows).

## Outlook

On the basis of the here discussed findings, I have the following recommendations for studies aiming to gain insights into abundances, effects and pathways of microplastics in the marine pelagic environment:

*Environmental Microplastic Contamination*

For future studies aiming to provide empirical evidence of microplastics contamination in marine pelagic species it will be crucial to focus efforts on economically, culturally and ecologically important species (see Figure I. 3 in Introduction). There is an urgent need to unify methodologies of microplastic extraction and analysis which has been called for several times (e.g. Stock et al., 2019). While no such widely accepted protocols exist at present, it remains to emphasise that 1) airborne contamination is a critical issue and should be mitigated at all costs through the use of air-filtration devices 2) the use of fine-meshed microplastic retention filters will allow the capture of the smaller size fraction of microplastics and 3) validation of the plastic nature of potential microplastics should be confirmed through appropriate analysis ((micro-)FTIR, Raman spectroscopy, hyperspectral imaging).

*Individual Effect Studies*

In a first instance, effect studies should consider whether an organism of interest is even likely to interact with microplastics in the environment, now or in the near future. To assess this, encounter rates should be estimated, taking into account different feeding modes, size of the organism and physical, chemical and biological characteristics of the environment it inhabits (see Table D. 1). If an organism is likely to encounter microplastics in the environment then it will make sense to proceed with exposure experiments and investigate any adverse effects. For these experiments, microplastics should be used which resemble those present in the environment. Protocols to produce such plastics exist (Cole, 2016; Coppock et al., 2019) or alternatively, they can be obtained from companies, such as Carat GmbH, specialising in their production and analysis (Chapter 2). In regards to microplastic concentrations to which organisms are exposed to, these need to be aligned with those reported in the environment but also include effect and lethal endpoints (see Introduction). Further, less direct impacts on organisms, as shown through responses by bacteria and phytoplankton in Chapter 3, should also be considered.

## Discussion

*Microplastic toxicity*

The study of toxins associated with and absorbed by microplastics present a complex field of research which does not fall in the scope of this thesis. Nevertheless, it should be emphasised that studies in this field will be of crucial importance and provide the much-needed evidence which will help to determine whether microplastics present a threat to organisms ingesting them and their predators (and the predator's predators), and as a consequence also present a threat to human health.

*Studying the impact of microplastics on POM sinking speed*

Future experimental studies investigating alterations to sinking and rising velocities of aggregates and faecal matter should aim to produce data which can feed into biogeochemical models to predict the over-arching effects of microplastics in the pelagic environment. In order to obtain viable data, experiments should include a range of microplastic types of different shapes, sizes and densities and expose them to varying physical and biological conditions (Table D. 1). For instance, this could mean exposure to high and low organic matter or prey concentrations and varying rates of turbidity. How organic matter content can impact on potential coagulation and biofilm formation rates has been demonstrated within Chapter 3, but also by Long et al. (2015) who report different effects on microplastic sinking rates for algae aggregates comprised of the two studied algal species. In many present studies, microplastics were presented to organisms alongside prey monocultures which is not representative, and this should be improved upon in future studies. As discussed above, during exposure experiments a range of concentrations should also be used, which align to those in the environment. For microplastics impacting faecal pellet sinking velocities, krill in particular, would be an interesting species to investigate in the future as their importance in carbon sequestration to the seafloor has recently been emphasised once more (Belcher et al., 2019).

For studies looking at the subsequent impacts on sinking speed of microplastic biofilm formation and their incorporation into aggregates, factors listed in Table D. 1 should be considered. Furthermore, a new device is currently being developed which will optimise the production of marine snow under laboratory conditions (Laurenceau-Cornec et al., 2019). This device is possesses features which allow for more environmentally relevant production of aggregates and also allow assessment of sinking speed and their safe retrieval for inspection. Introducing different types of microplastics into this apparatus at varying concentrations in order to assess

particle succession, makeup and sinking speed could provide some compelling insights.

*Microplastic cycling through the environment*

To investigate microplastic distribution and pathways it may be particularly interesting to sample (microplastic-)particles in areas of density discontinuities or in areas of high and low organic matter content. In order to do so researchers should utilise current methods available to study suspended particles in our seas. One promising and relatively novel tool which could be used are bottle-nets (Agusti et al., 2015). These can collect particles down to the size of 20 µm throughout the water column. Sampling can be combined with CTD-casts (CTD = conductivity-temperature-depth-probe) and bottle-nets can easily be attached to Niskin bottle rosettes. Of course, the deployment of more conventional tools such as sediment traps will also aid in providing empirical evidence of the distribution of microplastics and their association with organic matter in the water column. Lastly, in recent years some interesting new technologies have been developed to image plankton and suspended particles *in situ.* One promising example of this is the Underwater Vision Profiler 5 (UVP) which has successfully been deployed to study marine snow fall (Kiko et al., 2017; Picheral et al., 2010). While at present the UVP can only identify particles larger than 100 µm in size, with ever-improving camera chip technologies there may be great potential to utilise such methods for microplastic *in situ* assessment in the future.

## Perspective

Our planet is ~4.5 billion years old, whereas plastics have only been around for 112 years. Despite their brief existence, plastics are perceived by many as a major threat to our planet and have been proposed to be amongst the planetary boundary threats we face (Villarrubia-Gómez et al., 2018). While being durable on the one hand, plastics cycle through our environments in a highly dynamic way as has been demonstrated within this thesis. This dynamic cycling causes them to become widely distributed and makes their release into the environment irreversible (Villarrubia-Gómez et al., 2018). For this reason, and because of the vast amount of plastics which have been released into the environment (Geyer et al., 2017), plastics may represent a new, relatively stable carbon reservoir (Figure 3: $C_xH_y$) similar to calcium carbonate rocks (Figure 3: $CaCO_3$). While such reservoirs are interconnected with each other, the cycling of elements between them is limited to

the efficiency and timescales of biogeochemical processes (Garrels and Perry, 1974; Westbroek, 2010) (Figure D. 3). This is also true for the widely distributed and diverse plastic reservoir as the breakdown of plastics and flux of its building blocks into any of the other reservoirs is limited to physical, chemical and biological processes acting upon it (Shah et al., 2018). The timescales of plastic breakdown processes have many times been highlighted to be beyond that of many human generations and exceed the time plastics have been around for. As a consequence plastics have become the first stable reservoir on our planet which has been entirely shaped by humans. While at present many knowledge gaps exist which make it difficult to draw conclusions about the far-reaching environmental impacts of plastics, it should be widely recognised that society has created such a reservoir which may have unknown adverse effects in decades and centuries to come.

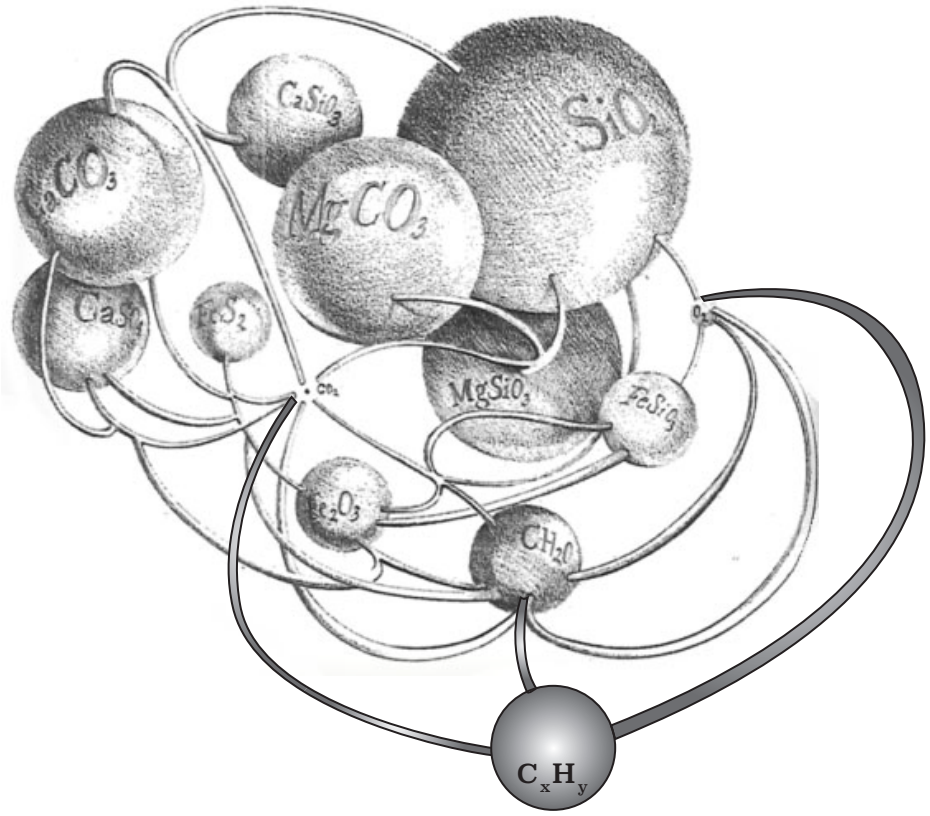


Figure D. 3: Earth's key reservoirs, including plastics as the first man-made reservoir and their interconnectivity. Adapted from Westbroek (1991).

## Discussion

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# Appendices

## Appendix 1 – Chapter 2

*Appendix 1.1: Method for UV exposure*

For UV exposure, microplastics were transferred into borosilicate scintillation vials and placed at 3.0 cm distance to a UV Pen-Ray® standard mercury lamp (190012-01). Microplastics were exposed to the mercury lamp for a total of 20 h in 1 h intervals to prevent any heating and thermodegradation of the microplastics. Temperature was measured after each hour by the use of a temperature gun and never exceeded 25 °C. According to Jacovides et al. (2009) the average daily UV flux in the eastern Mediterranean equals 0.5 MJ $m^{-2}$ $d^{-1}$. The UV Pen-Ray® standard mercury lamp emits approximately 74 µW $cm^{-2}$ (at 25 cm) and as a consequence an exposure at 1.5 cm distance from the lamp for 20 h is equivalent to 29.6 days at the sea surface of the Eastern Mediterranean. To ensure that no UVA and UVB light had been absorbed by the borosilicate glass vials, the transmission of the vials was measured using an Ocean Optics Maya 2000 Pro spectrophotometer (spectral range 165 -1100 nm) which showed that only light below 280 nm was absorbed and thus the vials were deemed suitable for the exposure experiment.

*Appendix 1.2: Derivation of the amount of microplastic in exposure experiment*

Plastic abundance data from the Mediterranean and the South Pacific Gyre for the size category 250 – 500 µm were taken from Cózar et al. (2015) and Eriksen et al. (2014). Cózar et al. (2015) report a maximum encounter rate of ~29,000 particles per $km^2$ for the Mediterranean. Eriksen et al. (2014) report maximum counts between 1,000 and 1,000,000 microplastics per $km^2$ in the South Pacific Gyre for the 330 – 1,000 µm size category. Thus 500,000 microplastics per $km^2$ was taken as a conservative estimate for the abundance of 250 – 500 µm microplastics in this region. Only one study so far has looked at the abundances of smaller microplastics (down to 53 µm) and has found a ratio of 35% smaller (53 - 250 µm) to 40% larger particles (250 – 500 µm) (Shaw & Day 1994). Even though it has been hypothesized that the ingestion by zooplankton (such as salps) may greatly reduce smaller particle numbers (Cózar et al., 2015), this ratio was taken as the best estimate at present for the smaller sized microplastics.

To work out how many microplastics a salp is likely to encounter within a 24 h period it is important to consider species-specific filtration rates. *Salpa fusiformis*

filtration rates depend on their size and whether or not they are part of an aggregate (Andersen, 1985). While the averaged sized *Salpa fusiformis* individual has been reported to filter 510 ml $h^{-1}$, Andersen measured filtration rates of 635 ml $h^{-1}$ per individual within a chain and 241 ml $h^{-1}$ per solitary individual (Andersen, 1985). An average of the three estimates was taken and used as a proxy for individual *Salpa fusiformis* filtration rates. For a 24 h period, 20 salps were thus estimated to filter 221.76 L $d^{-1}$ $(20\ salps)^{-1}$.

When measured using a Manta trawl, microplastic abundances are commonly given in $km^2$. Assuming that the highest concentration of plastics can be found in the top centimetre of the water an abundance per volume was estimated as follows:

$$1\ km^2 = 1\ 000\ 000\ m^2\ \times\ 0.01\ m = 10\ 000\ m^3$$

Using the average filtration rate, the likely microplastic encounter rates for 20 salps over a 24 h period was calculated as:

$$\frac{MP's\ km^2}{10\ 000\ m^3\ \times\ 0.22176\ m^3\ d^{-1}(20\ salps)^{-1}} = MP\ encounter\ d^{-1}(20\ salps)^{-1}$$

Estimated plastic encounter rates for the different scenarios are listed in Figure 2. 2: A.

*Appendix 1.3: Calculation of faecal pellet density*

It was assumed that the ellipsoidal pellets are roughly spherical in shape and an average of two axes measurements taken 90 degrees to one another was used as a diameter estimate. The unitless Reynolds number (Re) of the sinking faecal pellets was calculated using the following equation:

$$\mathrm{Re} = \frac{\mathrm{p_f dv}}{\eta}$$

where $p_f$ is the fluid density (1027.398 kg $m^{-3}$) and η the viscosity (0.0012563 kg $m^{-1}$ $s^{-1}$) computed from average temperatures and salinity taken from: www.st.nmfs.noaa.gov. The diameter and fluid flow speed of each pellet are given

as d (m) and v (m $s^{-1}$), respectively. Reynolds numbers of the faecal pellets lay between 0.3 and 103.6 and as a consequence the Newton-Rittinger equation was used to infer the densities of the pellets from their sinking speed:

$$v^2 = \frac{4}{3C_d}\left(\frac{p_s - p_f}{p_f}\right)d_g$$

OR

$$p^s = \left(\frac{v^2 3C_d}{4dg}\right)p_f + p_f$$

Where $p_s$ is the density of the particle (kg $m^{-3}$), g is gravity acceleration (9.8 m $s^{-2}$) and $C_d$ is the drag coefficient obtained for each faecal pellet by applying its Reynolds number to the following equation [6]:

$$C_d = \frac{24}{Re}(1 + 0.27Re)^{0.43}$$

For the above equation, a drag coefficient of a solid sphere was assumed as the water is unlikely to flow through the faecal pellets while they sink due to their tight packaging and high density. Further, the above calculation approximates the drag coefficient of a sphere at *Re* < 100 which was assumed to be close enough to those calculated for the faecal pellets (*Re*: 0.3 – 103.6).

*Appendix 1.4: Quality assessment of general linear model fit*

GLM with Sinking Speed as the response variable and with input variables being Type (factor at two levels: “control incubation” and “effect control”) and ESD as a covariate including a possible interaction term between Type and ESD

## Appendices

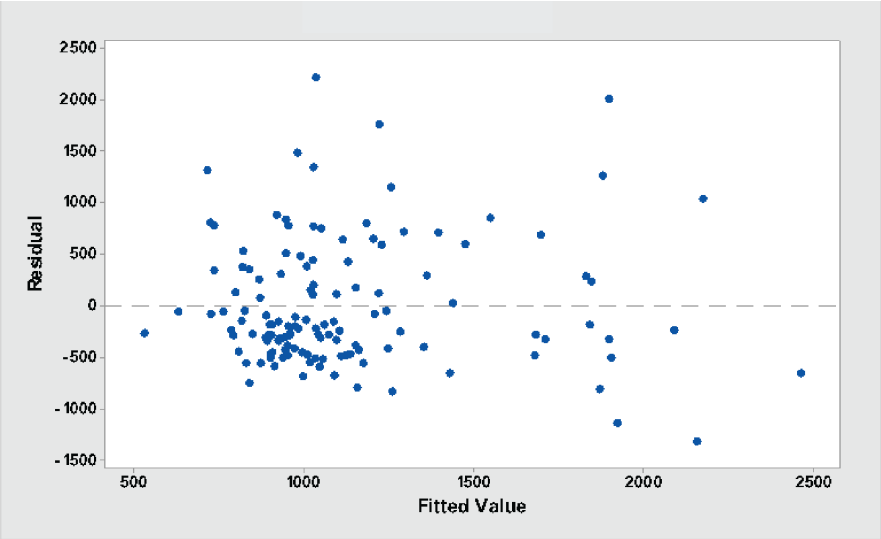


**Figure A.1. 1**: Plot of residuals versus fitted values for the GLM of Sinking Speed on Type and ESD.

**Table A.1. 1:** Root mean squared error ($s = \sqrt{MSE}$), coefficient of multiple determination ($R^2$), adjusted coefficient of multiple determination ($R^2$ adjusted) and predicted coefficient of multiple determination ($R^2$ predicted) for GLM with Sinking Speed as the response variable and with input variables being Type (factor at two levels: "control incubation" and "effect control") and ESD as a covariate including a possible interaction term between Type and ESD.

| | $s = \sqrt{MSE}$ | $R^2$ (%) | $R^2$ adjusted (%) | $R^2$ predicted (%) |
|---|---|---|---|---|
| • control incubation<br>• effect control | 622.853 | 22.99 | 23.81 | 20.45 |

GLM with Sinking Speed as the response variable and input variables being Type (factor at three levels: "control", "polyethylene" and "polystyrene") and ESD as a covariate

## Appendices

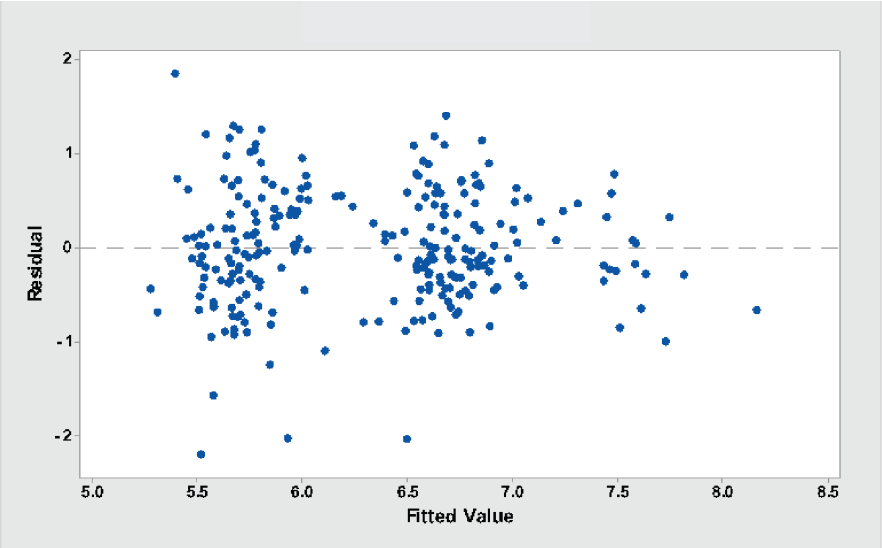


**Figure A.1. 2:** Graph of residuals versus fitted values for the GLM of LOG(Sinking Speed) on Type (at three levels) and ESD. (LOG is to base e=2.718…).

**Table A.1. 2:** Root mean squared error ($s = \sqrt{MSE}$), coefficient of multiple determination ($R^2$), adjusted coefficient of multiple determination ($R^2$ adjusted) and predicted coefficient of multiple determination ($R^2$ predicted) for GLM with Sinking Speed as the response variable and input variables being Type (factor at three levels: "control", "polyethylene" and "polystyrene") and ESD as a covariate

| | $s = \sqrt{MSE}$ | $R^2$ (%) | $R^2$ adjusted (%) | $R^2$ predicted (%) |
|---|---|---|---|---|
| • control<br>• polyethylene<br>• polystyrene | 0.614 | 50.31 | 49.7 | 48.56 |

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## Appendices

## Appendix 2 – Chapter 3

### *Appendix 2. 1: Sample time and dates*

**Table A. 2. 1:** *Sample dates and times of CTD casts in coordinated universal time (UTC) for samples taken to construct depth profiles and those used for incubations for each station.*

| | **Sample date and times (UTC)** | | | | | |
|---|---|---|---|---|---|---|
| | **Depth profile 10 m - 125 m** | | **Depth profile 150 m – 300 m** | | **Incubation sample** | |
| **Station 12** | 16.01.2016 | 21:01 | 16.01.2016 | 15:18 | 16.01.2016 | 15:18 |
| **Station 14** | 20.01.2016 | 19:20 | 21.01.2016 | 16:47 | 21.01.2016 | 16:47 |
| **Station 15** | 24.01.2016 | 02:13 | 23.01.2016 | 23:23 | 23.01.2016 | 12:56 |

### *Appendix 2. 2: Swimming motilities and sinking rates*

**Table A. 2. 2:** Swimming motilities taken from a: Visser & Kiørboe (2006), b: Jeong Hae (2007), c: Nielsen & Kiørboe (2015), d: Willey (67), e: Waterbury et al. (1981), f: Halsey et al. (69); and sinking velocities calculated for HNFs, *Synechococcus*, bacteria and FLBds.

| | **Swimming (m $s^{-1}$)** | | **Sinking (m $s^{-1}$)** |
|---|---|---|---|
| **HNF** | [a]*Bodo designis* | $3.90 \times 10^{-5}$ | $9.98 \times 10^{-7}$ |
| | [a]*Spumella* sp | $2.50 \times 10^{-5}$ | |
| | [a]*Heteocapsa triquetra* | $9.70 \times 10^{-5}$ | |
| | [b]*Cafeteria* | $1.06 \times 10^{-4}$ | |
| **Mixotrophs** | [c]*Akashiwo sanguinea* | $4.50 \times 10^{-5}$ | $5.18 \times 10^{-7}$ |
| | [c]*Dinophysis acuta* | $1.04 \times 10^{-4}$ | |
| | [c]*Lingulodinium polyedrum* | $1.05 \times 10^{-4}$ | |
| | [c]*Protoceratium reticulatum* | $1.45 \times 10^{-4}$ | |
| **Bacteria** | [a]Marine bacterium TW-3 | $4.40 \times 10^{-5}$ | $2.30 \times 10^{-7}$ |
| | [a]*Microscilla furvescens* | $3.20 \times 10^{-5}$ | |
| ***Synechococcus*** | [d]*Synechococcus* sp. | $1.40 \times 10^{-5}$ | $2.30 \times 10^{-7}$ |
| | [e]*Synechococcus* sp. -max | $2.50 \times 10^{-5}$ | |
| | [e]*Synechococcus* sp. -min | $0.50 \times 10^{-5}$ | |
| **Picoeukaryotes** | [f]*Micromonas pusilla* | $1.00 \times 10^{-4}$ | $2.30 \times 10^{-7}$ |
| | [f]*Heterosigma akashiwo* | $8.00 \times 10^{-5}$ | |
| **FLBds** | 0.76 µm FLBd | 0 | $6.89 \times 10^{-9}$ |
| | 0.5 µm FLBd | 0 | $2.81 \times 10^{-9}$ |

## Appendices

### *Appendix 2. 3: Dilution Series*

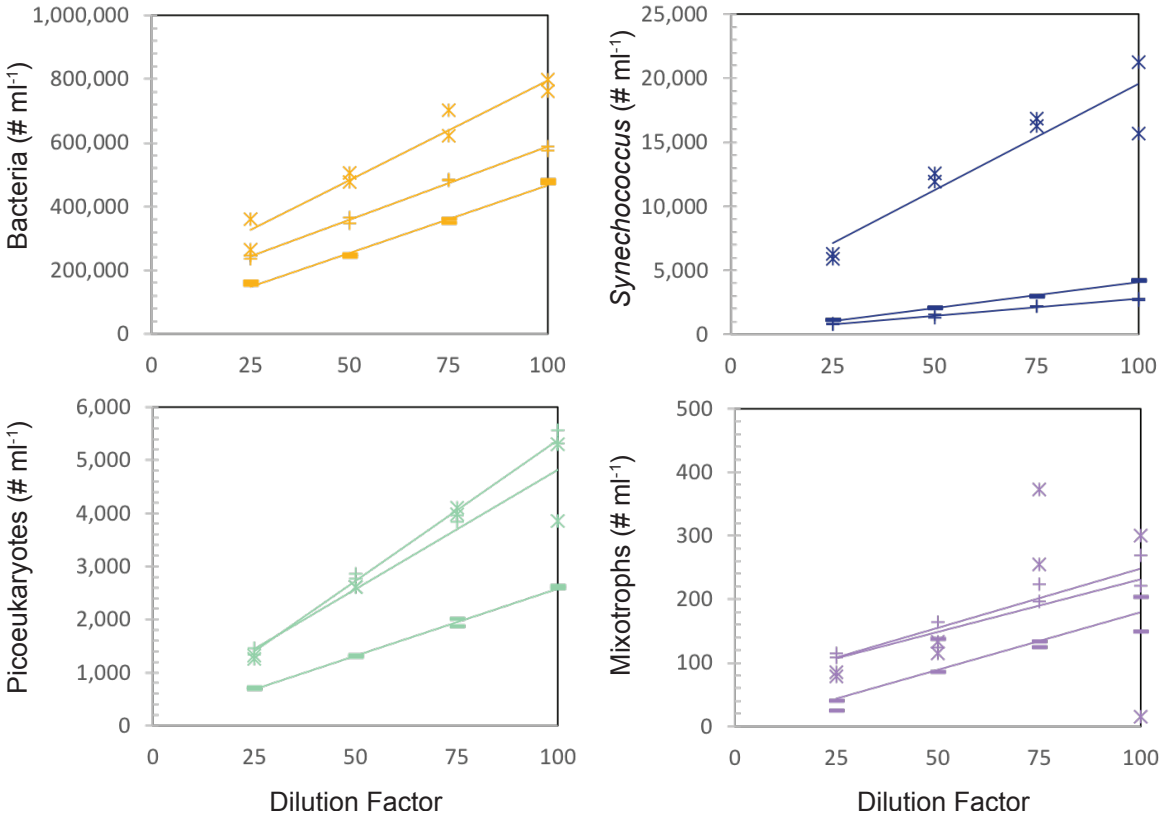


**Figure A. 2. 1:** Dilution series at start of each incubation series (t=0).

### *Appendix 2. 4: Nutrient Limitation Calculations*

**Table A. 2. 3:** Nutrient concentrations at depths from which incubation samples were taken and amount of bacterial and *Synechococcus* cells which could hypothetically be supported by these nutrient concentrations.

| | | $NO_3^-$ | $NO_2^-$ | $(PO_4)^{3-}$ | DON |
|---|---|---|---|---|---|
| **Station 12** | **Concentration (µmol/ml)** | 0.001505 | 0.000038 | 0.000284 | 0.005520 |
| | **SAR11 (cells/ml)** | 15,077,615 | 380,697 | 15,976,954 | 55301284.4 |
| | ***Synechococcus* (cells/ml)** | 30,647 | 774 | 103,576 | 112407.273 |
| **Station 14** | **Concentration (µmol/ml)** | 0.018110 | 0.000053 | 0.000323 | 0.005190 |
| | **SAR11 (cells/ml)** | 18,143,229 | 530,972 | 18,170,972 | 51995229.4 |
| | ***Synechococcus* (cells/ml)** | 36,879 | 1,079 | 117,800 | 105687.273 |
| **Station 15** | **Concentration (µmol/ml)** | 0.017664 | 0.022000 | 0.010146 | 0.004370 |
| | **SAR11 (cells/ml)** | 176,961,105 | 220,403,670 | 570,782,312 | 43780183.5 |
| | ***Synechococcus* (cells/ml)** | 359,697 | 448,000 | 3,700,306 | 88989.0909 |

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**Appendix 3 – Microplastics in the Arctic: A case study with sub-surface water and fish samples off Northeast Greenland**

# Microplastics in the Arctic: A case study with sub-surface water and fish samples off Northeast Greenland.

Silvia Morgana[1], Laura Ghigliotti[1], Noelia Estévez-Calvar[1], Roberto Stifanese[1], Alina Wieczorek[2], Tom Doyle[2], Jørgen S. Christiansen[3], Marco Faimali[1], Francesca Garaventa[1]

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**Role:** I supervised Silvia Morgana, visiting student to NUI Galway, during microplastic extraction in the laboratory and provided comments to the manuscript.

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# Microplastics in the Arctic: A case study with sub-surface water and fish samples off Northeast Greenland☆

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ABSTRACT

The Arctic is a unique and fragile ecosystem that needs to be preserved and protected. Despite its remoteness, plastic pollution has been documented in this region. In the coming years, it is likely to worsen since, with climate changes and the opening of new shipping routes, the human presence is going to increase in the whole area. Here, we investigated the presence of microplastics (MPs) in sub-surface water and in two mid-trophic level Arctic fishes collected off Northeast Greenland: the demersal bigeye sculpin, *Triglops nybelini*, and the pelagic polar cod, *Boreogadus saida*. Plastics debris were found in the water samples at a concentration of 2.4 items/m$^3$ ±0.8 SD which is higher than in most seas at lower latitudes. Both fish species had eaten MPs with different proportion among the species, 34% for *T. nybelini* (n = 71) and 18% for *B. saida* (n = 85). The significant difference in the occurrence of MPs between the two species is likely a consequence of their feeding behavior and habitat. Polyethylene was the main plastic polymer for water samples (41%, n = 17) and polyester (34%, n = 156) for fish samples as analyzed by Fourier Transformed Infrared (FT-IR) spectroscopy. Our data underscore that the Arctic regions are turning into a hotspot for plastic pollution, and this calls urgently for precautionary measures.


## 1. Introduction

Plastic pollution is an emerging and growing threat across world oceans (Andrady, 2017). Global plastics production has consistently increased over recent years and it currently stands at about 335 million tons per year (Plastics Europe, 2017). Thanks to its durability, low cost, and widespread application, plastic is a material of vast benefits to society (Andrady and Neal, 2009). Therefore, plastics production is likely to increase even further (Andrady, 2017).

Once in the environment, plastics tend to break down into smaller debris, namely microplastics (MPs). Most commonly, MPs have been defined as synthetic organic polymer particles, less than 5 mm in size that may differ in shape, color and chemical composition (Duis and Coors, 2016). However, there is no general consensus about MPs classification, with discrepancies between studies (Cesa et al., 2017; Hidalgo-Ruz et al., 2012).

MPs can originate from different sources, according to which they are classified into 'primary' or 'secondary' (Duis and Coors, 2016). Primary MPs are used in specific personal care products (hand cleaners, facial cleaners and toothpaste) or as raw materials used for manufacturing plastic products, namely plastic resin pellets or flakes and plastic powder or fluff (Duis and Coors, 2016). Secondary MPs generally result from larger pieces that gradually break up. About 75–90% of the plastic debris in the marine environment has been estimated to originate from land-based and about 10–25% from ocean-based sources (Andrady, 2011).

Once in the oceans, plastic particles are available for ingestion by a broad range of organisms from lower trophic level to top predators (Lusher, 2015). If ingested, plastics can cause adverse physiological effects, including injury or clogging of the digestive

☆ This paper has been recommended for acceptance by Maria Cristina Fossi.

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0269-7491/

tract and false feeling of satiation (Rummel et al., 2016; Tanaka and Takada, 2016). Floating debris can also act as a carrier of non-indigenous marine organisms such as bacteria, algae and invertebrates (Kiessling et al., 2015) that can be transported over long distances, thereby posing a threat to marine biodiversity and to the food web. Moreover, plastic polymers are often mixed with potentially toxic additives (i.e. dyes, POPs, flame-retardants and softeners used in plastic products), therefore MPs ingestion and the uptake of associated chemicals can cause adverse physiological effects, still largely uninvestigated (Rochman et al., 2016).

Over the last decade, the impact of plastic pollution has received growing attention, as an emerging threat for the marine ecosystem (GESAMP, 2015), while monitoring plastics in the environment is requested by the EU as part of the process to achieve good environmental status by 2020 (Marine Strategy Framework Directive, 2008/56/EC, Galgani et al., 2010).

Therefore, in recent years, studies about the occurrence of MPs have multiplied, and the presence of MPs has been documented in the oceans worldwide (Tekman et al., 2017a). Signs of plastic pollution have been detected in different environmental compartments of the Arctic region, despite its remote location, away from human activities. Cózar et al. (2017) reported high concentrations of floating plastics in the northernmost and easternmost areas of Greenland and Barents Sea. Buoyant plastics were recorded during ship and helicopter observation surveys in the Barents Sea and Fram Strait (Bergmann et al., 2016). A citizen-project study reported macroplastics on beaches of the Svalbard Arcipelago (Bergmann et al., 2017a). In 2018, MPs were identified in sub-surface waters of the Arctic Central Basin (Kanhai et al., 2018). Macro and microplastics have been found in deep sediment of the Fram Strait (Bergmann et al., 2017b; Tekman et al., 2017b). Regarding marine organisms, a few top consumers, including the Greenland shark *Somniosus microcephalus* (Nielsen et al., 2013), cetaceans (Finley, 2001; Lowry, 1993; Martin and Clarke, 1986), and sea birds (Amélineau et al., 2016; Provencher et al., 2010; Trevail et al., 2015) have been documented to have ingested plastic debris. Prior to our study, MPs have been reported in the stomach of juvenile polar cod *Boreogadus saida* specimens sampled from underneath the sea ice in the Eurasian Basin and in open waters around Svalbard (Kühn et al., 2018).

To date, little information is available on marine plastics pollution off Northeast Greenland, where anthropogenic litter on the seafloor (Schulz et al., 2010) and floating plastic debris have been reported (Christiansen et al., 2016). However, no systematic data are yet published.

Filling the gaps in the knowledge on plastics pollution in Arctic areas where information is poor or lacking is a priority to set baseline data, which are not only functional to the current understanding of MP distribution in the marine realm, but also important to correctly evaluate the effects of increased human activities in the area. In the forthcoming years, with global warming, well documented in the Arctic (Proshutinsky et al., 2015) and rapidly causing a significant sea-ice retreat (Polyakov et al., 2017), the anthropogenic presence in terms of shipping, tourism, fisheries and hydrocarbon exploration and, in turn plastic litter, is expected to increase (Stephenson and Smith, 2015).

In this paper we report on the investigation of plastics pollution along the Northeast Greenland shelf break. This high Arctic stretch of sea, dominated by the southward East Greenland Current (EGC), is also connected to the Spitsbergen area through the Return Atlantic Current (RAC) and the Greenland Sea Gyre (GSG). Therefore, even if remote and scarcely impacted by humans in direct ways, this area is a potential collector for plastic litter coming from Norway and northern Europe through the Western Spitsbergen Current (WSC) and the GSG. Indeed, plastics have been found to dominate marine floating litter drifting northward (Prokhorova, 2014), while buoyant plastics from highly populated areas are accumulating in the Greenland and Barents Seas, where any further spread is hindered by landmasses as well as by the polar ice cap (Cózar et al. (2017)). An area of plastics accumulation in Arctic waters, resulting from converging currents, has been recently assumed (Van Sebille et al., 2012). Moreover, the flow of melting sea-ice into the area should not be overlooked, following recent reports by Peeken et al. of very high MPs concentrations in landfast ice from NE Greenland (Peeken et al., 2018).

This study aims at expanding knowledge about MPs in Northeast Greenland, which is a barely studied Arctic area, where plastics debris have been recently observed, but never systematically investigated (Christiansen et al., 2016). Motivated by these sightings, we firstly evaluated the presence of plastics in sub-surface water samples. Then, we assessed plastics ingestion by two mid-trophic level fish species with different feeding models: the demersal bigeye sculpin, *Triglops nybelini* and the pelagic polar cod, *Boreogadus saida*.

## 2. Materials and methods

### 2.1. Study area and samples collection

Water and fish samples were collected at selected stations (Fig. 1, Table 1) during the TUNU-VI Expedition (5–17 August 2015) onboard the R/V Helmer Hanssen (UiT The Arctic University of Norway).

Sub-surface water samples were obtained at each station through continuous intake made of brass with rubber sealings located on the drop keel in the center of the vessel, at a depth of 6 m below surface. 1000 L of seawater were passed through a stainless steel sieve (80 μm mesh). Once the allotted volume was filtered, the sieve was washed with filtered water into 500 ml **plastic**

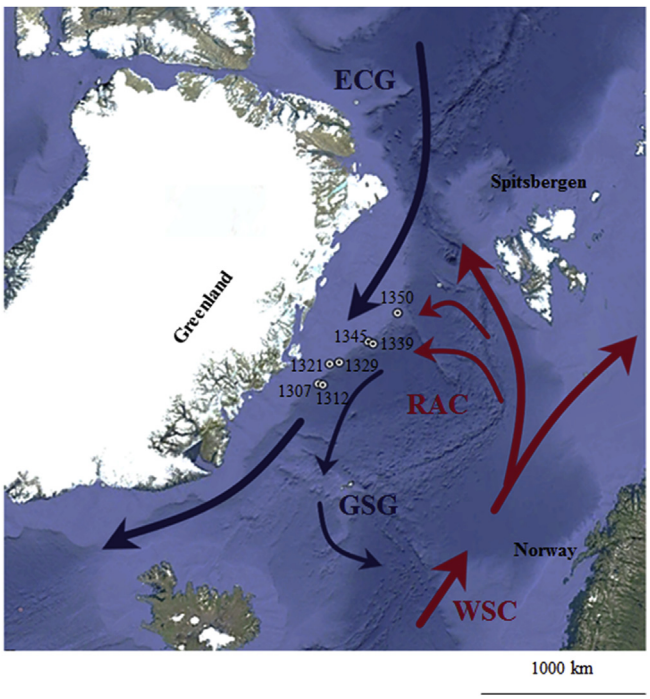


**Fig. 1.** General study area with stations (open numbered circles) sampled during the TUNU-VI Expedition August 5–17, 2015. ECG: East Greenland Current, WSC: West Spitsbergen Current, RAC: Return Atlantic Current, GSG: Greenland Sea Gyre.

**Table 1**
Station number, position (latitude and longitude), sampling date of water and fish samples.

| Station | Latitude °N | Longitude °W | Date | Water | Fishes |
|---|---|---|---|---|---|
| 1307 | 74.33 | 13.53 | 08.08.2015 | x | |
| 1312 | 74.33 | 14.08 | 09.08.2015 | x | |
| 1321 | 75.10 | 13.38 | 10.08.2015 | x | |
| 1329 | 75.23 | 12.23 | 11.08.2015 | x | |
| 1339 | 76.14 | 09.03 | 12.08.2015 | x | x |
| 1345 | 76.09 | 08.31 | 13.08.2015 | x | |
| 1350 | 77.28 | 05.50 | 14.08.2015 | x | |

**containers**, which were then stored in a −20 °C freezer until returned to the laboratory for analysis under controlled conditions.

For fish samples, two different species were investigated. The demersal bigeye sculpin, *Triglops nybelini*, and the pelagic polar cod, *Boreogadus saida*. Samples were collected from Station 1339 using a Campelen 1800/96 NOFI bottom green trawl (made of nylon and polyethylene) equipped with a cod-end inner lining of 10 mm mesh size. The trawl (door spread ~50 m) was towed for ~15 min at 280 m bottom depth at a speed of 2.3–3.4 knots. Soon after the net was back on deck, the catch was carried to a sorting room on the main deck where the fishes were rinsed, sorted and rapidly wrapped in aluminum foil in small groups of 10 specimens each and then stored at −20 °C.

### 2.2. Lab contamination control

To mitigate sample contamination in the laboratory, several precautions have been recommended in the scientific literature. All lab procedures were carried out under a laminar flow hood. Consumables were taken directly out from their packaging and all equipment was always rinsed with Milli-Q® before and after use. Fishes were rinsed in Milli-Q® water (Millipore, Bedford, USA) before the analysis. Samples and equipment were covered where possible, to minimize exposure periods. Filter blanks were run in parallel to verify contamination during both water and fish sample processing. Particles detected on filter blanks were analyzed for color, size and chemical composition and compared to particles from environmental samples in order to avoid false results. Clothing made of cotton was worn during the analysis.

### 2.3. Microplastics identification from sub-surface water samples

The water samples were analyzed for their plastics content at the CNR-ISMAR laboratory in Genoa (Italy). All samples were visually examined and sorted under a stereomicroscope (Olympus SZX7, 8x-56x) with attached digital camera (Nikon, DSL3). All potential MPs were manually sorted out and photographed. Particles were classified by shape (fiber, fragment, bead and foam), color and size (macroplastics: > 5 mm; large MPs: 5-1 mm; small MPs: 1–0.5 mm and <0.5 mm) (Imhof et al., 2012).

In order to confirm the polymeric nature of the items and identify their polymer composition, a PerkinElmer Spectrum Two Fourier Transform Infrared Spectroscopy (FT-IR) spectrometer was used equipped with Universal ATR (UATR) accessory with a 9-bounce diamond top-plate (Wave number range: 4000 and 450 $cm^{-1}$; 4 $cm^{-1}$ resolution; 32 scans). After measurement, the spectrum was compared to reference spectra through libraries supplied by Perkin Elmer, with a >70% similarity threshold.

### 2.4. Microplastics identification from fish samples

MPs presence in fishes were assessed at the National University of Ireland Galway laboratory. Plastics were investigated in 156 specimens in total: 71 bigeye sculpin *Triglops nybelini* and 85 polar cod *Boreogadus saida*. For each fish, basic sizes were recorded, including total and standard length, weight and gutted weight. Fish samples were rinsed with Milli-Q® water (Millipore, Bedford, USA) and weighed before being transferred into a borosilicate container inside a laminar flow hood (AirClean600 R: ISO class 5) where the gastrointestinal tract (GIT) was removed to be analyzed for MP content. An alkaline digestion was carried out according to Cole et al. (2014) and Wieczorek et al. (2018). Briefly, 20 mL of 1 M sodium hydroxide (Certified analytical reagent for analysis, Fisher Scientific, UK) were added to the GITs maintained in borosilicate scintillation vials at room temperature for 24 h. Post-digestion samples were vacuum-filtered onto 0.7 μm mesh filters **(Whatman glass fiber filters, 42 mm Ø, 0.7 μm mesh)** using a vacuum pump and Büchner flask. Retained biological material was copiously flushed with Milli-Q® water, then the filters were removed and analyzed for their plastic content under an Olympus SZX16 stereo microscope (Olympus, SZX16) with attached digital camera (Olympus, DP17). **Particles > 700 μm were then identified for their polymer nature using FT-IR analysis (PerkinElmer Spectrum Two).**

### 2.5. Statistical analysis

To determine the fitness of the analyzed specimens, K Fulton's Condition Factor (K) (Nash et al., 2006) was calculated as follows:

$$K = (W/L^3)*100$$

where: W = gutted weight (g); L = standard length (cm)

Statistical analyses were performed using R 3.2.2 (R Development Core Team, 2015). Significant differences were observed in the uptake of plastic items between *Triglops nybelini* and *Boreogadus saida*, and in CFs (*arcsin* $\sqrt{p}$ transformed). A *t*-test was run to assess any likely effect of plastic ingestion on fish fitness between fish with and those without ingested plastics, after testing for normality and homogeneity of variance with Shapiro-Wilk and Levene tests. When data failed to meet the previous assumptions, a Wilcoxon nonparametric test was used. Significance for all statistical analyses was determined at $\alpha = 0.05$.

## 3. Results

### 3.1. Quality control

During all lab analyses, procedural blanks were run. For sub-surface water sample analysis, one synthetic fiber (black, polyester, 0.35 mm) was found. For fish sample analysis, one synthetic fragment (transparent, rubber, 2.77 mm) and five fibers (blue, PS, 2.8 mm; black, PET, 0.22 mm; blue, PS, 1.25 mm; red, polyester, 0.45 mm; black, rayon, 0.45 mm) were identified. Items isolated from environmental samples with similar color and polymer to those found in the procedural blank were not considered. Two green PA fibers were isolated from fish GITs, but these were supposed to be residues of the trawl nets used for sampling. Consequently, also these items were excluded from the total count of detected MPs (Supplementary Tables S1, S2, S3).

### 3.2. Sub-surface water samples

Plastic items were found at all stations sampled. The number of MPs found in water samples was expressed as items/$m^3$. A high number of fibers was found, ranging from $4*10^4$ to $6*10^5$ items/$m^3$, but these items were not included in the final estimates of plastic

abundance since samples were likely to have been contaminated during onboard operations. The abundance of plastic fragments ranged between 1 and 3 items/$m^3$, with a mean value of 2.4 items/$m^3$ (±0.8 SD) (Fig. 2) (Fig. 3).

With an average size of 1.6 mm, MPs in the investigated water samples accounted for 94% of the total (n = 17). Small MPs amounted to 59% of the total amount of isolated items (n = 17). Most of them belonged to the <0.5 mm class size (Fig. 4A). Considering fragment color, blue (29%, n = 17) and white (23%, n = 17) were the most abundant colors (Fig. 4B). Following FT-IR analysis, sample fragments (n = 17) were composed as follows: 41.2% polyethylene, 23.5% polypropylene, 11.8% polyvinyl chloride (PVC), and <6% polystyrene, ethylene-vinyl acetate, polyurethane and polyamide, respectively (Fig. 4C). Other identified materials included chitin, cellulose and rayon, which however were not included in the estimate. Detailed data are available in Supplementary Table S1.

### 3.3. Fish samples

Plastic items were detected in 25% of the examined fish samples (n = 156) but with different proportions depending on the species – i.e., 34% for bigeye sculpin *T. nybelini* (n = 71) and 18% for polar cod *B. saida* (n = 85) (Table 2). Ingestion by demersal sculpin was significantly higher than in the pelagic polar cod (W = 2554, n = 156, p = 0.02835). No *T. nybelini* had more than one plastic items, whereas one to two items (an average of 1.1 (±0.3)) were found in the digestive tract of *B. saida*. The K values of fishes that had and had not ingested plastic items did not significantly differ for both investigated species (*T. nybelini*: *t*-test t = 0.11932, df = 37.66, p = 0.906; *B. saida*: *t*-test t = −1.066, df = 16.082, p = 0.304).

Fibers were the main identified plastic shape (88%, n = 41) and blue was the dominant color (49%, n = 41) with an average length of 1.6 mm (Fig. 4D and E). Large MPs (41%, n = 41) were the main size class recorded in fish samples followed by small 0.5–1 mm size MPs (27%, n = 41). Only >700 μm particles were subjected to FT-IR analysis (corresponding to > 73% of total items). **Particles lower than 700 μm (11 items) were considered as plastic.** Following FT-IR spectrophotometry, the recorded items were shown to derive from a variety of polymeric materials: polyester (34%, n = 30), acrylic (24% n = 30), polyamide (21% n = 30), polyethylene (17% n = 30), ethylene-vinyl acetate (EVA n = 30) (7%) (Fig. 4F). A summary of the main plastic characterization output species is shown in Table 3, whereas detailed data are available in Supplementary Table S2.

## 4. Discussion

This study reports on the presence of plastics in both sub-surface water and in fish species off Northeast Greenland, an area covered by sea ice for most of the year and still spared from fisheries and other human activities (Christiansen et al., 2016). Here, sub-surface water samples were found with plastics with an average abundance of 2.4 items/$m^3$ ± 0.8 SD. Most of the items (94%) were smaller than 5 mm, thus confirming MPs as the most abundant type of marine debris in sea water (Collignon et al., 2012; Desforges et al., 2014; Ivar do Sul et al., 2013; Lusher et al., 2014; Moore et al., 2001; Reisser et al., 2013; Thompson et al., 2004). The average estimate of plastic abundance in sub-surface waters reported in our study is similar to what was previously reported for southern locations off East Greenland (2.38 item/$m^3$), where water sampling was performed with 100–500 μm plankton net and plastics analyzed in water samples sieved on a 500 μm mesh (Amélineau et al., 2016). It is also similar to what was reported for sub-surface waters out of the Svalbard archipelago (2.68 items/$m^3$ ± 2.95 SD), sampled through continuous onboard seawater pump (Lusher et al., 2015).

In contrast to our mean value, the MPs content was very high in sea ice cores (4.5*$10^6$ items/$m^3$, Peeken et al., 2018) and deep sea sediment (>4300 items/kg, Bergmann et al., 2017b) in nearby regions. Even though sea ice and sediment are known to act as accumulation areas for MPs (Peeken et al., 2018), the different sampling and lab techniques used (e.g. 80 μm mesh size of sieve, visual inspection and sorting under stereomicroscope) might also explain the above discrepancy.

A huge amount of fibers was found at all stations, ranging from

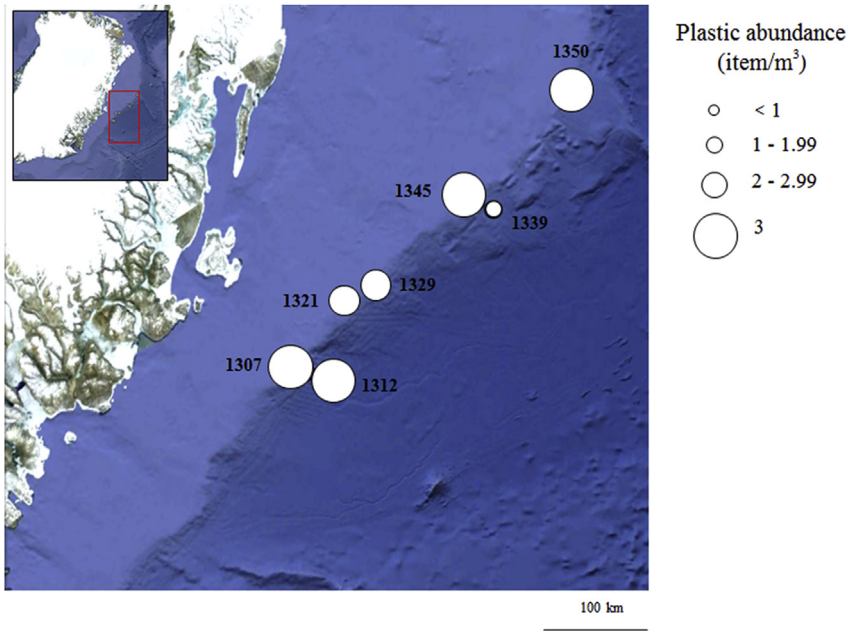


**Fig. 2.** Abundance of plastic fragments (items/$m^3$) at each sampling station.

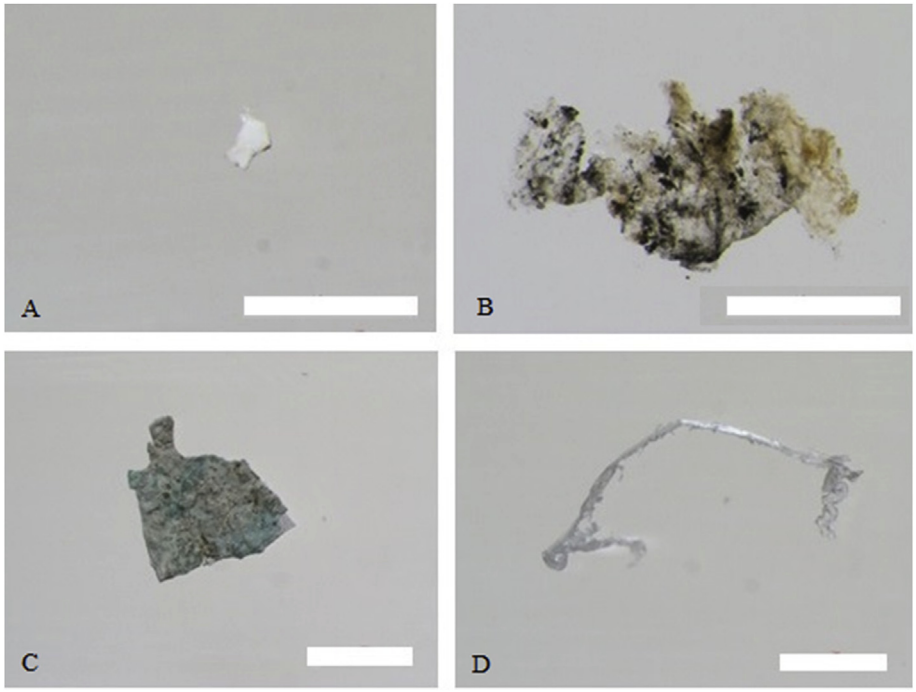


**Fig. 3.** Example of microplastics isolated from water samples and analyzed using FT-IR spectroscopy. A) white PVC fragment; B) PE-degraded fragment; C) grey/blue PE fragment; D) transparent polyamide fragment. Scale Bars = 1 mm. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

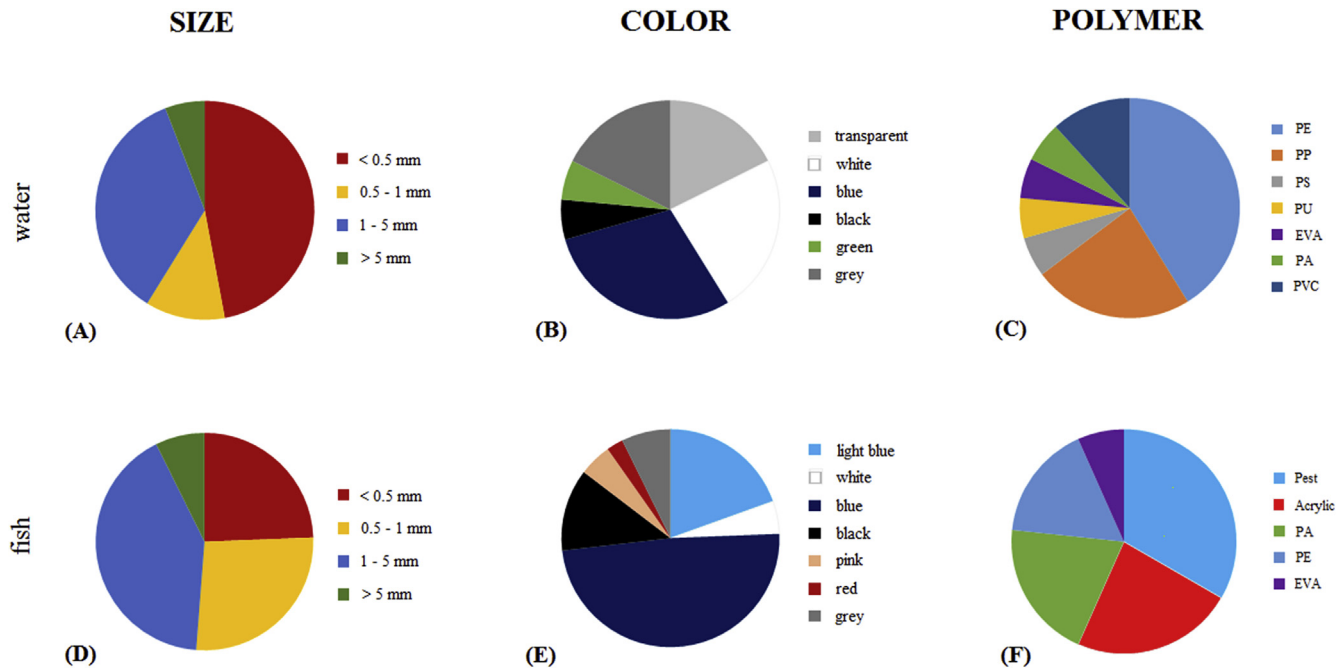


**Fig. 4.** Distribution of plastic size (A, D), color (B, E) and polymer (C, F) in water and fish samples. PE: polyethylene, PP: polypropylene, PS: polystyrene, PU: polyurethane, EVA: ethylene-vinyl acetate, PVC: polyvinyl chloride, Pest: polyester, PA: polyamide. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

**Table 2**
Fish data: species, sample size, average weight (W) and standard length (SL) and corresponding plastic ingestion as individual counts and percentages.

| Species | Sample size | AvgW (±Stdev) (g) | AvgSL (±Stdev) (mm) | Individual with ingested MPs | Overall % of ind. with ingested MPs |
|---|---|---|---|---|---|
| *T. nybelini* | 71 | 7.3 (±3.2) | 81.91 (±9.5) | 24 | 34 |
| *B. saida* | 85 | 8.5 (±3.3) | 97 (±11.5) | 15 | 18 |

**Table 3**
Mean length, shape, color and polymer of plastic items isolated from fish GITs.

| | Bigeye sculpin *T.nybelini* | Polar cod *B. saida* |
|---|---|---|
| Mean length (mm) | 1.4 | 1.8 |
| Main shape | Fiber (83%) | Fiber (94%) |
| Main color | Blue (58%) | Blue (35%) |
| Main polymer | Polyester (35%) | Polyester (31%) |

$4^{*}10^{4}$ to $6^{*}10^{5}$ items/$m^3$. Such a high quantity may be an indication of air-borne contamination. Several precautions were adopted during lab analysis (Wesch et al., 2017), thus making sample contamination during this step most unlikely, as also confirmed by procedural blank (Supplementary Table S1). Conversely, conditions during water sample collection were poorly controlled. Therefore, fiber contamination may have occurred during onboard procedures, which are impossible to quantify since no field blank was run. In the light of our experience, a better procedural check also during sampling activities is strongly recommended in any future studies. Fibers were excluded from our final MPs estimates in water samples. Nevertheless, the actual presence of fibers in the sampling area cannot be excluded, as fibers belong to a well-documented MP type found in the Arctic region (Barrows et al., 2018; Lusher et al., 2015) and in the marine environment in general (Cesa et al., 2017).

The mean MPs concentration reported for the Arctic region is higher than those in the North Pacific (0.116 items/$m^3$) (Goldstein et al., 2012), North Atlantic (0.01–0.04 items/$m^3$) (Thompson et al., 2004), and it is comparable to that recently reported by Suaria et al. (2016) for the Mediterranean Sea (3.13 items/$m^3$). All these areas are known to be hotspots for MPs accumulation. Our finds support the idea that the Arctic Ocean is indeed a hotspot for plastic pollution (Van Sebille et al., 2012).

A marine debris accumulation area may have developed due to ocean current dynamics (Bergmann et al., 2016; Christiansen et al., 2016), fed by anthropogenic debris drifted through the northward WSC and the southward EGC. Plastic debris is likely to come from the highly MP-contaminated North Atlantic region (Lusher et al., 2014) and riverine Siberian and Canadian discharge, whose plastic input is still unknown (Peeken et al., 2018). Furthermore, sea ice melting may contribute to plastic debris accumulation in this region. Indeed, very high quantities of plastic items have been reported to be trapped in the Arctic sea ice (Obbard et al., 2014; Peeken et al., 2018) which could be released, because of sea ice melting, into the underlying water column, thus increasing the levels of plastic pollution in the area. In light of sea-ice loss and the concomitant increase in human activities within the Arctic Ocean proper (Hoegh-Guldberg and Bruno, 2010; Proshutinsky et al., 2015; Christiansen, 2017) the accumulation of plastics is likely to be further strengthened.

This study documents plastic ingestion in two species of Arctic fishes, i.e. 34% of the bigeye sculpin *Triglops nybelini* and 18% of polar cod *Boreogadus saida* are contaminated. To date, the ingestion of plastics debris by marine animals from Arctic regions has been reported for a few top consumers, including the Greenland shark *Somniosus microcephalus* (Nielsen et al., 2013), cetaceans (Finley, 2001; Lowry, 1993; Martin and Clarke, 1986), sea birds (Amélineau et al., 2016; Lydersen et al., 1989; Provencher et al., 2010; Trevail et al., 2015) and the polar bear (Bergmann et al., 2017a). Our study reveals the presence of synthetic materials in the digestive tract of two Arctic mid-trophic level fish species, *B. saida* and *T. nybelini*. Both species are distributed in Arctic waters (Johannesen et al., 2017). The polar cod is frequently associated with the sea-ice (Ponomarenko, 1968), where MPs are particularly abundant (Obbard et al., 2014; Peeken et al., 2018), therefore this species can be particularly exposed to MP pollution. Ingestion of MPs can be affected by various factors such as MPs concentration in the environment and foraging behavior (Ryan et al., 2016). MPs can be taken in by fish directly, confusing plastics with prey, or indirectly by eating prey contaminated with MPs. The bigeye sculpin and the polar cod are mid-trophic level fish species that play a key role in the ecosystem by conveying energy from lower to higher trophic levels (Bowman et al., 2000; Christiansen et al., 2012; Christiansen, 2017). The herein documented MP ingestion by species that prey on zooplankton and are regularly consumed by top predators (Kühn et al., 2018) turns them into potential carriers of plastics in Arctic food webs.

Ingestion of MPs by polar cod has been recently documented by Kühn et al. (2018), reporting 2.8% of fish with non-fibrous plastic particles in their stomach. According to these authors, most synthetic fibers they found derived from secondary contamination, due to poorly controlled analytical procedures.

When working on MPs, fiber contamination of samples is a potential problem that needs to be addressed by specific protocols. In our study, most MPs isolated in fish GITs were fibers (88%), which we exclude to derive from secondary pollution because a series of measures and methods recommended in the scientific literature were strictly followed (Wesch et al., 2017). Due to the use of controls, we were able to identify items derived from background contamination (Foekema et al., 2013), which were excluded from the total count of detected plastic items. Furthermore, feeding while in fishing nets has been documented as a likely source of bias (Davison and Asch, 2011; Rummel et al., 2016) that may affect results. In this study, we may assume that net feeding occasionally occurred. Only 2 out of the 156 analyzed fishes (1.3%) were found with ingested green PA fibers which most probably derived from the net used during fishing and consequently these specific fibers were not included in the final MP estimate. Therefore, we can reasonably assume that fibrous-plastic particles identified in this study had actually been ingested by fish.

Synthetic fibers are a form of plastics frequently found in the guts of several marine animals, including fishes (Ryan et al., 2016). The occurrence of fibers has been reported in both pelagic and demersal fishes from the English Channel (Lusher et al., 2013), commercial Atlantic and Baltic fishes (Beer et al., 2017; Neves et al., 2015), and intertidal species showing distinct feeding habits (Mizraji et al., 2017). Most of the fibers found in the investigated species, including fish larvae from the western English Channel (Steer et al., 2017) were blue. Ory et al. (2017) in a Pacific fish (*Decapterus muroadsi*) observed a selectivity for blue MPs that are similar in color to the blue-pigmented copepod species they commonly prey upon, suggesting that fishes may ingest MPs resembling their natural prey. The prevalence of specific colored items in stomach contents reveals an interesting feeding behavior that deserves further investigations under controlled laboratory conditions.

Although many studies have documented the presence of plastics in the gastrointestinal tract of fishes (Foekema et al., 2013; Possatto et al., 2011; Rummel et al., 2016; Sanchez et al., 2014), little is known about the effects. In our study, the condition factor (K, used as a proxy for fitness) did not differ between specimens with and without ingested plastics. Hence, MPs items with an average size of 1.8 mm (*B. saida*) and 1.4 mm (*T. nybelini*) seem too small to affect fish conditions by causing false feelings of satiation, intestinal blockage, or digestive tract lesions (Foekema et al., 2013). However, evaluating MP effects remains difficult, because any detected MPs in fish digestive tracts while showing that this individual fish has recently ingested some plastic, it does not mean that it is structurally more exposed than others (Foekema et al., 2013). A comprehensive evaluation of MPs effects requires long-term controlled lab experiments rather than “snapshots” from the field.

Interestingly, demersal bigeye sculpin showed a significantly higher value (34%) of ingested MPs compared to the pelagic polar cod (18%). This difference probably reflects the feeding ecology in the two species. Considering that the fibers found in the fish GITs were mainly polyester, acrylic and polyamide - all polymers with higher than seawater density - (Cesa et al., 2017; Neves et al., 2015), sediments might be assumed to act as a collector for those MPs. Further, their ingestion by fish with demersal feeding habits, like the bigeye sculpin, might be more likely than by pelagic ones, such as the polar cod. In addition, deep Arctic sea has been identified as a major sink for MPs (Bergmann et al., 2017b; Tekmann et al., 2017b) that can be ingested by marine organisms, especially those with demersal habits, thus entering the Arctic food webs and becoming available to upper trophic levels, where MPs have been reported.

FT-IR and Raman spectroscopy are fundamental in MP studies in order to confirm the chemical composition of items isolated from environmental samples. **Without these analyses, plastic abundance may be strongly overestimated and the uncertainty of pure visual identification increases with decreasing particle size** (Löder and Gerdts, 2015).

In this study, FT-IR analysis has shown different polymer types in water and fish samples. Polyethylene was found to be the most representative plastic in water samples. This is consistent with other studies from nearby regions (Peeken et al., 2018) that report PE as the most abundant polymer type, belonging to the largest product group of plastics (Polyolefin), widely used as packaging material and discarded after a short lifetime (Plastics Europe, 2017). Of all synthetic items isolated from fishes, polyester was the prevalent type, followed by acrylic (24%) and polyamide (21%). PE accounts for 17%. Polyester, commonly used in textiles and fishing gear, has already been documented in Arctic polar waters and sea ice (Amélineau et al., 2016; Kanhai et al., 2018; Lusher et al., 2015; Obbard et al., 2014). **In our view, the difference in polymeric composition between water and fish samples could be explained by the different applied techniques: water samples were filtered (80 μm mesh) and then, without treatment, visually sorted at the stereomicroscope; differently, fish samples were subjected to alkaline digestion, vacuum-filtered onto 0.7 μm mesh filters and then inspected at the stereomicroscope.** The alkaline treatment we used could have affected our results by degrading Nylon fibers, melding polyethylene fragments, and yellowing PVC granules, as reported by Cole et al. (2014). **In comparison to the treatment with strong alkaline or acidic reagents, the recent purification of MP samples with different enzymes is an efficient alternative that conserves the whole range of plastic polymers. However, this approach is more elaborate as it requires several handling steps and longer incubation times** (Löder et al., 2017).

Generally speaking, when working on MPs we should always be careful when comparing and drawing conclusions, because of the lack of standardized MP analysis procedures. In the abundant literature on marine plastics pollution, several sampling and lab methods have been described (Hidalgo-Ruz et al., 2012; Löder and Gerdts, 2015; Mai et al., 2018 Shim et al., 2017). Our study confirms MP presence in the Arctic region, where a sixth plastic gyre has been supposed to exist. It also highlights how difficult it is to make spatial or temporal comparisons. In the absence of standardized protocols, any statements should always be carefully formulated, in order to avoid biased plastics pollution estimates and wrong assumptions about their alleged impact on marine organisms.

## 5. Conclusion

Our study underscores that plastic pollution is a real issue for the still under-investigated Arctic regions and we document that MPs are actually eaten by mid-trophic level fishes such as bigeye sculpin and polar cod. Plastic fibers have been observed in both water and fish samples. Nevertheless, plastic fiber presence in water samples could be due to air-borne contamination during sampling procedures. Conversely, fibers isolated from fish GITs are unlikely to originate from secondary pollution (airborne contamination, net feeding). Therefore, when working on MPs, a careful data interpretation as well as standardized methods during sampling and lab activities are mandatory; the need for unique and standardized protocols is strongly stressed. The present study highlights the vulnerability of the Arctic Ocean, which is not immune to the plastic threat, despite its remoteness and absence of direct human impacts.

## Acknowledgements

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## Appendix A. Supplementary data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.envpol.2018.08.001.

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