Exposure to low-density polyethylene microplastic particles: presence in *Mytilus edulis* tissues and pseudofeces

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Master thesis in marine biology

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Spring 2020

Acknowledgements

The work presented in this master thesis was conducted at NIVA Solbergstrand, the Department of Biosciences at the University of Oslo (UiO) and the Institute of Marine Research in Bergen (IMR).

First, I would like to thank my supervisor Ketil Hylland (UiO) and my co-supervisor Bjørn Einar Grøsvik (IMR). Ketil, thank you for agreeing to be my supervisor despite already helping plenty of other master students, and for helping me with my wish to do "something with microplastics". I am so thankful for your patience, your help with the practical work at NIVA Solbergstrand and your excellent feedback- it has been very valuable and highly appreciated. Bjørn Einar, thank you for your always positive outlook, your great reflections and feedback, support and welcoming me to IMR in Bergen with open arms.

I want to thank the amazing people who helped me at IMR in Bergen. Lisbeth Sælemyr, I am very glad we met at the Microplastics course in Bergen two years ago. You have been a great discussion partner and a great help at IMR, arranging for me to work in the histology lab. Of course, in addition to always being supportive and encouraging. Stein Mortensen, I am so grateful for all the exceptional knowledge of mussels you shared with me, your continuous help and feedback with writing, and your amazing illustrations. Ingrid Fiksdal, thank you for making me feel welcome in the histology lab, your positive feedback and leading the way from a lump of tissue to observing plastic particles through a microscope. Dawit Berhe Ghebretnsae, thank you for teaching me how to use the microtome and your always good mood. Anders Thorsen, thank you for your help with the fluorescence microscope- the amazing Nile Red photos would not exist without you.

I would also like to thank the staff at NIVA Solbergstrand, who were always friendly and accommodating, and especially Pål Fasseland, who "fed" my mussels with plastic and algae while I was counting salmon lice in Namsos.

I also want to give a big thank you to Jan Heuschele for getting engaged in my project and helping me with statistics, even on Zoom after the university closed in March. Without you I would still be lost in RStudio-help videos on Youtube.

Finally, I want to thank my parents for always encouraging my dream of becoming a marine biologist and taking great interest in my field of study, and my friends for their constant support. And of course, thank you Magnus for being you. And for letting me have the home office to myself these past months.

Abstract

The presence of microplastics in the marine environment is an emerging concern for marine life. This study investigated the presence of low-density polyethylene (LDPE) particles (20-25 µm) in the gills, intestinal lumen, digestive diverticula and pseudofeces of blue mussels (*Mytilus edulis*) following exposure, using histological procedures and two different staining and microscopy approaches. Mussels were exposed to microplastics at three concentrations (0 mg/L, 0.1mg/L and 5 mg/L) and two different concentrations of algae (0.7 mg/L and 2 mg/L) for 4 days and 56 days. A reference group not exposed to microplastics or algae was also included. Following exposure, tissues were dissected and embedded in paraffin, and histological sections were dyed using two different staining methods*;* Hematoxylin-Erythrosine-Saffron (HES) and Nile Red (NR). The HES-stained sections were examined using polarized light microscopy, and the NR sections were exposed to fluorescent light of wavelength 470 nm, using a FITC (fluorescein isothiocyanat) filter with detection wavelength of 525 nm. LDPE particles were detected in *M. edulis* tissues by the use of these procedures. The number of plastic particles in tissues was not affected by exposure time or algae concentration. The LDPE concentration in the exposure medium and matrix (gills, intestinal lumen, digestive gland, pseudofeces) affected the number of plastic particles observed. The largest number of particles were observed in the intestinal lumen, followed by in pseudofeces. Few particles were found in the digestive gland. These observations suggest that microplastics of this size and polymer type are not to a great extent translocated from the digestive system to other tissues, and that blue mussels are able to reject and discard unknown or unwanted particles as pseudofeces.

Contents

1. Introduction

1.1 The plastic predicament

Plastics are at present natural components in everyday life in all corners of the world. These materials, which did not even exist a century ago, are now seemingly indispensable. Packaging makes up 39.9% of the global total plastic demand and is unquestionably the largest end-use market (Plastics Europe, 2019). In the packaging segment, low-density polyethylene (LDPE) is in the highest demand, contributing to 17.5% of the total global plastic production (Plastics Europe, 2019). Plastic packaging is predominantly single-use, resulting in enormous amounts of plastic waste, with only 18% being recycled globally (Geyer et al., 2017). The generation of such large quanta of plastic litter has inevitably resulted in the release of plastic products into both the terrestrial and the marine environment. It is estimated that around 8 million tons of plastic debris are discharged in the ocean every year (Jambeck et al., 2015), and that 80% of plastic litter in the ocean originates from land-based sources. The slow degradation of plastic materials, and hence their persistence in the environment, has made the accumulation of plastic in nature one of the largest global environmental threats as of today (NOAA & UN Marine Debris Programme, 2011). Plastic debris has been documented in all areas of the marine environment, from the open oceans, to along coastlines, and even in remote locations and in the deep sea (Van Cauwenberghe et al. 2013; Woodall et al., 2014; Desforges et al., 2014; Fischer et al., 2015; Kim et al., 2015; Bergmann et al., 2017; Jamieson et al., 2019). Large pieces of plastic may entangle, strangle and be ingested by marine fauna (Laist, 1997; Sazima et al., 2002; Sheavly, 2005; Hofmeyr et al., 2006), destroy habitats, and smother macro algae and coral reefs (Richards & Beger, 2011; Pawar et al., 2016). However, plastic litter is also subject to fragmentation into smaller pieces, namely microplastics.

1.2 Microplastics

According to a 2016 report (Eunomia Research & Consulting Ltd), 950 000 tons of microplastics, which are plastic particles smaller than 5 mm (GESAMP, 2016), are released from terrestrial sources into the ocean every year. This is in addition to an annual estimate of 230 thousand tons of microplastic formed from degradation of macroplastic which is already in the marine environment (Booth et al., 2017). Plastic particles of very small sizes were reported in the ocean already in the 1970s (Rochman, 2018), but the term “microplastic” was first used scientifically by Thompson et al. (2004) and is now widely used and acknowledged.

1.2.1 Primary and secondary sources of microplastics

Microplastics enter the environment either from primary or secondary sources (Andrady, 2011). Primary microplastics are produced in microscopic sizes, and used in a large variety of products and processes ranging from microbeads in personal hygiene products such as face wash, scrubs, toothpaste and cosmetics (Zitko et al., 1991; Fendall & Sewell 2009; Rochman et al., 2015), to abrasive media in metal works such as plastic blasting grit for ships and offshore constructions (Song et al., 2014). Resin pellets, industrial raw materials of plastic made for molding larger plastic products (Peng et al., 2017; Talvitie et al., 2017) may also be referred to as primary microplastics, though these are sometimes categorized as mesoplastic (size 5-10 mm) (Gregory & Andrady, 2003; Isobe et al., 2014).

Secondary microplastics are derived from fragmentation of macroplastic objects, either through use, or once released into the environment. Microplastics may be by-products of products or industrial processes, e.g. plastic dust from polishing plastic items, from painting, and from maintenance work on painted metal constructions like bridges and buildings. Plastic dust may also originate from households, like textile fibers released in laundry machines and driers (Browne et al., 2011), and weathering from paint, furniture and other plastic items at home. Other sources of microplastic as inherent by-products are weathering of plastic ropes, commonly related to fisheries and aquaculture, degradation of plastic films used in agriculture, and abrasion of car tyres (Ohtake et al., 1998; Jang et al., 2014; Sundt et al., 2014; Kole et al., 2017). In the environment, plastic debris may be degraded to microplastics by UV radiation from the sun which oxidize the polymer matrix, weathering by waves and currents, and chemical oxidation (Barnes et al., 2009; Andrady; 2011; Andrady 2015; Gewert et al., 2015; Song et al., 2017). Microplastics can again be fragmented to nanoplastics of sizes smaller than 100 nm (Koelmans et al., 2015).

1.2.2 Microplastics in the Norwegian marine environment

It is estimated that 8000 tons of microplastic are released from Norwegian primary sources every year. A large proportion is likely to end up in the ocean, in addition to the microplastic being released directly into the ocean e.g. through sewage systems (Aniansson et al., 2007). Tire-dust is thought to be largest single source in Norway. There is not sufficient knowledge of the volume of release of microplastic in Norway, and hence the figures for secondary sources are uncertain. Indications are that around 10 000 tons of Norwegian macro litter enter the ocean annually, however the proportion of this being fragmented to microplastics is not

known (Sundt et al., 2014; Sundt et al., 2016). In addition to the micro- and macroplastic contributed by Norway, plastic debris is also transported from other areas, often long distances. The Norwegian coastal current, which runs along the entire Norwegian coastline, transports pollution and litter from the North Atlantic Current, the Baltic Sea and European rivers and coastal territories (Strand et al., 2015).

1.2.3 Presence of microplastics in marine organisms

The number of studies investigating presence and uptake of microplastics in marine organisms has been increasing the past ten years. The fragmentation of plastic to microplastic increases the bioavailability and risk of intake by marine organisms, both in the water column and in benthic communities (Wright et al., 2013). Due to their small size resembling that of plankton and other suspended material, microplastics are prone to be ingested by organisms ranging from small filter feeders, suspension feeders and detritivores to fish (Wright et al., 2013), and ingestion has been reported in marine organisms following both field studies and exposure experiments. Microplastics have e.g. been found in the intestine of the sea cucumber *Apostichopus japonicus* (Mohsen et al., 2019), in primary tubules of the sea scallop *Placopecten magellanicus* (Brilliant & MacDonaldt, 2000), in mesenterial tissue in the stomach of the scleractinian coral *Dipsastrea pallida* (Hall et al., 2015), in the intestine of the baleen whale *Megaptera novaeangliae* (Besseling et al., 2015), in the intestine and gills of the Korean rockfish *Sebastes schlegelii* (Yin et al. 2018), in the gill chamber of the shore crab (*Carcinus maenas*, Watts et al., 2016), and in pseudofeces of the clam *Atactodea striata* (Xu et al. 2017) and the blue mussel *Mytilus edulis* (Woods et al. 2018).

The intake and uptake of microplastic particles (MPs) by marine organisms will depend on the shape, size and type of plastic polymer (de Sá et al., 2018). De Sá et al. (2018) did a literature study and found that following field sampling, polyethylene was the most reported microplastic type in organisms (17%), followed by polypropylene (14%), polyester (13%), polyamide (10%) and polystyrene (9%). The remaining 36% of the studies had not specified the polymer type found. Still, polystyrene is used in 40% of laboratory exposure experiments, and polyethylene in 33% (de Sá et al., 2018). Spherical fluorescent polystyrene beads are the most commonly used (Phuong et al., 2016). Due to its extensive use in private homes and being the most commonly observed plastic polymer in the ocean (Suaria et al., 2016), as well as the most frequently observed polymer in organisms in natural ecosystems (de Sá et al., 2018), LDPE was chosen as the plastic polymer for this study.

1.2.4 Effects of microplastics on marine organisms

While entanglement, strangulation and ingestion of larger plastic debris leading to a false sense of fullness followed by starvation (Gregory, 2009) are the main hazards for larger marine organisms, smaller organisms are at risk of ingesting microplastics, which in addition to potentially physically blocking the intestinal tract and obstructing gill filaments, may cause toxicity. Bivalves such as *Saccostrea cucullata* (Li et al., 2018), *Corbicula fluminea* (Oliveira et al., 2018), and *Ennucula tenuis* (Bour et al., 2018); polychaetes like *Arenicola marina* (Besseling et al., 2013, Browne et al., 2013, Van Cauwenberghe et al., 2015); zebrafish (*Danio rerio*, LeMoine et al., 2018); the crustacean *Nephrops norvegicus* (Welden & Cowie 2016) and zooplankton (Cole et al., 2013) are only some examples of organisms in which studies have shown negative consequences of ingesting microplastics, either in laboratory studies or in nature.

During manufacture, chemicals and additives are added to improve the characteristics of the plastic (Hartmann et al., 2017; Peng 2017; Galloway 2017; Prokić 2019). Many of the additives are hazardous (Browne et al., 2007; Peng et al., 2017), and have the potential of leaking from the microplastics into the surrounding water or being released in the intestinal tract of organisms following ingestion (Cole et al., 2011). In addition to potentially containing toxic substances, microplastics also have the potential to adsorb contaminants from the surrounding environment, (Cole et al., 2011; Bakir et al., 2014). Due to their large surface area to volume ratio, microplastics tend to adhere to waterborne pollution substances (Thompson et al., 2007; Cole et al., 2011), making them sinks for potentially toxic chemicals which again may leak out in organisms. Besseling et al. (2013) observed an increase in polychlorinated biphenyl (PCB) in the lugworm *Arenicola marina* when polystyrene particles contaminated with PCB were placed in sediments during a laboratory experiment. The type of plastic will also affect its ability to adsorb toxic substances (Cole et al., 2011) and the health effects on organisms (Straub et al. 2017). Rochman et al. (2013) carried out a 12 months exposure experiment, where the sorption of PCBs and polycyclic aromatic hydrocarbons (PAH) to polyethylene terephthalate, polyvinyl chloride, polypropylene, high-density polyethylene (HDPE) and LDPE was recorded. These are the five types of plastic used in packaging, and the most frequently observed in the marine environment (Erni-Cassola et al., 2019; PlasticsEurope, 2019). LDPE and HDPE had the highest sorption rates and concentrations of PCBs and PAHs, suggesting that these pose a greater threat to marine

organisms than the other polymers. These types of chemicals are often concentrated in the sea-surface layer, where also microplastic types of low densities, such as LDPE, are typically found (Teuten et al., 2009).

The consequences of organisms ingesting contaminated MPs is not yet fully understood (Gall & Thompson, 2015), and definite evidence that it affects the health of marine organisms over long-term periods is lacking (Pittura et al., 2018). However, observed effects include disruption of endocrine processes, reduced reproduction, oxidative stress, immunity dysfunction, reduced development and mobility, genotoxicity and carcinogenesis (Barnes et al., 2009; Lithner et al., 2009; Wright et al., 2013; Avio et al., 2015; Canesi et al., 2015; Galloway et al., 2017). It is not always clear which effects are caused by the microplastic in itself or the leakage of toxic chemicals. Nevertheless, the tendency of LDPE to adsorb toxic substances, and the risk of leakage of these substances as well as its inherent toxic additives, makes obtaining information on the presence and behavior of these microplastics in marine organisms like mussels, who are prone to ingest them, a noteworthy study objective.

1.3 The model species: *Mytilus edulis*

The blue mussel, of species *Mytilus edulis,* was chosen as model species for the study. Morphological similarity between the three *Mytilus* species *edulis*, *trossulus* and *galloprovincialis* makes visual identification challenging. All three species have been found along the Norwegian coast (Brooks & Farmen, 2013) and a high level of hybridization has been observed (Mathiesen et al., 2016). However, *M. edulis* was the only species found in the inner and outer Oslofjord by Brooks and Farmen (2013) and is hence the species used in the present study. *Mytilus* are key species in coastal marine ecosystems. They are widely distributed and sessile filter-feeders with high filtration rates. Their main source of nutrition is phytoplankton, which are often in the same size range as microplastics (Wright et al., 2013). The size range *Mytilus* will filter is not universally agreed upon. A particle size range of 4-100 μm is reported (Bayne et al., 1976, Kautsky & Evans, 1987). However, when Davenport et al. (2000) offered *M. edulis* nauplii of the brine shrimp (*Artemia* sp.) of size 300 μm and adults of the copepod *Tigriopus brevicornis* of size 1000 μm, 90% of *Artemia* sp. and 34% of *T. brevicornis* were ingested. Filter-feeders are highly susceptible to ingesting microplastics as they filter large water-volumes, and efficiently trap particles (Browne et al., 2013; Wright et al., 2013, Setälä et al., 2016; Détrée & Gallardo-Escárate, 2017) or take up chemicals in the

water. Due to these traits, blue mussels are frequently used as bioindicators, enabling monitoring of marine pollution and variations in environmental conditions (Brooks & Farmen, 2013; Farrington et al., 2016; Thushari et al., 2017; Beyer et al., 2017). Mussels have been used in a range of environmental monitoring programs, like OSPAR's Coordinated Environmental Monitoring Program (CEMP), US Mussel Watch Project, and Assessment and Control of Pollution in the Mediterranean region (MEDPOL) (Beyer et al., 2017).

1.3.1 *Mytilus edulis* particle intake

Figure 1.1 (A) shows intake, uptake, processing and release of particles in *Mytilus* mussels, and figure 1.1 (B) shows digestive processes taking place in the digestive gland.

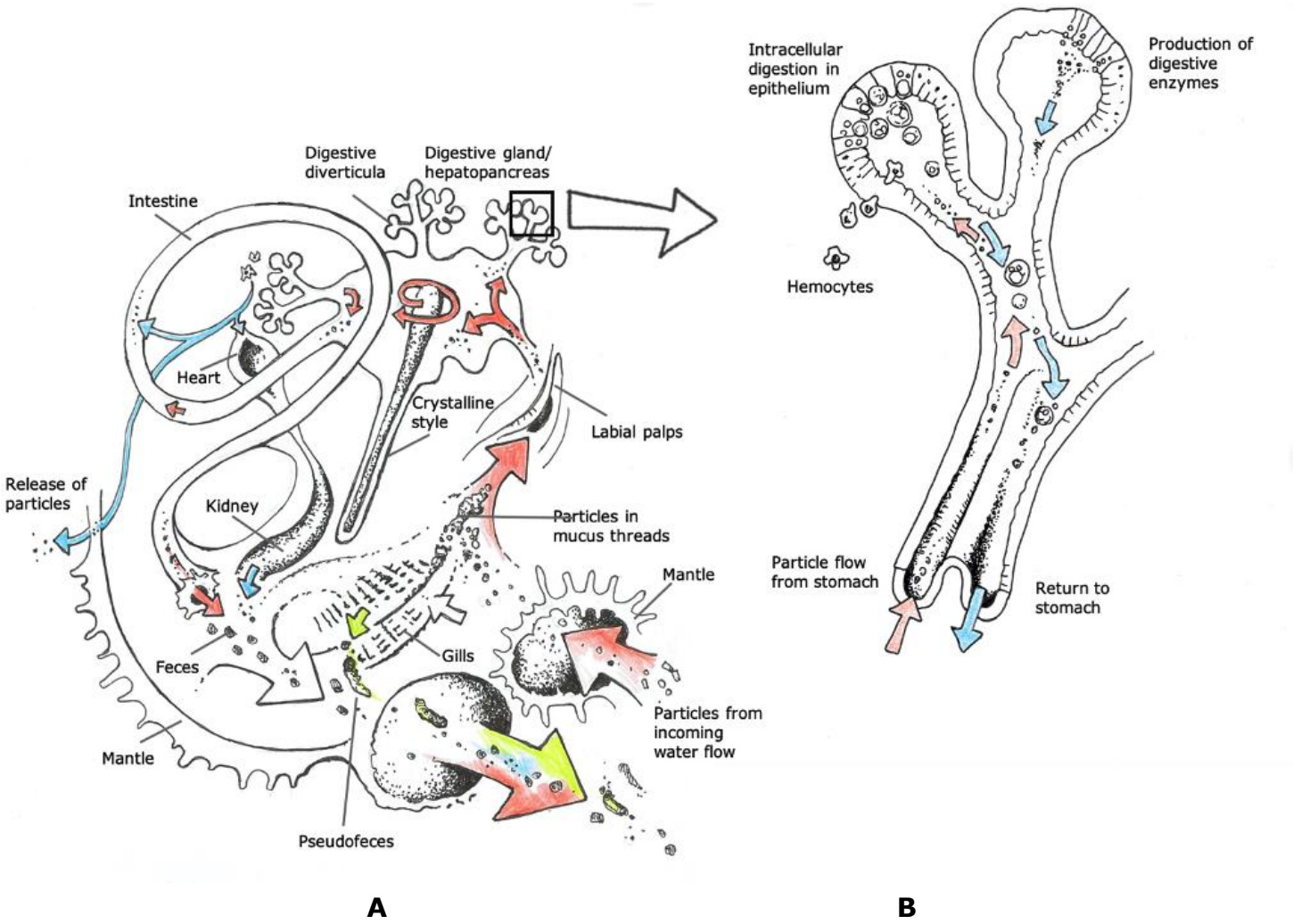

Figure 1.1. Intake, processing and release of particles in mussels (A) and digestive processes in digestive gland of mussels (B). (Illustration by Stein Mortensen made for this study, 2020).

Blue mussels filter large volumes of water to trap food particles. The gills play an important role in the intake of particles. The folded structure of the gills creates a large surface, enabling filtration of large volumes of water. Ribbons of cilia on the surface of gill filaments create water currents across the gill surface, and particles in the current are trapped by cilia and mucus produced by cells on the gills. Facilitated by cilia, the trapped particles are then transported in mucus to the labial palps, the mussels' foraging apparatus, into the mouth, through the esophagus and into the stomach (Figure 1.1 (A)).

The stomach is connected to the digestive gland, also called the hepatopancreas, which consists of tubules leading to smaller channels and to digestive diverticula, which are small sacks where food particles are taken up by endocytosis and digested by specialized cells (Figure 1.1 (B)). Mobile hemocytes are also active in this process, transporting food material by endocytosis. The part of the digestive process taking place in the hepatopancreas is called the intracellular digestion (Hovgaard et al., 2001; National Research Council, 2003). Which particles are taken up in the intracellular digestion depends amongst other factors on particle size. Browne et al. (2008) exposed *M .edulis* to two different sizes of MPs (3 and 9.6 μm) and found that more than 60% more of the smaller (3 μm) particles had translocated to the hemolymph after 3 days, suggesting that the uptake of MPs into organism tissues increases with decreasing MP size. Flows of food particles are driven into the digestive tubules by cilia and the rotating crystalline style, which is a reservoir for digestive enzymes. Particles not taken up into the tissues in the digestive diverticula, as well as waste from the intracellular digestion, are released back into the digestive tubules and to the stomach. The extracellular digestion takes place in the intestinal lumen (National Research Council, 2003). Both digested and undigested material, such as plastic particles, may be present in the digestive gland, in the stomach and in the intestine. The residence time in the gut and intestinal lumen seems to also depend on the size of the particles. Ward et al. (2019) exposed *M. edulis* to polystyrene microspheres of sizes 19, 113, 287, 510 and 1000 μm over a 2-hour period followed by 1 hour of depuration. *M. edulis* did not ingest any of the 1000- μm spheres, and of the ingested spheres, a larger proportion of 510-μm spheres were egested in <3 hours than of the other size classes. Fernandéz & Albentosa (2019), who exposed *M. galloprovincialis* to a mixture of different sized (2-22 μm) HDPE particles for 4 hours found that larger MPs (>10 μm) were eliminated from the mussels faster than the smaller ones. The intestine makes a loop in the visceral mass and ends in the rectum on the dorsal side of the rear adductor muscle (Hovgaard

et al., 2001). Von Moos et al. (2012) also observed uptake of microplastics particles of size 0-80 μm through the gill surface, by transport of particles into the gills via endocytosis.

1.3.2 MPs in *Mytilus* pseudofeces

If the particle concentration in the water is too high, or the particles are unknown or un-nutritious material, mussels may get rid of the excess particles before they are ingested. Fragments of the mucus with trapped particles, which is being transported from the gills to the labial palps by cilia, are detached, released into the mantle cavity and led out with the outgoing water (Figure 1.1 (A)). These mucus fragments with rejected particles are called pseudofeces (Hovgaard et al., 2001), and are a way for bivalves to regulate the intake of particles when there are large amounts of particulate matter in the surroundings, and to hinder particles that may not be used as food from entering (Ward & Shumway, 2004).

Although microplastics may be taken up and internalized in tissues, such as in the Browne et al. (2008)- study, where polystyrene particles of sizes 3 and 9.6 μm were translocated to the hemolymph of *M. edulis*, MPs often just pass through the intestine and are egested in feces or they are rejected in pseudofeces before entering the mussel. *M. edulis* exposed to polyethylene terephthalate microfibers (460 μm length) rejected 71% of the fibers in pseudofeces, and only 9% were ingested (Woods et al. 2018). MPs were also found in pseudofeces in *M. edulis* (and *Crassostrea virginica*) following exposure to polystyrene microspheres of sizes 19, 113, 287, 510 and 1000 μm over a 2-hour period and 1 hour of depuration (Ward et al., 2019). The study found that the number of microspheres that were rejected in pseudofeces, compared to the number of microspheres ingested, depended on particle size; the number of particles rejected in pseudofeces increased with increasing particle size. *M. edulis* rejected significantly lower amounts of 19 μm and 113 μm -spheres compared to ingested, but they rejected all 1000 μm spheres.

Microplastics in pseudofeces or feces may increase the density, causing the aggregates to sink towards the bottom (Galloway et al., 2017). Both feces and pseudofeces could be a pathway for microplastics into the marine food web, as this material may be ingested by suspension feeders and detritivores (Wright et al., 2013). This makes it interesting, and possibly essential, to study the fate of microplastics when in contact with filter-feeders such as blue mussels.

1.3.3 *Mytilus* in microplastic studies

Mytilus species, and especially *M. edulis*, have been used in several studies of ingestion, uptake and effects of microplastics (Browne et al., 2008; Von Moos et al., 2012; Van Cauwenberghe et al., 2015; Paul-Pont et al., 2016; Détrée & Gallardo-Escárate, 2017; Bråte el al. 2018; Green et al., 2018; Woods et al., 2018; Ward et al., 2019). Microplastics are often found in the intestine or intestinal lumen of marine organisms, which is also the case for blue mussels (Browne et al., 2008; Von Moos et al., 2012; Van Cauwenberghe & Jannsen, 2014; Avio et al., 2015; Pittura et al., 2018, Gonzáles-Soto et al., 2019). Most microplastic studies with *Mytilus* focus on physiological health effects, and not necessarily on the fate of the MPs. Laboratory exposure of mussels to microplastics has been shown to induce inflammatory responses, granulocytoma formation and lysosomal membrane destabilization (Von Moos et al., 2012), oxidative stress, increased immune response and apoptosis in the mantle and digestive gland (Détrée & Gallardo-Escárate, 2017), reduced number of byssal threads and attachments strength (Green et al., 2018), as well as increase in hemocyte cytotoxicity and changes in cellular oxidative balance (Paul-Pont et al., 2016). Von Moos et al. (2012) found histological alterations in digestive cells, in inflammatory responses and formation of granulocytomas following exposure of *Mytilus* to 0-80 μm HDPE particles for up to 96 hours. Few other studies have however included histology of mussel tissues following microplastics exposure, and there is a clear need for development of histological analyses for this purpose.

1.4 Histological analyses of microplastics

Assessment tools and methods are continuously being developed for studying the presence, behavior and effect of marine litter in the marine environment, and methods for detection, identification and quantification of microplastics in organisms vary between studies. This poses a challenge with obtaining comparable data, meaning there is a need for standardization (Löder & Gerdts 2015). Histological studies are useful for detecting microplastics in tissues and to visualize potential damage to tissues and organs simultaneously. Tissues are stained to emphasize key elements of the tissue, and to differentiate between them (Alturkistani et al., 2016). Several different staining techniques exist, directed at specific cells or tissue features (Black & Black, 2012). The present study used two staining methods: Hematoxylin-erythrosine-saffron dye (HES) and Nile Red (NR).

Hematoxylin-erythrosine-saffron-dyeing and polarized light microscopy

HES-dyeing is carried out to differentiate between tissues during microscopy. Hematoxylin is used to dye cell nuclei blue, erythrosin is used to dye cytoplasma and muscle fibers red, and saffron is used to dye connective tissue yellow. HES-staining does not stain plastic, however, in combination with polarized light microscopy MPs may be observed in tissue sections (Von Moos et al., 2012). Polarized light microscopy may be used to detect the birefringent properties of plastic polymers (Lusher et al., 2016). The chemical structure and manufacturing of plastic materials result in different optical properties in different directions (Von Moos et al. 2012; Lusher et al., 2016), and when particles are illuminated, polarized light makes the particles light up in different colors (Abbasi et al., 2017). However, as some other materials occasionally light up under polarized light, such as wood and paper (Abbasi et al., 2017), validation of the method with e.g. NR has been recommended, especially when investigating field samples where the presence of microplastics is not expected or a visual pattern is not established.

Nile Red-dyeing

NR is a hydrophobic, lipophilic dye, which may be used to dye plastics, which are also hydrophobic (Andrady, 2011; Shim et al., 2016; Erni-Cassola et al., 2017). Shim et al. (2016) and Erni-Cassola et al. (2017) found that NR effectively stained LDPE particles, and that the particles could be observed with green fluorescence, at the same time preventing observation of contaminants and background noise. NR has previously been cross-validated with FT-IR microscopy for detection of microplastics (Maes et al., 2017; Shim et al., 2017). The NR-staining method is inexpensive, efficient and allows for easy visualization under a microscope with fluorescent light.

1.5 Aims of the study

The aim of the study was to quantify low-density polyethylene (LDPE) microplastic particles in *Mytilus edulis* following exposure through water for two periods of time, at three exposure concentrations and two algae concentrations. The study required validating histological methods to detect the presence of LDPE particles in *M. edulis.*

Specific objectives were to clarify:

- The presence and quantity of LDPE microplastics in gills, digestive diverticula, intestinal lumen and pseudofeces.
- Whether the exposure time, algae concentration in the exposure medium or LDPE concentration in the exposure medium affected the presence of LDPE microplastic particles in gills, digestive diverticula, intestinal lumen and pseudofeces.
- Whether the chosen dyeing and microscopy method was suitable for observing microplastics in tissues during histological assessments.

2. Materials and methods

2.1 Sampling of *Mytilus edulis*

Mussels were sampled from the shore outside NIVA (Norwegian Institute for Water Research) at Solbergstrand, located in the Oslofjord in Norway (59.61575, 10.65281, figure 2.1). On 15.04.19, 65 individuals of similar size (50-60 mm) were collected by hand, placed in buckets with seawater and brought directly to the wetlab at NIVA. 30 individuals were collected for the short-term exposure experiment, 30 for the long-term experiment and 5 were kept as a reference. The mussels were placed in a 50-L aquarium with a constant flow of seawater from the shore outside NIVA Solbergstrand and left for acclimatization without food supply for two weeks. Later, on 26.06.19, 20 extra reference individuals were collected and dissected.

Figure 2.1. Sampling location – the shore outside NIVA Solbergstrand. Map modified from Google Maps.

2.2 LDPE solutions

The plastic used for the exposure experiment was low-density polyethylene (LDPE) microparticles produced as part of EPHEMARE (Ecotoxicological effects of microplastics in marine ecosystem), an EU-project funded by JPI Oceans. The microplastic was provided as dry powder with irregular fragments of size 20-25 µm and density 0.96 g/cm3, produced by Micropowders Inc; NY, USA (Bour et al. 2018). LDPE solutions were made in the chemistry lab at the University of Oslo. Two glass bottles were filled with 900 mL of seawater from the media lab. The remaining 100 mL were added later at NIVA Solbergstrand to avoid spillage. 1500 mg of 20-25 µm LDPE were added to one of the bottles to create a stock solution of 1500 mg/L which was later used to create 5 mg/L solutions in the aquariums. 30 mg of 20-25 µm LDPE were added to the other to create a 30 mg/L stock solution which was later used to create 0.1 mg/L solutions in the aquariums. Calculations are in Appendix A. The microplastic was weighed in a weighing room, and no synthetic clothing was worn. The bottles were shaken lightly and placed on a plate shaker to weather for two weeks. After two weeks, one drop of Tween 20 was added to each of the bottles and they were placed in an ultrasound waterbath for 15 minutes. The process was repeated 2 weeks into the long-term exposure setup, 2 weeks before the first batch of solutions was exhausted.

2.3 Aquaria set-up

Thirty 4-L aquaria were set up in the wetlab at NIVA Solbergstrand (figure 2.2) and filled with 3 L of seawater from 60-m depth. The mean water temperature and salinity in the aquariums were 8.3 ◦C and 33.3 ppm during the short-term exposure, and 8.2 ◦C and 33.8 ppm during the long-term exposure. One circulating pump was placed in each aquarium. The aquaria were numbered from 1 to 30. One mussel was placed in each aquarium. 10 mL of the 1500-mg/L stock solution were added to ten of the aquariums, creating ten aquariums with nominal concentrations of 5 mg/L LDPE. 10 mL of the 30-mg/L stock solution were added to ten other aquariums, creating ten aquariums with nominal concentrations of 0.1 mg/L LDPE. The volumes of stock solution were added to the aquariums using glass measuring cylinders. No LDPE solution was added to the remaining ten aquariums. The mussels were fed with two different concentrations (0.7 mg/L and 2 mg/L) of Shellfish Diet 1800 (Reed Mariculture Inc, USA) using an Eppendorf pipette.

The experiment was a full factorial design, with two exposure durations (short-term, 4 days, and long-term, 54 days), two algae concentrations (0.7 mg/L algae biomass and 2 mg/L algae biomass), and 3 LDPE concentrations (0 mg/L, 0.1 mg/L and 5 mg/L). This resulted in 6 different treatment combinations, each with 5 replicates of each (figure 2.3, table 2.1). 0 mg/L algae biomass was not included, as it would not be a realistic scenario. The reference group consisted of 25 mussels taken directly from the sea and fixed without exposure to either algae or plastic. Figure 2.3 shows how the aquariums were arranged in order during the long-term experiment. The actual experiment had 4 rows with 8-7-8-7 aquariums in each, but the same order as in the diagram. The aquarium numbers and corresponding treatments were taken note of.

Figure 2.2. Aquaria set-up in the wetlab at NIVA Solbergstrand.

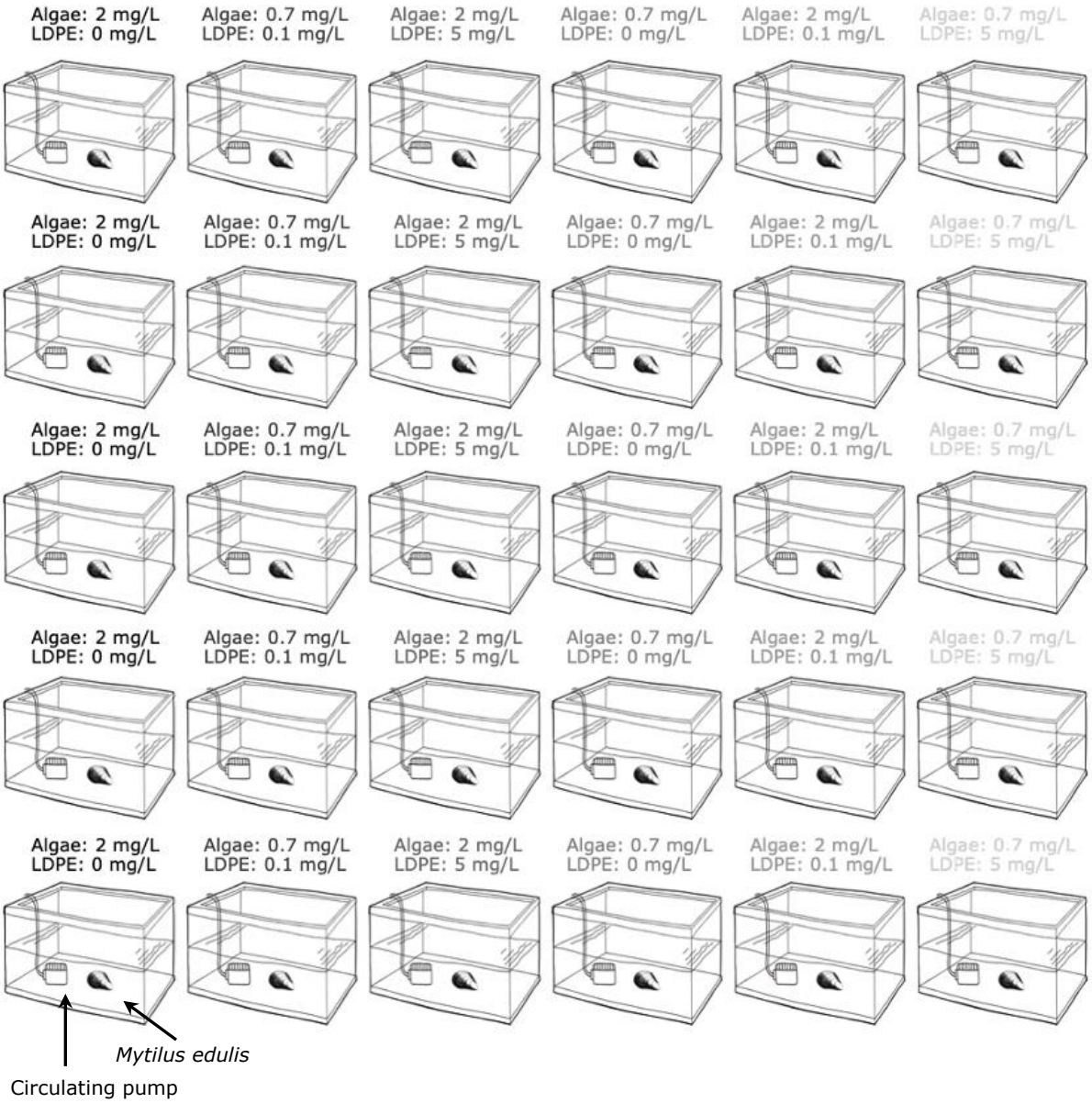

Figure 2.3. Diagram of experiment setup for long-term exposure, showing how the six different treatments were spread out across aquariums. The replicates exposed to the same treatment are marked with text in the same color. The aquariums were numbered from 1-30. (Figure modified from netclipart.com)

Table 2.1 Cross-section of treatments and number of replicates with the same treatment of LDPE concentration and algae concentration

	Algae concentration	
LDPE concentration	***0.7 mg/L***	***2 mg/L***
0 mg/L	5	5
0.1 mg/L	5	5
5 mg/L	5	5

2.3.1 Diet

For both the short-term and the long-term exposure experiments, the mussels were fed with Shellfish Diet 1800 from Reed Mariculture Inc, USA. This is a mixture of six marine microalgae; *Isochrysis* sp., *Pavlova* sp., *Tetraselmis* sp., *Chaetoceros calcitrans*, *Thalassiosira weissflogii* and *Thalassiosira pseudonana*. Feeding was carried out at the same time as replenishing the aquariums with LDPE, and was carried out using an Eppendorf pipette. Half of the mussels were fed 0.7 mg/L (35 µL) Shellfish Diet, and the other half were fed 2 mg/L (100 µL). The amounts were measured in dry-weight of algae and added in mL. Reed Mariculture specify the concentration of Shellfish Diet by dry weight and not cell number due to the large difference in cell volume and biomass with a small difference in cell size. Calculations of concentrations are in Appendix A.

2.3.2 Short-term exposure

The short-term, four days exposure experiment, was started on 26.04.19. Thirty mussels were placed in separate aquaria, and the six different treatments were spread out evenly, similar to figure 2.3. The mussels were left for four days with the lights off. The mussels were then dissected and fixed on 30.04.

2.3.3 Long-term exposure

The long-term, 56 days exposure experiment, was set up on 01.05.19. The design was the same as for the short-term exposure, with small changes in the order of the treatments (figure 2.3). The water in the aquaria was changed and plastic solution and Shellfish diet were added every 2-4 days. The aquaria were more thoroughly cleaned with seawater and a dish brush once a week. In total, the process was carried out 15 times including the first day of the experiment. After 56 days, on 26.06.19, the mussels were dissected and fixed.

2.4 Laboratory processing

Detailed list of laboratory equipment is in Appendix B, and list of solutions and chemicals are in Appendix C.

2.4.1 Dissection and fixation

The mussels were taken from the aquaria five at a time and placed in a box with numbered squares. The lengths of the mussels were measured from the hinge to the edge of the shell and

noted down. The mussels were opened by holding them between the thumb and the forefinger, pushing the valves in opposite directions so they were shifted parallel to each other. A scalpel was drawn along the inside of the valves to cut the posterior adductor muscles, and care was taken to avoid damaging the visceral mass inside the mussel. The mussel was then opened carefully, making sure that all of the visceral mass was placed in the valve with the hinge on the left side and the curved part of the mussel facing up. A cross-section of tissue was cut out dorsoventrally with a scalpel and placed in a numbered 15-mL Falcon tube with 7 mL Davidson's fixation solution (Appendix C.2) using forceps. Each sample was transferred to the fixation solution immediately after dissection. The remaining mussel tissues were placed in small, separate glass containers in the freezer. The scalpel and the tweezers were sterilized in distilled water for each new mussel and the blade on the scalpel was changed for every fifth animal. The dissection and fixation were carried out at NIVA Solbergstrand, and the tissues were transferred to 15-mL Falcon tubes with 7 mL 70% ethanol in the toxicology lab at the University of Oslo after 48 hours. More detailed equipment list is found in Appendix B.1.

2.4.2 Dehydration and paraffin infiltration

The fixed tissues were transferred from the Falcon tubes with ethanol to cassettes marked with the corresponding aquarium number. Marking was done using a pencil, as ink would be removed during the infiltration process. The fixed samples were dehydrated and infiltrated in paraffin using a Histokinette Leica TP1020 in the Histology lab at the Institute of Marine Research in Bergen (Havforskningsinstituttet). The program consists of 12 baths, each containing approximately 1.3 L of its respective liquid (Appendix B.2, Table B.1). The cassettes were placed in the first bath and the Histokinette was turned on. The Histokinette was operated by Histology lab responsible Ingrid Fiksdal and head engineer Dawit Berhe Ghebretnsae. The process ran automatically, with a duration of 19 hours.

2.4.3 Paraffin embedding

The embedding machine and the forceps heater were turned on the day prior to embedding. Before turning it on, it was made sure that there was enough paraffin wax in the chamber, and that the temperature of the wax was between 60 and 63°C. The metal molds were placed on the heating plate of the machine to be warmed up before adding the wax. The container with cassettes was removed from the Histokinette, and the cassettes were immediately spread out on the heating plate. One mold was filled with ¼ of wax and placed back on the heating plate

to make sure it was in liquid form when adding the tissue. One cassette was opened, the lid was disposed of and, using warm forceps, the tissue was transferred to the mold. The tissue was placed with the desired orientation for observation facing down. The mold containing ¼ of wax and tissue was placed on a cooling element while slightly pushing down on the tissue with warm forceps. The labeled part of the cassette was placed on top of the mold, and the mold was filled with enough warm paraffin wax to fill the plastic cassette. The molds were placed on a cooling plate immediately after adding the hot wax. When the cooling plate was full, it was put in a -20°C freezer for 30 minutes. Then, the paraffin blocks were easily taken out of the molds, and excess paraffin wax around the plastic cassettes was removed with a knife. Equipment list is found in Appendix B.2.

2.4.4 Sectioning

The embedded tissues were sectioned using a Microm HM355S (figure 2.4). The water bath with deionized water was heated to 40-45°C. The paraffin block was clasped to the cassette clamp. The angle of the knife blade was set to 10 degrees. The microtome was set to 3um thickness, and trim mode. By turning the hand-wheel handle, it was made sure that the blade was not positioned too close to the paraffin block. The mechanical hand wheel brake was

Figure 2.4. Sectioning of tissue using the Microm HM355S

flipped, and the velocity was slowly increased. The block was first trimmed until the desired section of the tissue within the block was reached. Then the mode was changed from “trim” to “fine”. When an adequate section was reached, it was discarded into the water bath, where it was picked up with a microscope slide marked with the respective sample number, by descending it under the section and lifting it up vertically. This was repeated twice, to obtain one tissue section for HES-dyeing and one section for NR-dyeing. The microscope slide with

the tissue section was placed in a holder to dry. When all sections were cut and placed on microscope slides, they were dried in an incubator at 56°C for 20 minutes. Detailed equipment list is in Appendix B.3.

2.4.5 Deparaffinization and dyeing

Earlier, there have been issues with degradation of plastic when using histological methods including xylene for deparaffinization. Gonçalves et al. (2017) replaced xylene with isopropanol, which eliminated the degradation issue for polystyrene. Before teaming up, Lisbeth Sælemyr at the Institute of Marine Research conducted experiments to evaluate the effect of xylene on various polymers of plastic. LDPE proved not to be affected by the organic solvent, and preparation of tissue sections for histological analysis could be carried out using the protocol at the Institute of Marine Research, *with* xylene.

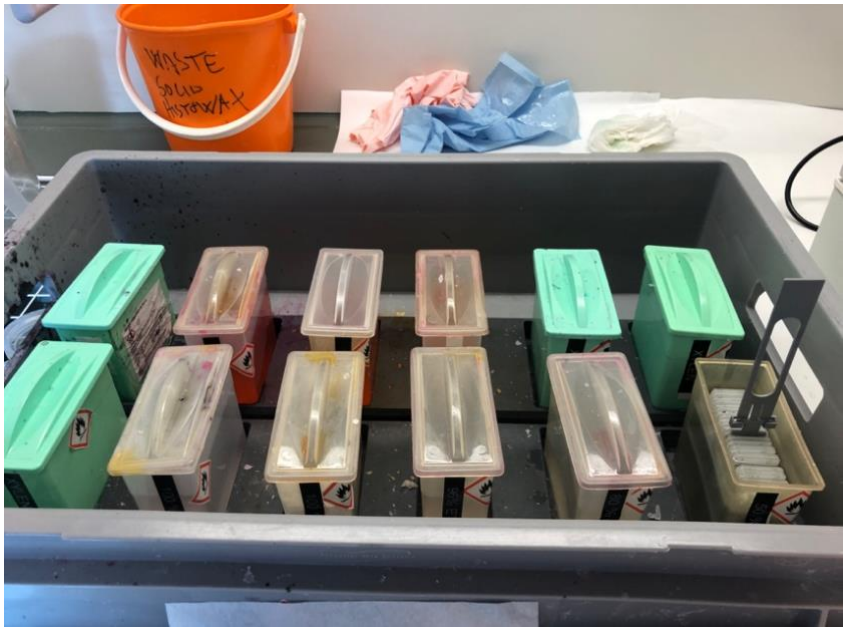

Figure 2.5. A) Baths for deparaffinization and HES-dyeing. Here depicted with tissue sections placed in bath 6 (50% ethanol).

HES-dyeing

The dyes were prepared by Histology lab responsible, Ingrid Fiksdal. Before staining, the paraffin must be removed from the tissue sections in a xylene bath. Subsequently, sections are hydrated in a water bath to allow the hydrophilic dye to react with the tissues. The HES-dyeing process consists of 12 baths (Appendix B.4, Table B.2). The baths were plastic containers with lids, placed in a fume hood in the Histology lab. Baths for cleansing with

water, erythrosine and alcoholic saffron were placed outside the box. The dyes were added to separate containers and poured back into their respective bottles after use. It was made sure that there was more than 200 mL ethanol and xylene in the containers. The hematoxylin was filtered right before each dyeing process. The microscope slides with the tissue sections were placed in a holder with a handle. The sections were then placed in consecutive deparaffinization- and dyeing/infiltration baths. Twenty-four sections were dyed simultaneously. The microscope slides with tissue sections were dipped a couple of times in the bath before they were left for the time period corresponding with the bath. When rinsing with water, it was made sure that the water beam did not hit the sections. The microscope slides in the last xylene bath were carefully lifted one by one from the holder using forceps and placed with the side with the section facing upwards. A drop of Histokit glue was added to a cover glass, which was carefully placed over the tissue section. The cover glass was carefully pushed down with a finger to avoid air bubbles. The microscope slides were left in a fume hood for 48 hours for evaporation of xylene. Equipment list is in Appendix B.4 and chemical list in Appendix C.1.

NR-dyeing

The microscope slides were added to baths 1-6 before proceeding to dyeing with NR stain. NR (Sigma-Aldrich), is commonly dissolved in acetone (Rumin et al. 2015, Erni-Cassola et al. 2017), which dissolves some plastics, but for this study, methanol was chosen, because common plastics are resistant to it (Erni-Cassola et al. 2017). Working solution was prepared by dissolving NR in methanol to a concentration of 0.1 mg/mL. It was then maintained at room temperature protected from light. The slides were left in the dye for ten minutes and were then rinsed quickly in distilled water, to remove excess NR solution. Glycerol was added to a cover glass, which was placed over the tissue section and pushed down lightly. The edges of the cover glass were sealed with nail polish to prevent air from entering. The slides were then put in a box in a fume hood to avoid light affecting the dye. The use of NR staining, followed by fluorescence microscopy, was used to validate the observations made when using HES-staining and polarized light microscopy in this study, which has previously only been done on cryotome (frozen) sections of mussel tissue (Von Moos et al., 2012; Pittura et al., 2017).

2.5 Visual microscopy analysis

2.5.1 HES-dyed sections

The histological analysis in the present study included studying in which matrices within the mussels MPs were present. This was done by using polarized light microscopy, a relatively novel method for detection of microplastics in HES-dyed tissue sections using a microscope. The HES-dyed sections were observed using a Leica DMRBE microscope, using both normal microscopy and polarized light microscopy. Polarized light made it possible to very easily localize the MPs in the mussel tissues, and to use ImageJ/Fiji to determine the number of particles in different parts of the tissue section. The magnifications were from 2.5X to 40X. The camera used was a Leica Wild MPS 52 Analog Microscope Camera. In addition to pictures, notes were taken of the location of plastic particles (intestine, digestive diverticula, gills and pseudofeces).

2.5.2 NR-dyed sections

The sections dyed with NR were examined with fluorescence microscopy, using a Nikon Eclipse 80i microscope with a Nikon Intensilight C-HGFI lightsource and a ET/GFP (FITC/Cy2) filter cube from Chroma (Chroma.com). The sections were exposed to blue light (excitation wavelength 470 nm), and green light (525 nm) was emitted from the microplastic particles. The camera used was a Spot Flex Color from Spot Imaging. The same sections stained using different techniques were compared visually to validate the method of using polarized light.

2.6 Matrices present on sections

After microscopy analyses of sections from all mussels from the short-term experiment, the long-term experiment and the reference group, it was found that not all four matrices were present on slides for all individuals. To facilitate the interpretation of the graphs in the results chapter, table 2.2 shows an overview of which matrices were present on the sections taken from the mussels in each treatment group.

Table 2.2: The number of available mussel sections for the various matrices, categorized for the 3 treatment levels (exposure time, algae concentration and plastic concentration). The percentages of mussels with available matrix samples within each treatment group are also included.

	Gills		**Intestinal lumen**		**Digestive diverticula**		**Pseudofeces**	
Short-term	**30**	**100%**	**26**	**86,7%**	**26**	**86,7%**	**18**	**60%**
0.7 mg/L algae	***15***	***100%***	***14***	***93.3%***	***14***	***93.3%***	***6***	***40%***
0 mg/L LDPE	5	100%	5	100%	5	100%	1	20%
0.1 mg/L LDPE	5	100%	4	0.8%	4	0.8%	3	60%
5 mg/L LDPE	5	100%	5	100%	5	100%	2	40%
2 mg/L algae	***15***	100%	***12***	***0.8%***	***12***	***0.8%***	***12***	***80%***
0 mg/L LDPE	5	100%	3	0.6%	3	0.6%	5	100%
0.1 mg/L LDPE	5	100%	4	0.8%	4	0.8%	2	40%
5mg/L LDPE	5	100%	5	100%	5	100%	5	100%
Long-term	**28**	**93.3%**	**27**	**0.9%**	**28**	**93.3%**	**24**	**80%**
0.7 mg/L algae	***13***	***86.7%***	***13***	***86.7%***	***15***	100%	***12***	***80%***
0 mg/L LDPE	3	0.6%	4	0.8%	5	100%	4	80%
0.1 mg/L LDPE	5	100%	4	0.8%	5	100%	3	60%
5 mg/L LDPE	5	100%	5	100%	5	100%	5	100%
2 mg/L algae	***15***	100%	***14***	***93.3%***	***13***	***86.7%***	***12***	***80%***
0 mg/L LDPE	5	100%	4	0.8%	4	0.8%	4	80%
0.1 mg/L LDPE	5	100%	5	100%	5	100%	4	80%
5 mg/L LDPE	5	100%	5	100%	4	0.8%	4	80%
Reference	**24**	**96%**	**22**	**88%**	**23**	**92%**	**25**	**100%**

2.7 Data analysis

2.7.1 ImageJ/Fiji

The use of polarized light microscopy combined with the Particle Analysis tool in ImageJ/Fiji (version 2.0.0-rc-69/1.52p) enabled identification and quantification of LDPE plastic particles in the mussel sections. With the Particle Analysis tool in ImageJ/Fiji, particles of a set size were tracked using color threshold, then counted by the program.

2.7.2 Statistical analyses

Statistical analyses were carried out in the statistics software RStudio (Version 1.2.5033 © 2009-2019 RStudio, Inc.). Statistical analyses of effects of the different variables (exposure time, algae concentration and LDPE concentration) on the number of plastic particles were

carried out based on the full data set, comprising all treatment groups for both the short-term exposure and the long-term exposure. The matrix in which the plastic was observed was also included in the analyses. Analyses for the control group were performed separately. As the dataset includes a large number of 0-values, finding the appropriate way to statistically analyze it was quite a challenge. However, two different models were tested for the best fit for the data. One of these models were chosen, followed by testing of four different variations of the chosen model. The two models tested were a generalized mixed effects model with incorporated random effects (glmer) and a generalized additive model (gam), which is a generalized linear model, but with smoothing functions. The model chosen for analyses of effects was a gam model with a negative binomial family. This model uses only parametric parameters, and only one smoothing function- the random factor for the different mussel individuals. The random factor was included as the data was taken from four different matrices from the same individual mussel.

Four different model combinations were tested to find the one with the best fit for the data. This was done by looking at the AIC (Akaike Information Criterion) and ΔAIC values. The model with the combination of variables that gives the lowest AIC value was the best fit for describing and explaining the variation in the number of plastic particles. Generally, models are considered equally effective if the ΔAIC value between the model in question and the model with the lowest AIC is < 2. The combinations tested were (Model 1) interactive effects of "algae" and "plastic concentration" and additive effects of "matrix" and "exposure" on the number of particles, (Model 2) additive effects of "algae", "plastic concentration", "matrix" and "exposure" on the number of particles, (Model 3) additive effects of "plastic concentration", "matrix" and "exposure" on the number of particles, and (Model 4) additive effects of "plastic" and "matrix" on the number of particles. There were no big differences in AICs between the models (Appendix D.2.5), which indicates that they fit equally well to estimate the effects of the variables on the number of plastic particles in the mussels. Model 1 was used for plotting partial effects, and the model that included only additive effects of plastic concentration and matrix was chosen for summary analysis (Model 4). This was also the model with the lowest AIC. Despite using a gam-model, the variables are fitted as in a generalized linear mixed effect (glme)-model as only one smooth function is used (the random factor mussel-ID). The summary results for the variables may be used as in any other lme-model. The model was calculated with a 95% confidence interval for the effect of the variables. The test of models is included in Appendix D.

3. Results

3.1 Presence of LDPE particles in tissues and pseudofeces

3.1.1 Presence of LDPE particles in gills

LDPE particles were observed on the gills in some of the mussel sections. In several of the sections, plastic was detected in mucus around the gills (figure 3.1 (B)). Figure 3.2 and 3.3 present the number of particles in the gills of mussels after the short-term and long-term exposure experiment, respectively. The graphs portray results for the replicates within each treatment. Only 6 of the mussels in the short-term exposure and 4 of the mussels in the long-term exposure were observed to have plastic particles on the gills. The highest numbers of particles were detected in individuals that had been exposed to a LDPE solution concentration of 5 mg/L. Although a few more of the individuals from the short-time exposure had plastic on the gills, there was no distinct pattern or difference in number between the two exposure time groups or algae concentration groups, and no distinct similarities between replicates.

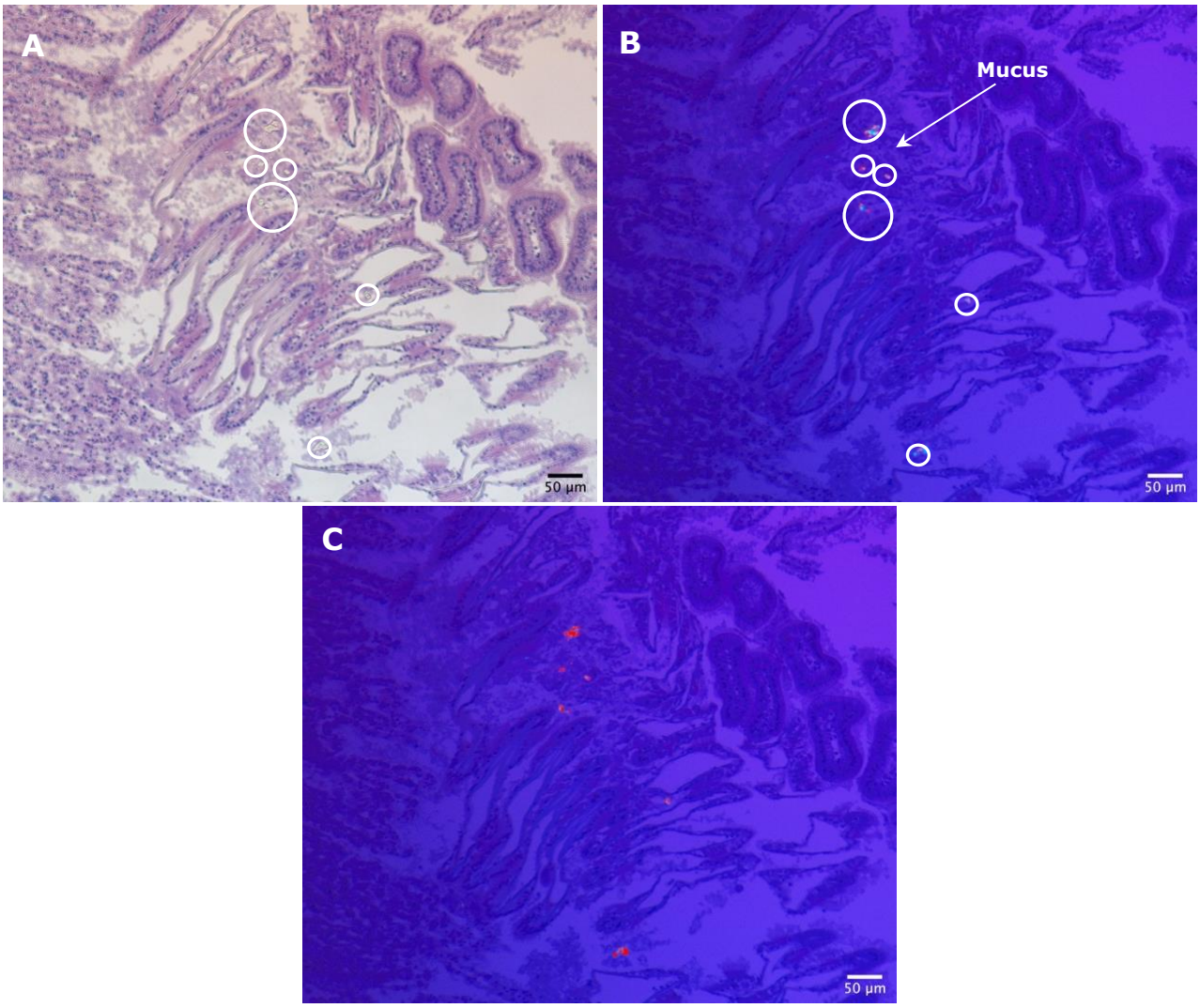

Figure 3.1. HES-stained tissue showing microplastic particles in mucus on gills with (A) normal filter, (B) polarized light filter, and (C) polarized light filter and ImageJ Particle Analysis tool. White circles in A mark the areas where microplastic particles were identified using polarized light (B and C).

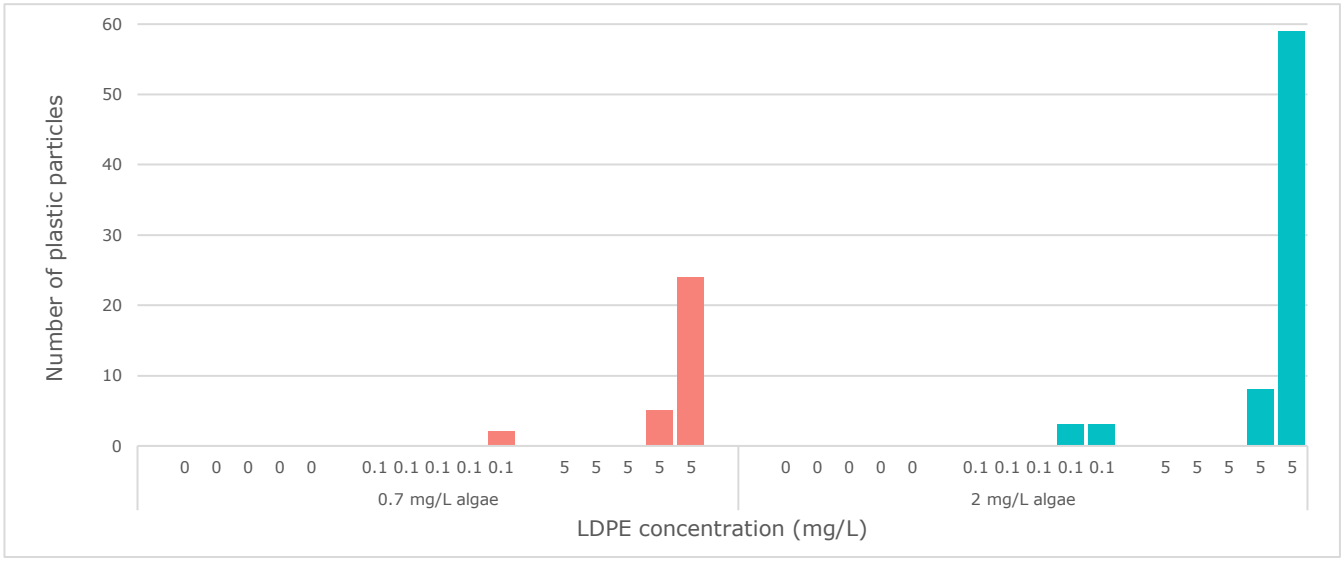

Figure 3.2. Number of LDPE particles in gill tissue after short-term exposure for replicates of each algae concentration (0.7 mg/L and 2 mg/L) and each LDPE solution concentration (0 mg/L, 0.1 mg/L and 5 mg/L). n/a- not available.

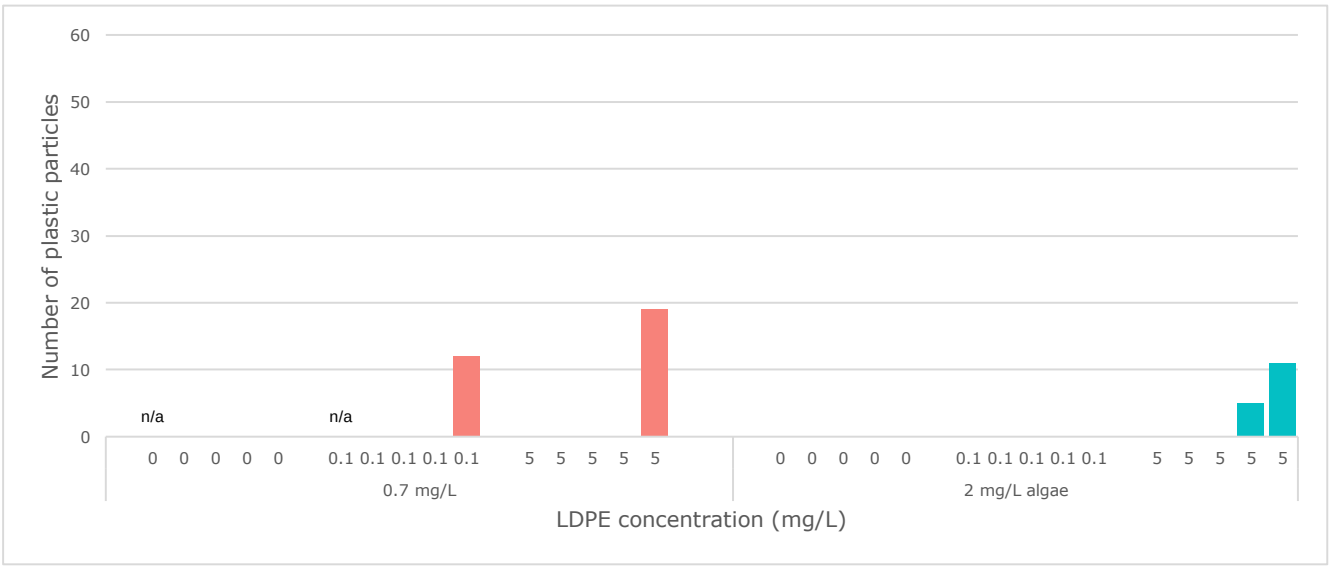

Figure 3.3. Number of LDPE particles in gill tissue after long-term exposure for replicates of each algae concentration (0.7 mg/L and 2 mg/L) and each LDPE solution concentration (0 mg/L, 0.1 mg/L and 5 mg/L). n/a- not available.

3.1.2 Presence of LDPE particles in intestinal lumen

Generally, the intestinal lumen had the highest number of LDPE particles of the four matrices. The particles were observed in clusters, close together (figure 3.4). Plastic particles were observed in the intestinal lumen in all treatment groups, however not for all replicates within the groups. There was some variation between the replicates. Figure 3.5 and 3.6 show that the amount of plastic was much higher in the mussels exposed to 5 mg/L LDPE solution for both exposure times than in mussels exposed to 0.1 mg/L. Some particles were found in the intestinal lumen of mussels that had been exposed to 0 mg/L LDPE. Note that the scale on the Y-axis varies between matrices.

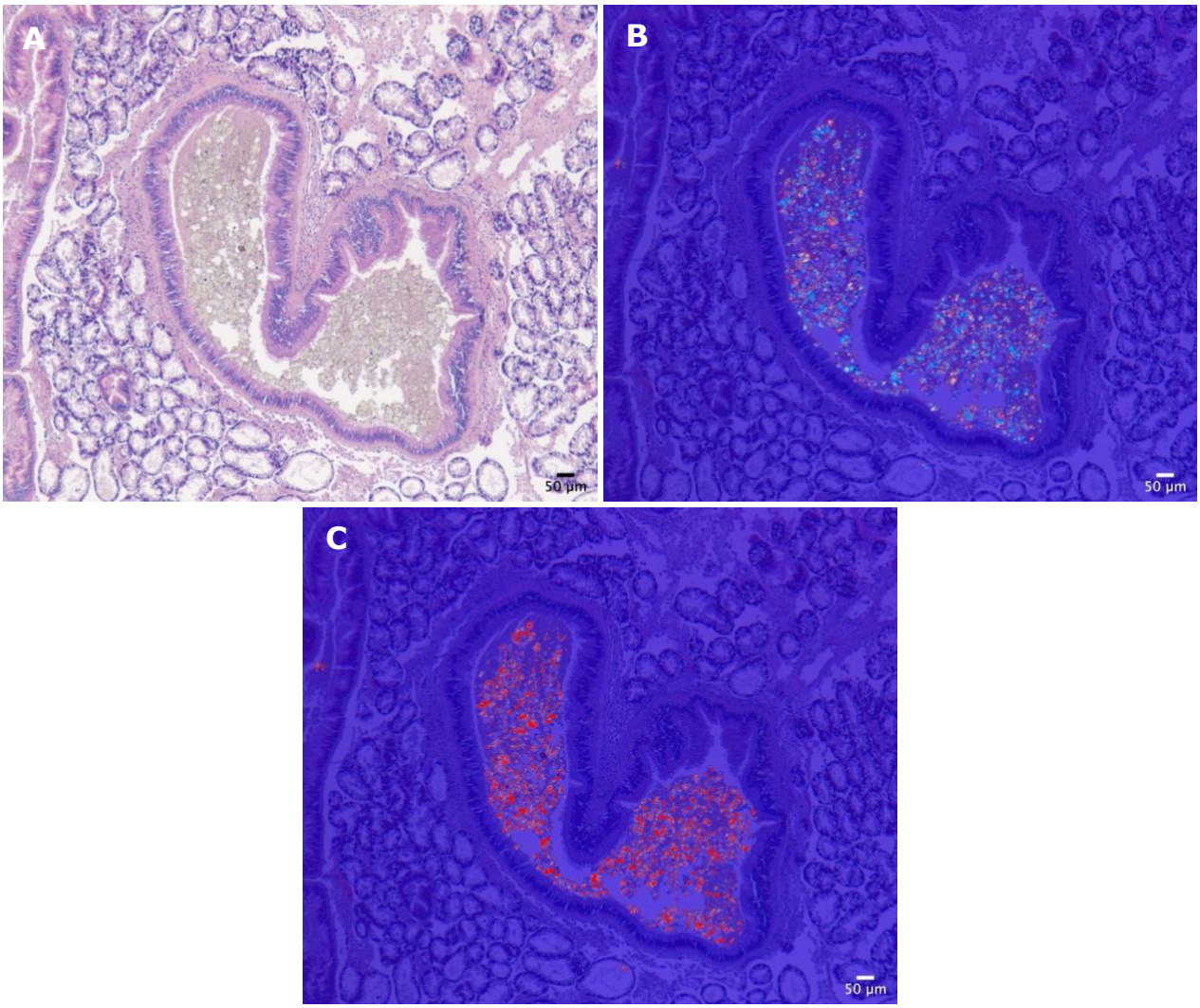

Figure 3.4. HES-stained tissue showing microplastic particles in intestinal lumen with (A) normal filter, (B) polarized light filter, and (C) polarized light filter and ImageJ Particle Analysis tool.

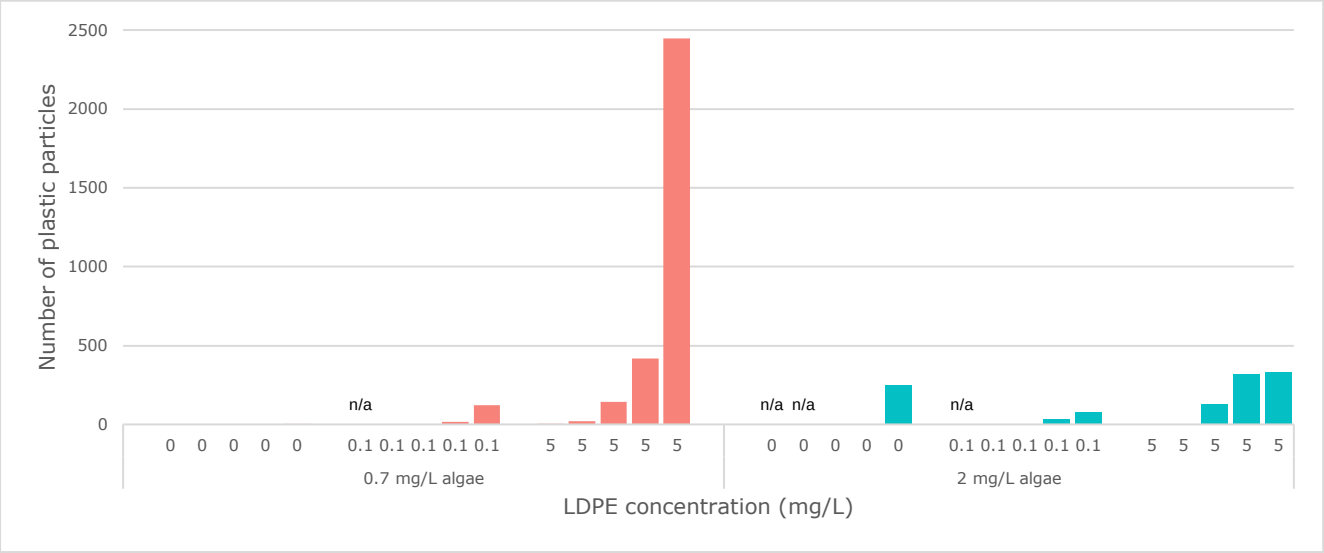

Figure 3.5. Number of LDPE particles in intestinal lumen after short-term exposure for replicates of each algae concentration (0.7 mg/L and 2 mg/L) and each LDPE solution concentration (0 mg/L, 0.1 mg/L and 5 mg/L). n/a- not available.

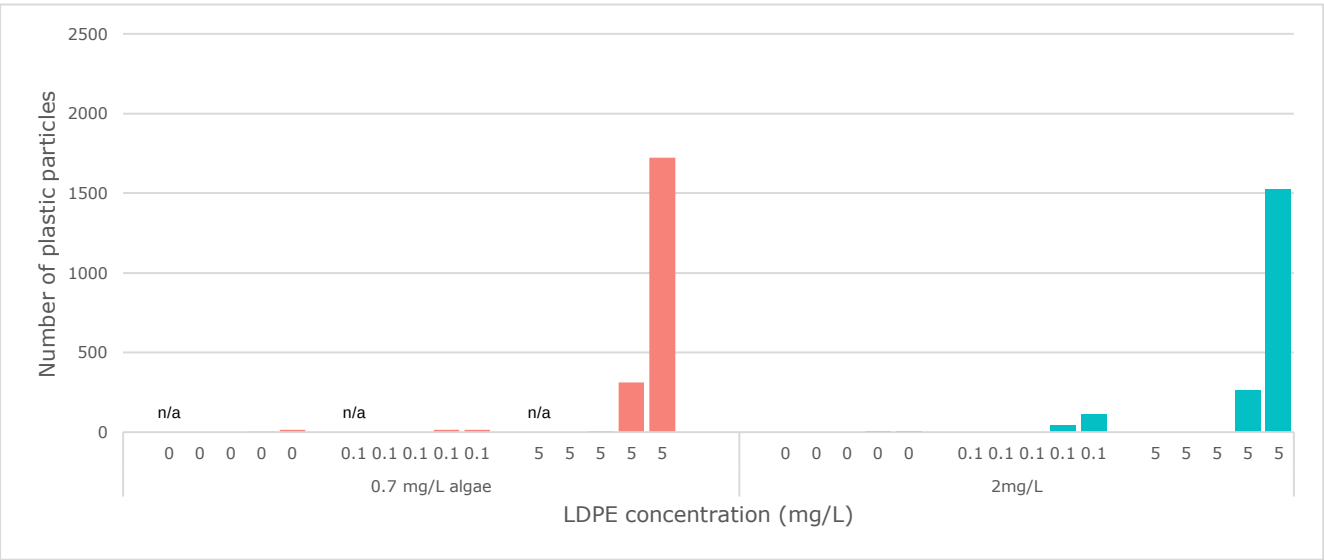

Figure 3.6. Number of LDPE particles in intestinal lumen after long-term exposure for replicates of each algae concentration (0.7 mg/L and 2 mg/L) and each LDPE solution concentration (0 mg/L, 0.1 mg/L and 5 mg/L). n/a- not available.

3.1.3 Presence of LDPE particles in digestive diverticula

LDPE particles were observed in the digestive diverticula of only a few of the mussels. In the mussels where MPs were seen, there were no aggregates, only particles spread out, as seen in figure 3.7. Although the digestive diverticula were present in the sections of all mussels of the short-term exposure, and all sections except for two from the long-term exposure, plastic particles were observed in the lumina of digestive diverticula in only in three individuals from the short-term (figure 3.8), and one individual from the long-term (figure 3.9). MPs were only observed in mussels exposed to 5 mg/L LDPE concentration for both exposure times.

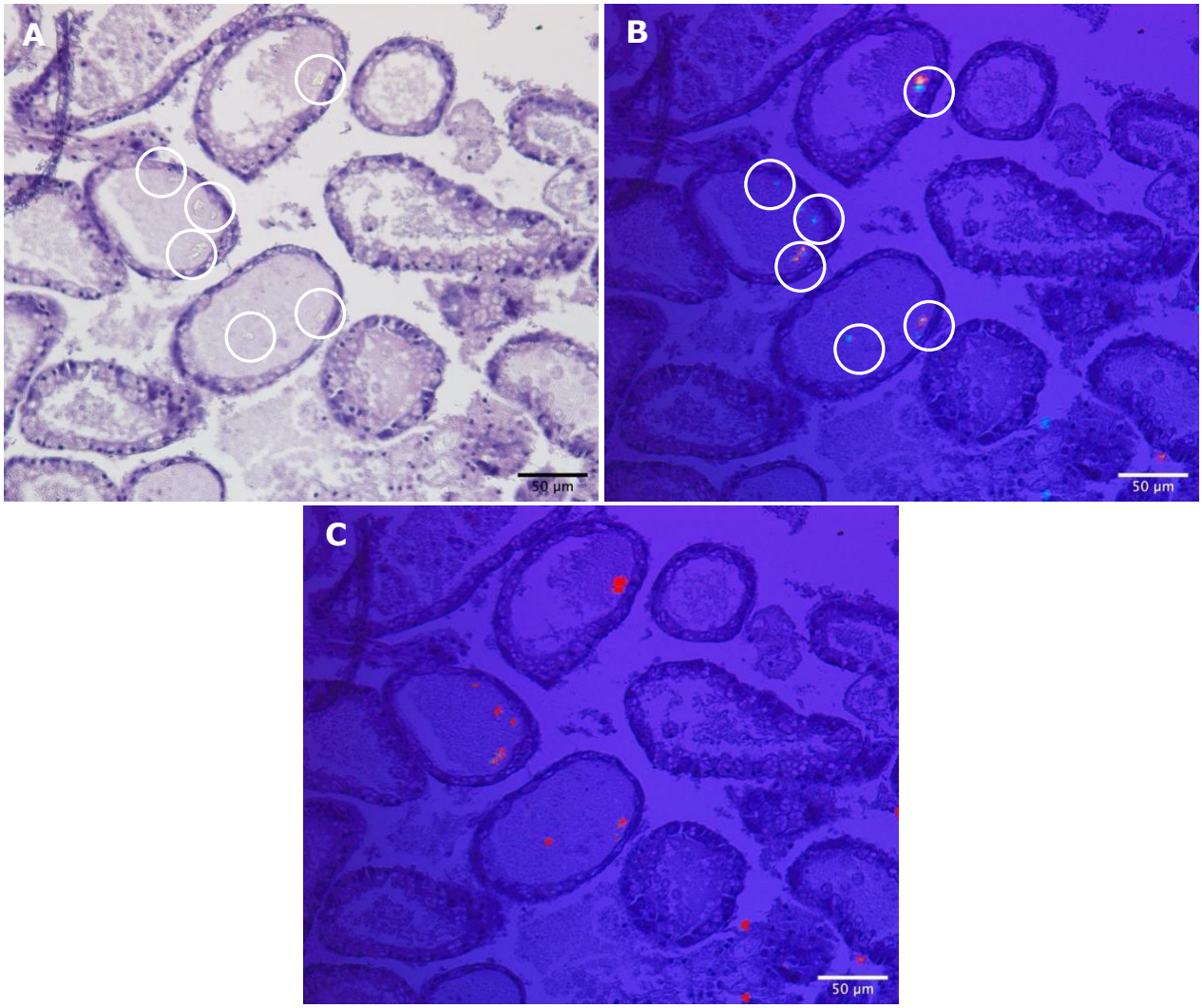

Figure 3.7. HES-stained tissue showing microplastic particles in digestive diverticula with (A) normal filter, (B) polarized light filter, and (C) polarized light filter and ImageJ Particle Analysis tool. Particles outside of diverticula were excluded from the count.

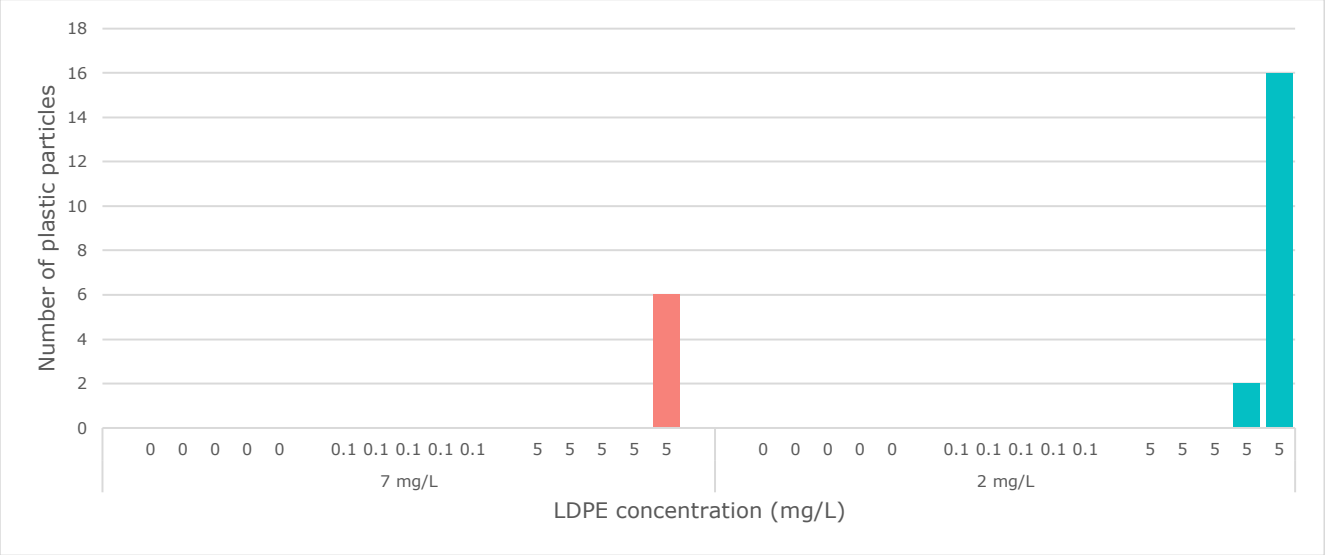

Figure 3.8. Number of LDPE particles in digestive diverticula after short-term exposure for replicates of each algae concentration (0.7 mg/L and 2 mg/L) and each LDPE solution concentration (0 mg/L, 0.1 mg/L and 5 mg/L). n/a- not available.

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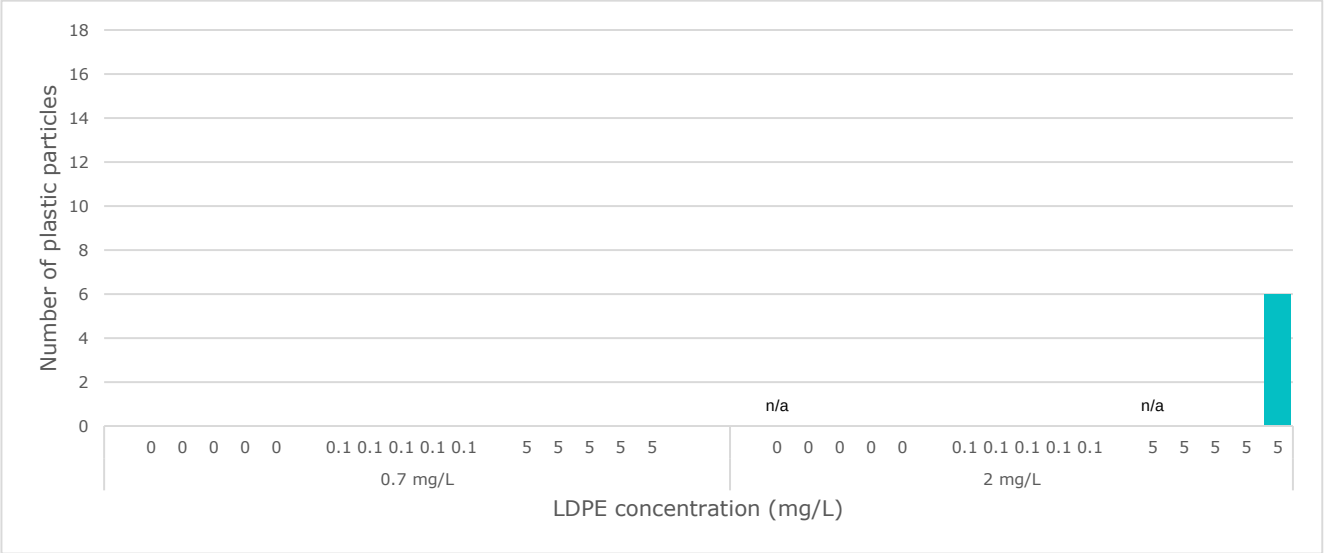

Figure 3.9. Number of LDPE particles in digestive diverticula after long-term exposure for replicates of each algae concentration (0.7 mg/L and 2 mg/L) and each LDPE solution concentration (0 mg/L, 0.1 mg/L and 5 mg/L). n/a- not available.

3.1.4 Presence of LDPE particles in pseudofeces

MPs in pseudofeces were often observed clustered together on a small area, typically near the gills (figure 3.10). Mussels exposed to 5 mg/L LDPE concentration in the exposure medium had the highest number of plastic particles in pseudofeces. There were no clear differences between exposure groups or algae concentration groups. Pseudofeces was the matrix with the highest number of slides with no observations, as seen in table 2.2 and from the n/a marks in figure 3.11 and 3.12. This was mostly the case for treatments of 0 mg/L LDPE and/or 0.7 mg/L algae in the exposure medium.

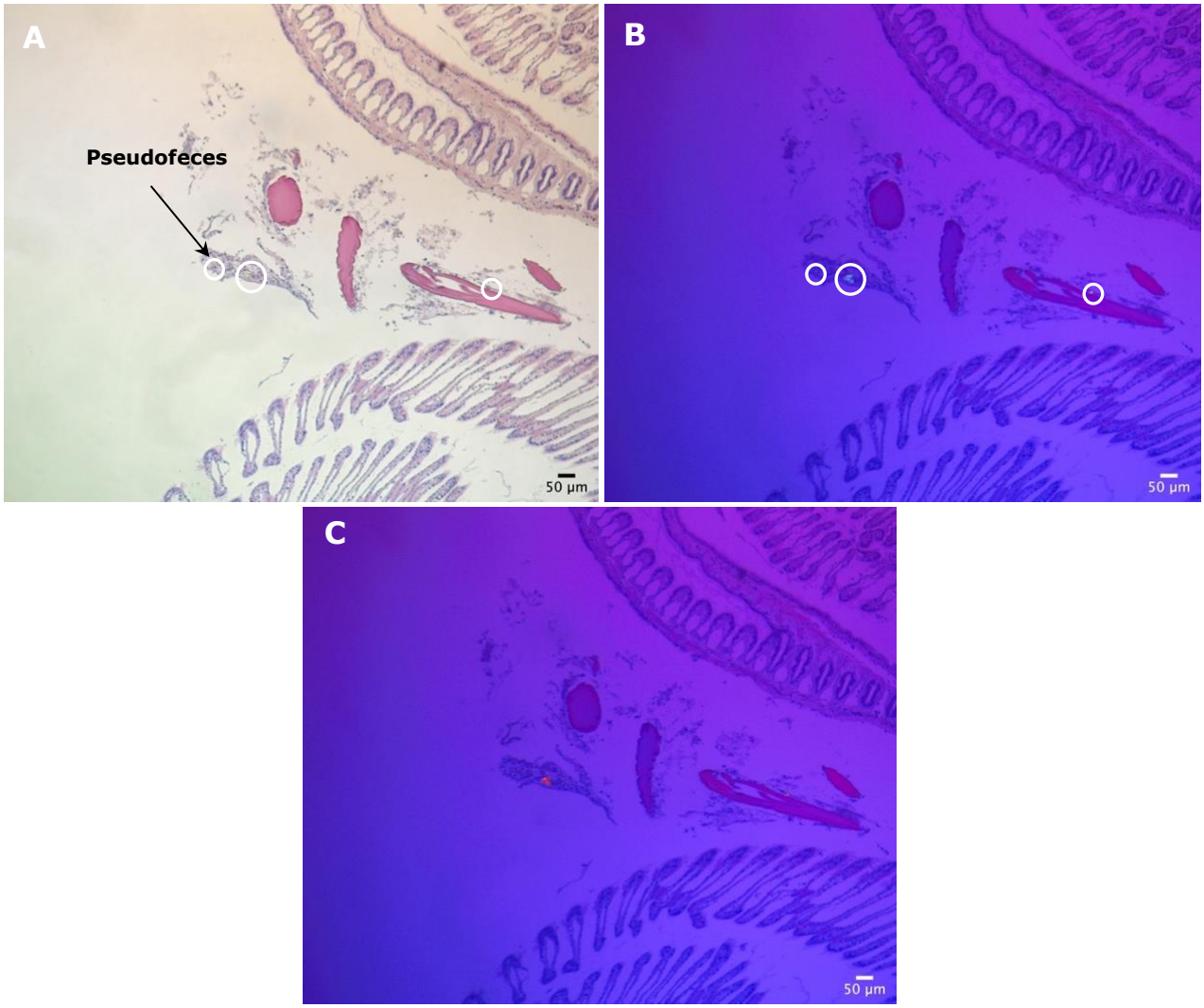

Figure 3.10. HES-stained section showing microplastic particles in pseudofeces with (A) normal filter, (B) polarized light filter, and (C) polarized light filter and ImageJ Particle Analysis tool.

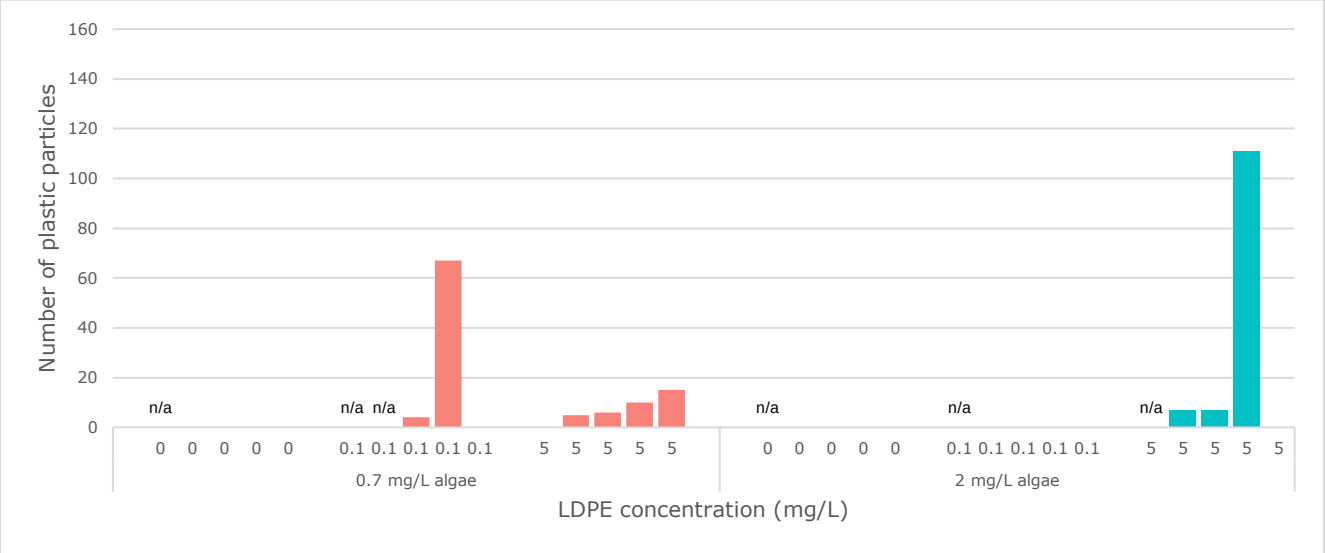

Figure 3.11. Number of LDPE particles in pseudofeces after short-term exposure for replicates of each algae concentration (0.7 mg/L and 2 mg/L) and each LDPE solution concentration (0 mg/L, 0.1 mg/L and 5 mg/L). n/a- not available.

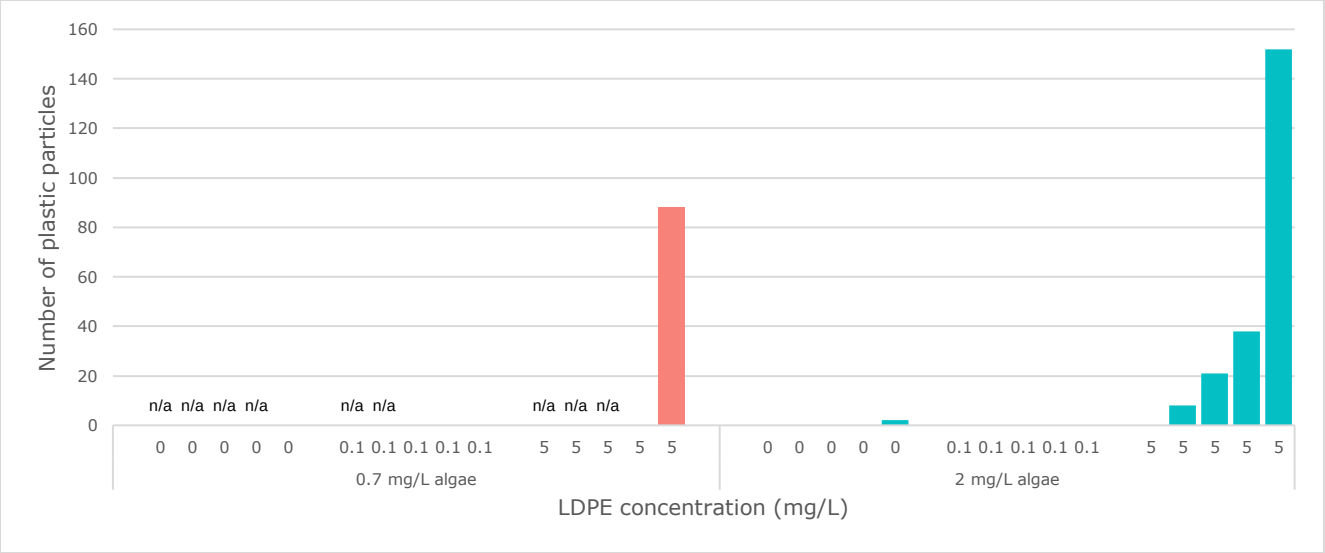

Figure 3.12. Number of LDPE particles in pseudofeces after long-term exposure for replicates of each algae concentration (0.7 mg/L and 2 mg/L) and each LDPE solution concentration (0 mg/L, 0.1 mg/L and 5 mg/L). n/a- not available.

3.2 Effect of exposure time, algae concentration, plastic concentration and matrix on the amount of plastic particles

Model 1 showed no significance of the interactive effects of algae concentration and plastic concentration (Appendix D.2.1), model 2 showed no significance of the algae concentration, (Appendix D.2.2), and model 3 showed no significance of exposure time (Appendix D.2.3). Model 4 included only plastic and matrix and showed significance for these (Appendix D.2.4).

The partial effects of the model which included all variables (exposure time, algae concentration, plastic concentration and matrix), were plotted (figure 3.13). The plots of partial effects describe how much each variable contributes to predicting the number of particles. The plots show very little effect of exposure time and algae concentration, as the partials for both concentrations and both exposures are nearly at zero, and there is no variation between treatment levels. The dotted lines represent confidence intervals, and figure 3.13 (A) shows that the confidence intervals of 2 mg/L algae concentration include the prediction for 0.7 mg/L. This is also the case for short-term exposure and long-term exposure (figure 3.13 (D). The partial plots for plastic concentration and matrix show a larger effect on the number of particles. The model predicts different amounts of plastic particles in the four different matrices. With regard to the LDPE concentration, the plot and model results show that plastic level at 0.5 mg/L is different from at 0 mg/L. There is some overlap in the confidence intervals between 0.1 mg/L and 5 mg/L, and thus one cannot dismiss the possibility that they are similar. However, figure 3.13 (B) indicates that the level is likely higher at 5 mg/L than 0.1 mg/L. They differ enough to conclude that the concentration of LDPE in the exposure medium was significant.

The model that included only additive effects of plastic concentration and matrix was chosen for summary analysis. In addition to the plots of partial effects, table 3.1 also shows that the effects of both plastic concentration and which matrix was being observed were significant for the particle number, with significances higher than 95% and 99%. The P-values of the table indicate that the effect of a concentration of 0.1 mg/L is not significantly different from a concentration of 0 mg/L. However, the 5 mg/L LDPE level is significantly different. The matrix type being observed had the highest significance for the number of particles.

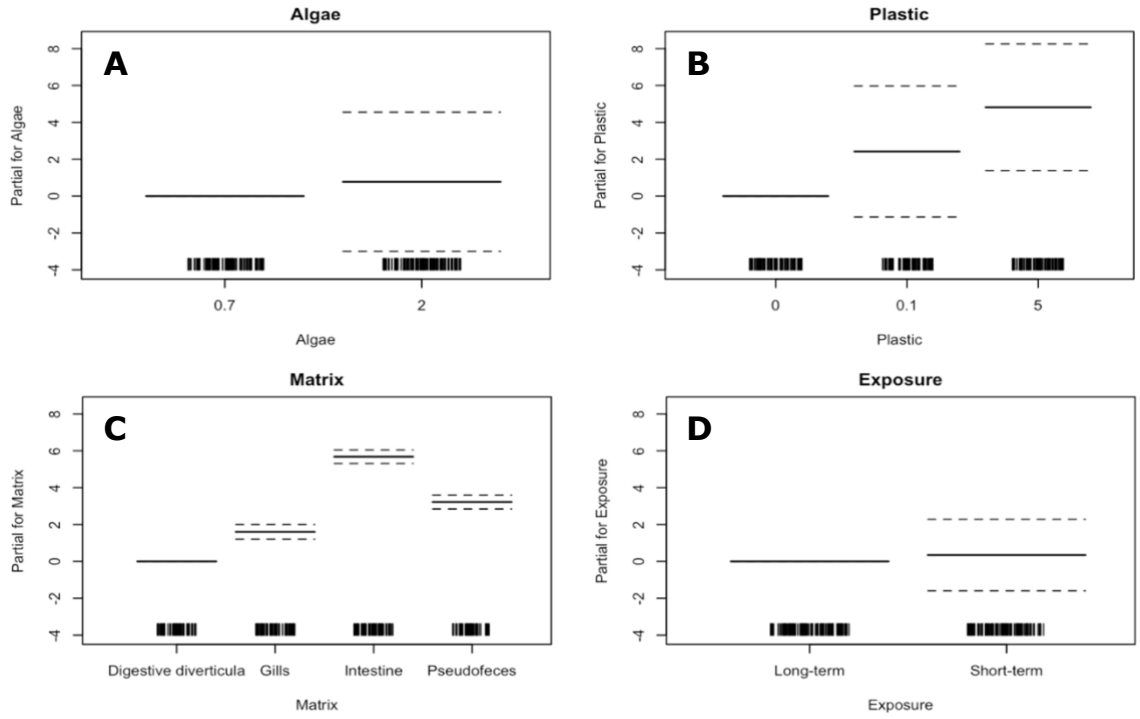

Figure 3.13. Plots of partial effects of (A) algae concentration, (B) plastic concentration, (C) matrix and (D) exposure time on the prediction of number of plastic particles

Table 3.1: Summary results of the model used to test the variance in number of particles observed against the variables plastic concentration and matrix. Significant effects are marked in bold. (The intercept represents digestive diverticula and a 0 mg/L plastic level).

Parametric coefficients	Estimate	Std. Error	Z value	P-value
Intercept	-6.1847	0.9051	-6.833	**8.32e-12 *****
0.1 mg/L LDPE	1.8316	1.1796	1.553	0.12
5 mg/L LDPE	4.6598	1.1403	4.086	**4.38e-05 *****
Gills	1.6027	0.1999	8.018	**1.08e-15 *****
Intestinal lumen	5.6769	0.1832	30.989	**< 2e-16 *****
Pseudofeces	3.2183	0.1877	17.143	**< 2e-16 *****

3.3 Validation of analytical method

All samples were dyed with HES and then analyzed using polarized light. A selection of 15 slides were dyed with NR to validate the method. As shown in figure 3.14, there was a good correlation between sections stained with NR and examined with fluorescent light, and sections stained with HES and examined in polarized light. Some minor deviations may have occurred, as sections may change visually as the microtome cuts deeper into the paraffin blocks, but generally the observations made using the different techniques corresponded with each other.

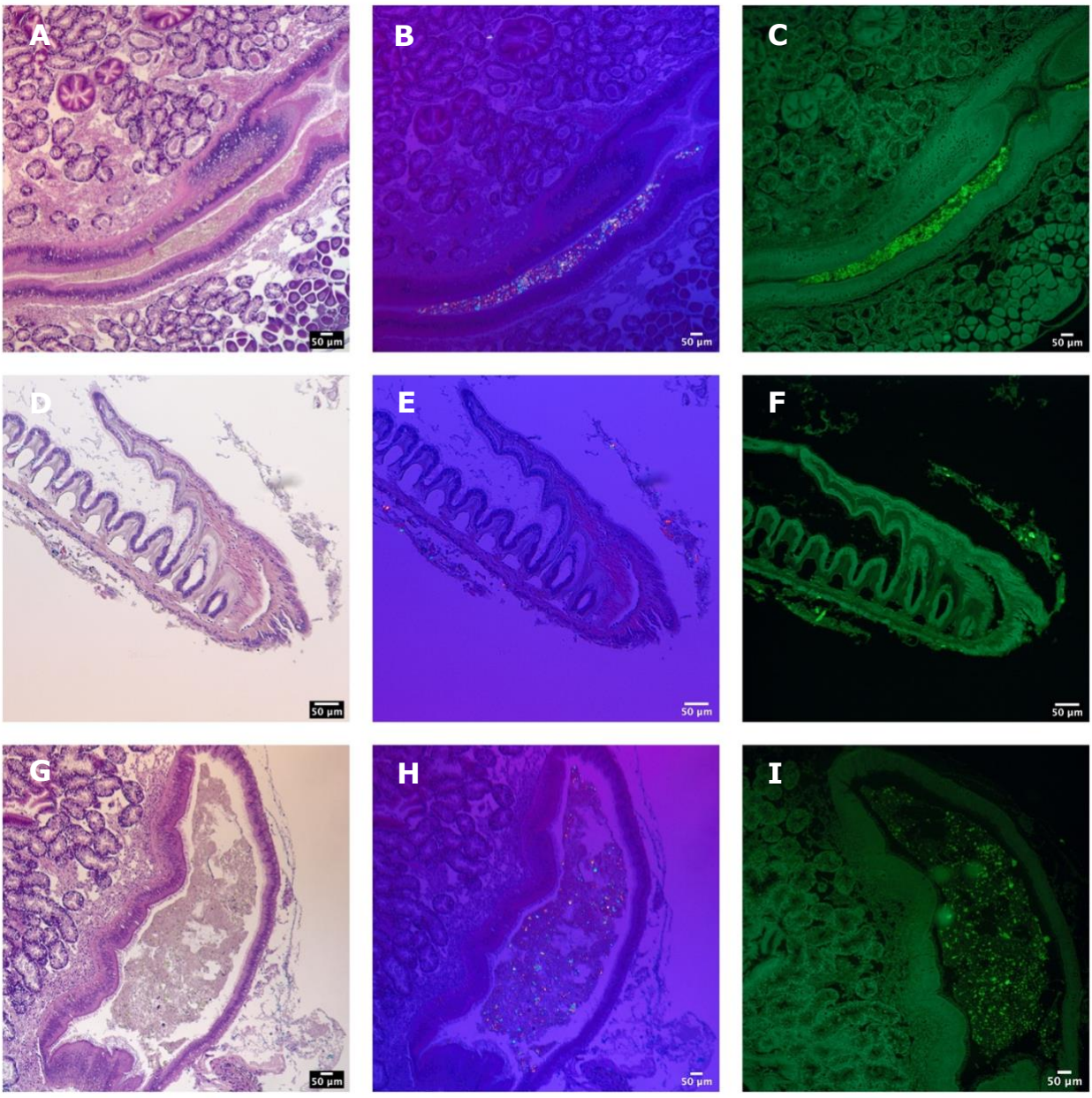

Figure 3.14. HES-stained tissue with microplastic particles observed with normal filter in (A) intestinal lumen following short-term exposure, (D) mucus around gills following short-term exposure and (G) intestinal lumen following short-term exposure. HES-stained tissue with microplastic particles observed in polarized light in (B) intestinal lumen, (E) mucus around gills and (H) intestinal lumen. NR-stained tissue with microplastic particles observed in fluorescent light in (C) intestinal lumen, (F) mucus around gills and (I) intestinal lumen.

3.3.1 Presence of LDPE in reference group and 0 mg/L group

The reference group included 25 mussels taken straight from the shore outside NIVA Solbergstrand and dissected and fixed without being exposed to either algae diet or plastic. When examining HES-stained sections from the reference group with polarized light microscopy, plastic particles were observed histologically in five of them. Only a few particles were observed, and these looked visually different from the particles in the mussels that had been exposed to LDPE. In four of the mussels the particles were observed in the intestinal lumen, and in one of the mussels particles were observed in pseudofeces. However, when examining the same sections, but stained with NR and under fluorescent light, no plastic particles were observed (figure 3.15 (C) and (F)), as no particles emitted green light as seen in figure 3.14 (C), (F) and (I).

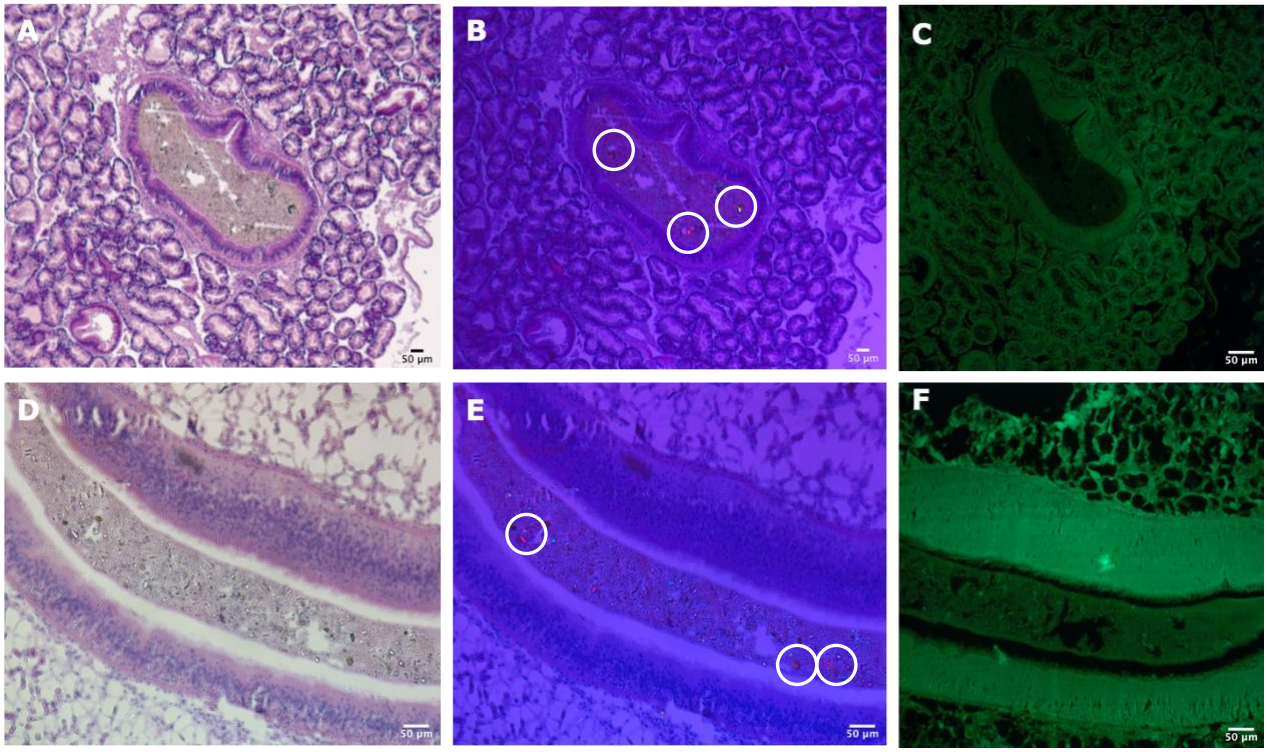

Figure 3.15. (A) and (D) show some particles in intestinal lumen of HES-stained tissue of two different reference mussels. (B) and (E) show the same HES-stained tissue examined under polarized light, with some particles lighting up in the intestinal lumen. (C) and (F) show no particles lighting up in the same tissue section of the two same mussels, dyed with NR and examined under fluorescence microscopy.

Figure 3.16 shows MPs in two different locations in the intestinal lumen of a mussel from the short-term exposure experiment which were not exposed to LDPE solution, and exposed to 2 mg/L algae. The number of particles in the intestinal lumen of this mussel was quite high despite the absence of plastic solution exposure. This was confirmed by the sections dyed with NR and observed with fluorescent light (figure 3.16 (C) and (F)).

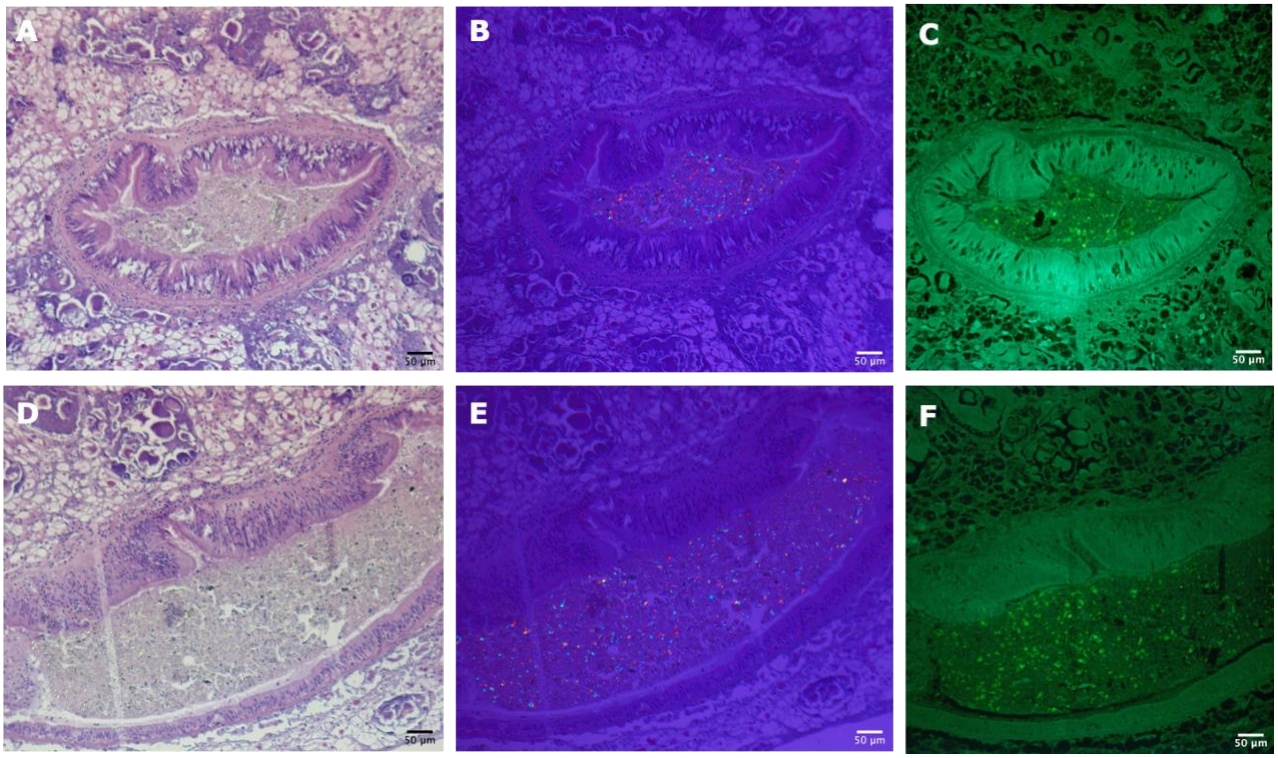

Figure 3.16. Microplastic particles in the intestinal lumen of an individual from the short-term exposure to 0 mg/L LDPE concentration. (A) & (D) show HES-stained tissue with normal filter, (B) & (E) show HES-stained tissue with polarized light, and (C) & (F) show NR-stained tissue with fluorescent light.

4. Discussion

This study aimed to quantify low-density polyethylene (LDPE) microplastic particles (MPs) in gills, digestive diverticula, intestinal lumen and pseudofeces of blue mussels (*Mytilus edulis*) after exposure through water to three concentrations of 20-25 μm particles (0 mg/L, 0.1 mg/L and 5 mg/L) and two concentrations of algae (0.7 mg/L and 2 mg/L) for 4 and 56 days. Further objectives were to analyze the effect of exposure time, algal concentration and LDPE concentration in the exposure medium on the number of particles in matrices (tissues and pseudofeces). The last aim was to validate whether the chosen dyeing and microscopy method (HES-dyeing and polarized light microscopy) was suitable for observing microplastics in tissues during histological examination of tissues.

4.1 Presence of LDPE microplastic in mussels

4.1.1 Presence of LDPE particles in gills

Ten mussels, out of the total of 58 from the short-term and long-term exposures where the gills were present on the histological section, had MPs in or around the gills after the exposure experiments. Most of the plastic particles appeared to be trapped in mucus around the gills. Mucus is produced by cells in the gills, and food particles are trapped and brought to the mouth and digestive system with the mucus (Hovgaard et al., 2001). The low number of individuals with particles on the gills suggests that the particles have either been brought to the mouth and further into the digestive system, or that the particles may have been expelled from the mussel via pseudofeces before sampling and dissection of the mussels.

Avio et al. (2015) exposed *M. galloprovincialis* to 100-μm polyethylene and polystyrene particles at 1.5 g/L for 7 days. Histological analyses of the mussels showed some MPs in gills, but much less than in the intestinal lumen, epithelium and tubules. Woods et al. (2018) exposed *M. edulis* to polyethylene terephthalate microfibers of length 460 μm and observed that the microfibers found in the gills were ejected from the mussels faster than from the digestive gland. After one hour of depuration, the number of microfibers in the gill had been reduced significantly. A 7-day exposure of *Mytilus* spp. to a mixture of polystyrene MPs of sizes 2 and 6 μm with a concentration of 32 μg/L was carried out by Paul-Pont et al. (2016). After the exposure, polystyrene particles were found mainly in the digestive tract, in the intestinal lumen. A few particles were observed in the mucus of the outer side of the gill epithelium. Polystyrene particles were not observed in other tissues. Revel et al. (2019)

exposed *Mytilus* spp. to three different concentrations (0.008 µg/L, 10 µg/L and 100 µg/L) of a microplastic mixture of polyethylene and polypropylene of a mean size of 204-287 µm for 10 days, followed by 10 days depuration. MPs were only observed with µFITR in the digestive glands of the mussels that had been exposed to 100 µg/L (0.75 particles/mussel), and no particles were observed in or on the gills at any of the concentrations. The findings of the present study of there being few to none MPs in or on the gills, are hence in accordance with previous studies. Uptake into gill cells seems to not be a common pathway, and particles are likely transported to the mouth. It is also worth noting that it is challenging to separate mucus threads produced by the gills from pseudofeces when observing the sections, meaning some of the particles detected around the gills could possibly be pseudofeces. Another possible explanation could be that particles were washed off from the gills during fixation, as they generally did not seem to be taken up into gill cells.

4.1.2 Presence of LDPE particles in intestinal lumen

The largest number of LDPE particles was observed in the intestinal lumen, after both short-term and long-term exposures. The statistical analysis used a model with a random factor for mussel ID. The significance of matrix type suggests that there was not necessarily a clear pattern in particle number in the different matrices of the same individual, as there was variation (Appendix D.2.8). For instance, high intestinal lumen content of microplastic did not indicate a high digestive diverticula content in the same mussel. The large number of particles in the intestinal lumen compared to the low number of particles in the digestive diverticula following both exposures suggests that the LDPE particles were not taken up and deposited in tissues of the mussels but transported through the intestine and egested as feces. Several aggregates of MPs were observed in intestinal lumens of mussels, while less MPs were observed in the digestive tubules and in the intestinal epithelium. It seems MPs are not frequently found in the intestinal epithelium cells of mussel after exposure. Sudies mostly report finding MPs in digestive diverticula, intestinal lumen and the stomach. In the present study, particles were often observed clustered in the intestinal lumen, which may have led to an underestimation of particle numbers by the Particle Analysis tool in ImageJ/Fiji, and hence one could expect an even higher number of particles in the intestinal lumen than used in the study analysis.

Pittura et al. (2018) examined the presence of LDPE particles in *M. galloprovincialis* after a 4-weeks exposure to LDPE (20-25 µm, 10 mg/L), and they too found the highest number of

particles in the intestinal lumen using polarized light microscopy. Several aggregates of MPs were observed in the intestinal lumen, while less MPs were observed in the digestive tubules and in the intestinal epithelium. Gonzáles-Soto et al. (2019) studied uptake and effects of exposure of *M. galloprovincialis* to PS MPs (0.5 μm and 4.5 μm). They predominantly found MPs in the lumen of the intestine and stomach, but they sporadically detected individual MPs in epithelial cells of the digestive tract, ducts and digestive tubules. The present study found no cases of MPs in epithelial cells, which could be due to the larger size of plastic particles used. In the Avio et al. (2015) study previously mentioned in 4.2.1, histological qualitative analyses observed aggregates of MPs in the intestinal lumen and digestive tissues post exposure. This supports the findings of clustered MPs in the present study. Kolandhasamy et al. (2017) exposed *M. edulis* to MPs at a concentration of 2000 microfibers/L, and a microplastic analysis was done after 48 hours. The microfibers were man-made, by cutting, plastic materials into tiny pieces, and of size 100 μm. They found that of the different organs they examined, the highest level of microplastic fibers per weight was in the intestine (171 items/g). Previous studies are hence in accordance with the findings of the present study, where the highest number of MP was in the intestinal lumen following exposure experiments.

4.1.3 Presence of LDPE particles in digestive diverticula

Although digestive diverticula were observed histologically for 26 (86.7%) of the mussels from the short-term exposure, and 28 (93.3%) of the mussels from the long-term exposure, plastic particles were only detected in the diverticula in 3 and 1 mussels, respectively. A maximum of 16 particles in one individual (short-term, 2 mg/L algae, 5 mg/L LDPE) were counted by ImageJ/Fiji. It is difficult to draw any conclusions from the fact that the highest number of particles was observed in a mussel exposed to the highest algae and plastic concentrations, as the basis for comparison was so low. As the digestive diverticula are the site for the intracellular digestion, the lack of plastic particles in this tissue indicates that they were not taken up into the digestive system and further into the tissues of the mussels. Possible fates of the MPs could be that 1) they were rejected before ingestion as pseudofeces, 2) they were transported directly from the stomach, through the intestine and egested as feces, 3) they were brought into the digestive tubules, but not internalized, and brought back to the stomach and intestine with the return-flow, or 4) a combination. This is also supported by the number of microplastics in pseudofeces, which was higher than in the digestive diverticula. An important factor in particle selection by mussels is particle-size. Uptake of particles through the epithelium in the intracellular digestion is seemingly size-dependent. Browne et

al. (2008) tracked polystyrene MPs in the hemolymph of *M. edulis* after exposure two different sizes of MPs (3 and 9.6 μm, 0.51 g/L) and found > 60% more of the smaller (3 μm) particles in the hemolymph than the bigger (9.6 μm) particles. They suggest that the accumulation of MPs in organism tissues increases with decreasing MP size. Van Cauwenberghe et al. (2015) exposed *M. edulis* to three different sizes of polystyrene MPs (10, 30 and 90 μm, 110 particles/mL) for 14 days, and carried out chemical digestion, following gut clearance to ensure only measuring uptake. They found that *M. edulis* had only taken up 10-μm MPs, and not any of the 30- or 90- μm MPs. They suggest a size limit for particle in mussel tissues must lie at 10 μm or somewhere between 10 and 30 μm. Von Moos et al. (2012) exposed *M. edulis* to non-uniformly shaped grains of HDPE MPs of size 0-80 μm for 3, 6, 12, 24, 48 and 96 hours and conducted histological analyses using polarized light microscopy on cryotome sections. They found MPs in the intestine, in the lumina of primary and secondary ducts of the digestive gland, as well as in vacuoles of digestive epithelial cells. The latter observation suggests that the MPs were internalized. Von Moos et al. (2012) were not able to quantify the particles or differentiate between sizes post exposure, and as the particles they used had a large size span also including very small MPs (0-80 μm), it is likely that the particles in the lower size boundary were the ones to be taken up into the digestive system. On the contrary, Gonçalves et al. (2019) exposed *M. galloprovincialis* to 6 μm and 10 μm polystyrene microplastic spheres at concentrations of 1000 particles/L for 90 minutes, and microplastic was only observed histologically in the intestinal lumen. No particles were found in gills or digestive gland diverticula. Nevertheless, studies generally show significance of particle size concerning translocation of MPs from the gut and into tissues. The MP size being too large in the present study could be a possible theory as to why the particles were not taken up.

4.1.4 Presence of LDPE particles in pseudofeces

After the intestinal lumen, plastic particles were found in the highest numbers in pseudofeces. Mussels get rid of excess particles through pseudofeces when the concentration of particles in the surrounding water is high, and when the particles are unknown or non-nutritive to the mussel (Jørgensen, 1981, Ward & Shumway 2004). This could explain why the highest number of particles were observed in pseudofeces in mussels exposed to 2 mg/L algae and 5 mg/L, as this is the treatment with the highest combined particle concentration. This agrees with the findings in a laboratory experiment carried out by Xu et al. (2017) who exposed striate beach clams (*Atactodea striata*) to 63-250 μm polystyrene microgranules and

quantified the number of MPs in pseudofeces after exposure to three different microplastic concentrations (control group, 10 items/L and 1000 items/L). The number of MPs in pseudofeces after exposure to the high MP concentration (1000 items/L) was larger than after exposure to the low MP concentration (10 items/L) and the control group. The level of MPs in pseudofeces is also dependent on particle size. Ward et al. (2019), who exposed *Mytilus* to polystyrene microspheres (19, 113, 287, 510 and 1000 μm) found that *Mytilus* rejected a large amount of the polystyrene microplastic spheres they were exposed to in pseudofeces. All 1000- μm spheres were rejected, and a significantly larger proportion of 510-μm spheres were rejected than ingested. Less of the 19-μm spheres were rejected in pseudofeces than ingested. 19 μm is close to the particle size used in the present study, and hence the observations of higher numbers of MPs in the intestinal lumen than in pseudofeces is in accordance with the Ward et al. (2019)-observation for this size class.

Environmental implications of MPs in pseudofeces

Despite mussels being able to reject some of the MPs from entering their digestive system by transporting them out in pseudofeces before ingestion, the MPs are not removed from the environment. By collecting plastic particles in mucus, the mussels are contributing to concentrating the particles, possibly making them bioavailable to other organisms when expelling pseudofeces. Due to traditionally large numbers of blue mussels in Norwegian coastal waters, they are important in the turnover of organic material in shallow waters. Both deposit-feeders in the benthos and other organisms may ingest the mussel pseudofeces containing concentrated amounts of MPs. When these organisms are eaten by larger organisms, this could be a path for MPs into the marine food web, and once in the food web, the plastic may be biomagnified with each step, potentially reaching humans at harmful concentrations (Wright et al., 2013; Santillo et al., 2017). No studies are yet done on the transfer of MPs from pseudofeces through the marine food web. Santana et al. (2017) suggest that the fast depuration of MPs in mussels, through feces and pseudofeces, reduces the threat to the higher trophic levels that have mussels included in their diet. However, the implications of MPs in the feces and pseudofeces are not considered. *Mytilus* spp. are widely used indicator species for monitoring of pollutants and changes in environmental conditions due to being sessile filter-feeding organisms unable to escape unfavorable circumstances (Bråte et al., 2018, Beyer et al., 2017). However, the detection of MPs in pseudofeces shows that mussels are able to refuse unwanted particles, which could challenge the perception of mussels as bioindicators. Ward et al. (2019) suggest in their study that bivalves are poor

bioindicators for microplastics, and that due to previous microplastic studies not quantifying particles in pseudofeces, studies have falsely reported corresponding microplastic concentrations inside bivalves and the surrounding environment.

4.2 Effects of exposure time, algal concentration, plastic concentration and matrix on the number of LDPE particles

There were no significant effects of the exposure duration or the concentration of algae in the exposure medium on the number of LDPE particles observed in the mussels. However, significant effects of the concentration of LDPE in the exposure medium and the matrix type being observed were found.

4.2.1 Effect of exposure time on the number of LDPE particles

There was no difference or pattern in presence of LDPE particles between the mussels from the short-term exposure and the long-term exposure. The mussels did not seem to accumulate more plastic particles with time. This suggests that the mussels handled the exposure, either by rejecting the particles in pseudofeces, or egesting them through feces. The present study is in accordance with the findings in some previous studies. Pittura et al. (2018), who, although the histological analysis was qualitative, observed no differences in the amount of LDPE MPs (20-25 μm, 10 mg/L) in *M. galloprovincialis* at different exposure times (7, 14 and 28 days). Von Moos et al. (2012) exposed *M. edulis* to non-uniformly shaped grains of HDPE MPs of size 0-80 μm for 3, 6, 12, 24, 48 and 96 hours, but found no effect of exposure duration on the intake of particles. Woods et al. (2018) exposed *M. edulis* to 460 μm polyethylene terephthalate microfibers at concentrations ranging from 3 particles/mL to 30 particles/mL, and particle-concentration in mussels were measured after 0, 1, 3, 6, 12, 24 and 72 hours. Maximum number of particles in *Mytilus* tissue occurred between 3 and 6 hours of exposure, however, with increasing depuration time, the particles in tissues decreased. After 6 hours in MP-free water, around 63% of the previously accumulated plastic was removed. This could be a an explanation as to why the present study found no effect of exposure time. The mussels were exposed to LDPE only once in the short-term exposure (3 days before dissection and fixation), and 2 days before dissection and fixation in the long-term exposure. It is possible that a large proportion of accumulated particles were egested, either as pseudofeces or feces, before the mussels were dissected and fixed, and that the result might have been different if the mussels in the long-term exposure were replenished with plastic every day. However, as

no significance of exposure time was in the Pittura et al. (2018)-study either, it is difficult to draw a clear conclusion.

4.2.2 Effect of algal concentration in exposure medium on the number of LDPE particles

When analyzing the whole dataset, the concentration of algal diet supplied in general had no effect on the presence of MPs in the mussels. Under conditions where food supply is optimal, the filtration of seawater by mussels will still continue at maximum speed, however, the filtration rate is reduced when the food concentration is too high. There is no universal agreement on which algal concentration induces reduced filtration due to saturation, however Pascoe et al. (2009) observed valve closure and reduced filtration rate in *M. edulis* of size 35 mm after feeding at 6 µg chlorophyll a/L for more than two hours. Bayne and Newell (1983) reports that pseudofeces production is induced in bivalves at a concentration of 2-10 mg/L of suspended particles. These are not definite numbers, but they do indicate that the particle concentrations in the exposure experiments are generally high enough to trigger filtration saturation. The excess food particles will likely be expelled as pseudofeces particles (Riisgård et al., 2011). The highest numbers of particles did occur in the intestinal lumen of mussels that had been exposed to 0.7 mg/L LDPE for both exposure times, which could indicate that a higher density of algae (2 mg/L) may have reduced the filtration rate or triggered a production of more pseudofeces. However, this did not apply to the other matrices, and no significance was found for algae concentration, which makes this observation too weak. The quantity of food needed to saturate the intake will differ depending on type, size and composition of the food source. This may have had an impact on the presence of MPs in the mussels, causing them to expel particles, and it may be that, in general, all mussels exposed to both algae and plastic particles experienced an excess of particles, reducing the variation in response.

Few studies describe the relation between food intake and accumulation of LDPE or other MPs in mussels, as most studies have either not fed the mussels continuously, there is no variation in food concentration, or the exposure time is too short. Chae and An (2019) examined the impact of algal food presence on ingestion and egestion rates of polyethylene MPs in *M. galloprovincialis,* by calculating the rates after depuration. They exposed mussels to three different treatments: (1) 10 mg/L PE and algal food (the green microalgae *Dunaliella salina,* $1x10^7$ cells/mL), (2) only 10 mg/L PE and (3) no PE and no algal food. The mussels were exposed for one hour before being transferred to clean seawater. Ingestion and egestion rates were measured after 6, 12, 18 and 24 hours of depuration. They found no significant

effect of the presence of food on ingestion rates of microplastic, but significant effects on egestion rates were observed. They observed that mussels exposed to only microplastics excreted the microplastics much faster than mussels exposed to microplastics and nutritional algal cells. The study suggests mussels are able to egest non-nutritional particles such as microplastics. This is supported by Jobling et al. (1987), who found that non-useful particles were egested faster than useful, nutritional particles in fish, which may account for mussels as well. Connor et al. (2016) suggested that intake and residence time of food in the intestine of *M. californiaus* was greater when the ingestion rates were low, similar to the scenario observed for microplastics in the intestine in the present study. However, due to differences in behavior of various materials and substances, and different reactions to these in mussels, these conclusions may not necessarily be transferred to discuss the effect of diet concentrations on microplastic behavior in mussels.

4.2.3 Effect of LDPE concentration in exposure medium on the number of LDPE particles

The concentration of LDPE in the exposure medium, as well as which matrix was being observed, had a significant effect on the number of plastic particles counted. In general, mussels exposed to 5 mg/L LDPE had the highest numbers of particles present in matrices, particularly in the intestinal lumen. This observation confirms findings from previous studies. Qu et al. (2017) exposed *M. edulis* to three types of MPs (beads, fragments and fibers) of two concentrations (100 particles/L and 1000 particles/L) for five days. They found that the abundance of microplastics was much higher in mussels which had been exposed to high concentrations of microplastics than in mussels in the low concentration treatment group. Woods et al. (2018) exposed *M. edulis* to 460 µm polyethylene terephthalate microfibers at concentrations ranging from 3 particles/mL to 30 particles/mL. Mussels exposed to 30 particles/mL ingested and retained significantly more particles than mussels exposed to the lower concentrations of 15 particles/mL and 3 particles/mL. Following exposure of *Mytilus* spp. to three different concentrations (0.008 µg/L, 10 µg/L and 100 µg/L) of a mixture of polyethylene and polypropylene MPs of a mean size of 204-287 µm for 10 days, followed by 10 days depuration, MPs were only observed with µFITR in the digestive glands of the mussels that had been exposed to the highest concentration, 100 µg/L (Revel et al., 2019). On the contrary, when Rist et al. (2019) exposed *Mytilus* to PS particles of size 50 µm at either 5 PS beads/L (low plastic concentration) or 100 beads/L (high plastic concentration), they found a similar distribution of PS beads inside the mussels and the water at both concentrations.

They suggest that the mussels did not adapt the filtration to the concentration of particles in the exposure medium. According to Bayne and Newell (1983) pseudofeces production is induced in bivalves at a concentration of 2-10 mg/L of suspended particles, and Riisgård et al. (2006) report that the valve gape of mussels is reduced at very high (and very low) concentrations of particles. They do not specify "very high", but it does indicate that the number of particles ingested by mussels would be reduced at a high concentration. However, despite the findings by Rist et al. (2019) that plastic concentration was not significant, the mussels in this study seem not to have been saturated by the LDPE concentration, and a higher concentration of plastic in the exposure medium leading to a higher number of MPs inside mussels seems to be an acceptable result.

4.3 Validation of analytical method

Histological studies of tissues and pseudofeces, and quantification of MPs were done by HES-dyeing histological sections followed by visual analysis using a microscope with polarized light and quantification of plastic in sections with ImageJ/Fiji. Validation was done by dyeing sections with NR followed by visual analysis using fluorescent light microscopy. The good correlation between plastic particles observed using the two techniques following the exposures confirmed that the use of polarized light on these sections was appropriate. The approach of using a microscope with a polarized light filter to visually observe HES-stained histological sections has previously only been applied to identify MPs histologically in mussels by Von Moos et al. (2012), Avio et al. (2015) and Pittura et al. (2018), where, in all three cases, this was done on cryotome (frozen) sections. This study used a formalin-based fixation and embedment in paraffin histowax, which enables archiving the material for later use in time series or environmental studies over time. The polarized light method also opens new possibilities of analyzing already archived paraffin blocks, for example to study microplastics. In this case, one must make sure to verify which types of plastic are degraded by xylene. The use of polarized light facilitates the analysis of microplastics in normal HES-dyed histological sections by making it possible to easily alternate between studying the presence of microplastics and studying potential effects in tissues. The use of ImageJ/Fiji has previously been used to measure MPs (Zhao et al., 2018) and fibers (Scott et al., 2019), but not to quantify MPs in biological tissues on histological sections. This proved an effective way to count small particles, which would have been challenging to do visually.

4.3.1 Presence of LDPE in reference group and 0 mg/L group

Plastic particles were not observed in any of the matrices of the reference group mussels. Some particles in the HES-dyed sections lit up with polarized light microscopy, however, no particles were observed with fluorescent light on sections dyed with NR. When visually analyzing field samples, the use of only polarized light may cause overestimation of plastic particles, as other types of materials, such as wood, paper, starch, mineral fibers and cement composites (Abbasi et al., 2017; Robinson & Davidson, n.d.), may light up as well. Several species of mantis shrimps have been shown to have body parts that reflect polarized light (Chiou et al., 2005), which may complicate the use of polarized light on organisms with unknown stomach contents which have not endured a depuration period before fixation. In field sampling, identification of plastic polymers should be of equal interest as quantification. As NR is a previously confirmed method, the counts done on the HES-sections from the reference mussels in the present study were dismissed to avoid overestimation of plastic particles.

Plastic particles were generally not observed with the applied method in mussels exposed to 0 mg/L LDPE. However, quite a few particles were observed in the intestinal lumen of one of the non-exposed mussels from the short-term exposure experiment, and this was confirmed by both HES-staining and polarized light, and NR and fluorescent light. This may be due to plastic already ingested in its natural environment before the start of the exposure, or due to human error when adding plastic solution to the aquaria.

4.4 Comparison with natural conditions

Exposure experiments to investigate occurrence, uptake, deposition and effects of microplastics in *Mytilus* spp. have previously been carried out in several studies (Thompson et al., 2004; Browne et al., 2008; Von Moos et al., 2012; Farrell and Nelson 2013; Van Cauwenberghe et al. 2015; Green et al., 2018; Pittura et al., 2018; Chae & An., 2019), with a variety of plastic polymers, plastic concentrations, exposure times and other adjustments in variables. Microplastic exposure experiments tend to use concentration levels which are much higher than in the environment. There is a limited amount of data on the concentrations of microplastic in the global marine environment. However, Gilfillan et al. (2009) measured a concentration 5.33 mg/L in the Californian Current System, while 3.02 mg/L is said to be the microplastic concentration in the North Pacific Central Gyre (Moore et al., 2001; Sussarellu et

al., 2016). These areas are hotspots for accumulation of marine debris (Lebreton et al., 2012), and thus the average concentration of microplastics in the global oceans will be lower than this. The Norwegian Environment Agency estimates that the microplastic concentration in Norwegian waters are comparable or lower than the global average. Although possibly too high, the microplastic levels used in this study (0.1 mg/L and 5 mg/L) are closer to environmental levels than in many other studies. A study by Paul-Pont et al. (2016) is an exception, as they exposed *Mytilus* spp. to a PS concentration of 32 μg/L. Concentrations are normally too high in these types of experiments. Von Moos et al. (2012) exposed mussels to 2.5 g/L HDPE for 96 hours, which is 500 times higher than the highest exposure level in this study (5 mg/L), and 25 000 times higher than the lowest exposure level (0.1 mg/L). Browne et al. (2008) observed translocation of MPs to the circulatory system in *M. edulis* following 3- and 12- hour exposures of mussels to 0.51 g/L polystyrene, which is 100 and 5000 times higher than the highest and lowest concentrations used in this study, respectively. Pittura et al. (2018) exposed *M. galloprovincialis* to 10 mg/L LDPE (20-25 μm) for 4 weeks. Although not an extremely high concentration compared to some other studies, they used a concentration twice as high as the highest concentration in the present study. Lower microplastic concentrations and longer periods of exposure are more realistic conditions. The present study managed this to a greater degree than many other research projects.

5. Conclusions

LDPE microplastic particles were observed histologically in all four matrices (gills, intestinal lumen, digestive diverticula and pseudofeces) following exposure to two plastic concentrations. The highest number of ingested MPs were observed in the intestinal lumen, followed by in pseudofeces.

Exposure time (4 and 56 days) and algae concentration (0.7 mg/L and 2 mg/L) in the exposure medium had no significant effect on the number of plastic particles observed in the mussel matrices. LDPE concentration (0, 0.1 or 5 mg/L) in the exposure medium affected the presence and quantity of LDPE particles in the matrices. A higher number of LDPE particles were observed in mussels exposed to the high LDPE concentration (5 mg/L) than in mussels exposed to the low LDPE concentration (0.1 mg/L).

The use of polarized light microscopy on HES-dyed histological sections, followed by quantification in ImageJ/Fiji was a suitable and useful method for identifying MPs in *M. edulis* matrices after exposure experiments. The use of NR-stain and fluorescent light microscopy validated the results.

6. Future research

Microplastics have become ubiquitous in the marine environment, and methods for examination of intake, uptake, presence and impacts of microplastics in these organisms vary greatly, creating a need for standardization.

More knowledge of the fate of microplastics in marine organisms is needed. Future research should focus on factors impacting whether particles are taken up in the digestive gland and translocated to tissues or pass through the digestive tract and are egested as feces. Further work should also be done on which types of materials, and which shapes and sizes of microplastics are expelled in mussel pseudofeces, the proportion of plastic in exposure media that is rejected in pseudofeces, as well as the trophic transfer of plastic through pseudofeces. If observations are that large proportions of plastic particles encountered by mussels are rejected before ingestion and uptake, it would indicate a mismatch between the inside of the mussel and the surrounding environment. These types of studies could possibly question the position of *Mytilus* spp. as a key bioindicator.

7. Reference list

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Appendix A: Calculations of algae volumes

Dry weight of Shellfish Diet is always 8%. 100 grams of concentrate will yield 8 grams of dry residue (Rikard & Walton, 2012).

$1\ ml\ Shellfish\ Diet\ concentrate$

$$= 750\ mg\ (weighed\ in\ laboratory\ at\ NIVA\ Solbergstrand)$$

$$1\ mg\ concentrate = \frac{1\ ml}{750\ mg} = 0.00133\ ml/mg$$

$2\ mg/L\ algae\ concentration$

$3\ L\ water\ in\ aquaria$

$6\ mg\ algae\ in\ each\ aquaria$

$$\frac{6\ mg}{0.08} = 75\ mg\ concentrate\ in\ each\ aquaria$$

$$75\ mg\ concentrate\ x\ 0.00133\ ml/mg = 0.1\ ml\ concentrate = \mathbf{100\ \mu l\ concentrate}$$

$0.7\ mg/L\ algae\ concentration$

$3\ L\ water\ in\ aquaria$

$2.1\ mg\ algae\ in\ each\ aquaria$

$$\frac{2.1\ mg}{0.08} = 26.25\ mg\ concentrate\ in\ each\ aquaria$$

$$26.25\ mg\ concentrate\ x\ 0.00133\ ml/mg = 0.035\ ml\ concentrate =$$

$35\ \mu l\ concentrate$

Appendix B: Laboratory equipment

B.1 Equipment for opening shells and extraction of tissue

- Scalpel
- Scalpel blades
- Anatomic forceps
- Falcon tubes for Davidson's fix
- Pen
- Gloves
- Tissue paper
- Bench coverage paper
- Ruler

B.2 Equipment for dehydration and paraffinization

- Cassettes with lid
- Molds in stainless steel
- Nitrile gloves (TouchNTuff, VWR)
- Forceps
- Paraffin wax molding machine with forceps warmer (Kunz instruments)
- Cold plates (TissueTek II cold plate, Lab-Tek Division Miles/Sakura)
- Histokinette (Leika TP1020)
- Bin for chemicals
- Freezer cabinet

Table B.1 Standard protocol for dehydration and paraffin infiltration of tissues in Histokinette (Leica TP 1020) (Norwegian Institute of Marine Research).

Bath	Solution	Time (minutes)
1	Empty	Start
2	70% ethanol	1 hour
3	70% ethanol	1 hour
4	80% ethanol	1 hour
5	96% ethanol	2 hours
6	96% ethanol	2 hours
7	100% ethanol	2 hours
8	100% ethanol	2 hours
9	Xylene	2 hours
10	Xylene	2 hours
11	Paraffin (Histo-wax)	2 hours
12	Paraffin (Histo-wax)	2 hours

B.3 Equipment for sectioning

- Microtome (Microm HM355S)
- Blades for microtome (Accu-Edge® (S35))
- Objective glasses (ThermoScientific, 76x26mm)
- Small paintbrush
- Knife

- Water bath (Leica HI 12010)
- Drying cabinet (Labnet)
- Gloves (Blue Nitrile gloves Semperguard, Thermo Fisher)
- Fridge
- Microscope
- Lens paper

B.4 Equipment for dyeing

- Fume hood
- Cover glass (24x50mm, VWR)
- Nitrile gloves (TouchNTuff, VWR)
- Forceps
- Containers for baths
- Objective glass-holders
- Stop-watch

Table B.2 Baths for deparaffinization and dyeing with time (minutes) in each bath (Norwegian Institute of Marine Research)

DEPARAFFINIZATION		
Bath	**Solution**	**Time (minutes)**
1	Xylene	10
2	100% ethanol	5
3	100% ethanol	5
4	96% ethanol	5
5	80% ethanol	5
6	50% ethanol	5
Container under water tap	Water from tap	5
DYEING		
Bath	**Solution**	**Time (minutes)**
7	Newly filtrated Hematoxylin	1.5
Container under water tap	Water from tap	4
Separate container	1% Erythrosin	1.5
Container under water tap	Water from tap	Dip to remove excess dye before transferring to ethanol
8	96% ethanol	45 seconds
9	100% ethanol	1
Separate container	Alcoholic Saffron solution	20 seconds
10	100% ethanol	1
11	Xylene	5 (minimum)
12	Xylene	5 (minimum)
(13)	Xylene	5 (minimum)

Appendix C: Chemicals, solutions and reagents

C.1 Chemicals

- Milli-Q water
- Absolute alcohol prima (100%) (Antibac)
- 96% ethanol (Antibac)
- Acetic acid, 99-100% (Merck)
- Filtrated seawater (From wet laboratory)
- Formaldehyde, 37% (Merck)
- Glycerol (Sigma-Aldrich)
- Paraffin, Histowax (Histolab Products)
- Xylene (AnalaR NORMAPUR, VWR)
- Shandon Instant
- Shandon Instant Hematoxylin (Thermo Fisher Scientific, Nerliens)
- Erythrosin B (C.I. 45430, Merck, artikkel nr 115936, VWR)
- Safran ekstrakt, Alkoholisk (RAL diagnostics, VWR).
- Histokitt (Hech assistent, Chemi-Teknik AS).
- Hemacolor® Rapid staining of blood smear (Merck)
- NR
- Methanol

C.2 Davidsons's fixation solution

Reagent	Formula	Supplier	Volume	Final concentration	Notes
Glycerol	$C_3H_8O_3$	Sigma	500 ml	10%	The 4 first reagents can be mixed and stored for up to 6 months before use
37% Formaldehyde	HCHO	Merck	1000 ml	7.4%	
96% Ethanol	C_2H_5OH	Antiback	1500 ml	28.8%	
Filtrated seawater, room temperature			1500 ml	30%	
Acetic acid*	CH_3COOH	Merck	500 ml	10%	* Do not add before use of solution

Appendix D: Statistical analyses

Testing of models

```
library(tidyverse)
library(readxl)
library(mgcv)
library(lme4)
library(MuMIn)

MusselData<- read_excel("MP Blue mussels_Full dataset.xlsx", col_types =
"text", na = "NA")

#Assigning the type of data in each column
MusselData$Algae<-as.factor(MusselData$`Algae`)
MusselData$Plastic<-as.factor(MusselData$`Plastic`)
MusselData$Matrix<-as.factor(MusselData$`Matrix`)
MusselData$Exposure<-as.factor(MusselData$`Exposure`)
MusselData$ID<-as.factor(MusselData$`ID`)
MusselData$Particles<-as.integer(MusselData$`Particles`)
```

D.1 lmer models with poisson distributed data

```
m1 = glmer(Particles ~ Algae * Plastic + Matrix + Exposure + (1|ID), family
= poisson, data = na.omit(MusselData))

## Warning in checkConv(attr(opt, "derivs"), opt$par, ctrl =
control$checkConv, :
## Model failed to converge with max|grad| = 0.16367 (tol = 0.001,
component 1)

anova(m1)

## Analysis of Variance Table
##               Df Sum Sq Mean Sq   F value
## Algae          1    0.1    0.13    0.1258
## Plastic        2   27.0   13.52   13.5199
## Matrix         3 5654.6 1884.88 1884.8755
## Exposure       1    0.0    0.02    0.0178
## Algae:Plastic  2    0.3    0.16    0.1617

m2 = glmer(Particles ~ Algae + Plastic + Matrix + Exposure + (1|ID), family
= poisson, data = na.omit(MusselData))

## Warning in checkConv(attr(opt, "derivs"), opt$par, ctrl =
control$checkConv, :
## Model failed to converge with max|grad| = 0.0197735 (tol = 0.001,
component 1)

anova(m2)

## Analysis of Variance Table
##          Df Sum Sq Mean Sq   F value
```

```
## Algae     1    0.2    0.20    0.2022
## Plastic   2   27.1   13.54   13.5373
## Matrix    3 5646.0 1882.01 1882.0104
## Exposure  1    0.1    0.06    0.0616
```

```
m3 = glmer(Particles ~ Algae + Plastic + Matrix + (1|ID),family = poisson,
data = na.omit(MusselData))
anova(m3)
```

```
## Analysis of Variance Table
##         Df Sum Sq Mean Sq   F value
## Algae    1    0.2    0.21    0.2051
## Plastic  2   26.9   13.44   13.4421
## Matrix   3 5645.6 1881.85 1881.8522
```

```
m4 = glmer(Particles ~  Plastic + Matrix + (1|ID),family = poisson, data =
na.omit(MusselData))
anova(m4)
```

```
## Analysis of Variance Table
##         Df Sum Sq Mean Sq  F value
## Plastic  2   26.8   13.42   13.422
## Matrix   3 5645.5 1881.83 1881.828
```

D.1.1 Checking AIC for the best fit model:

```
AICc(m1,m2,m3,m4)
```

```
##    df     AICc
## m1 11 3474.483
## m2  9 3470.376
## m3  8 3468.248
## m4  7 3466.216
```

m4 has the best AIC.

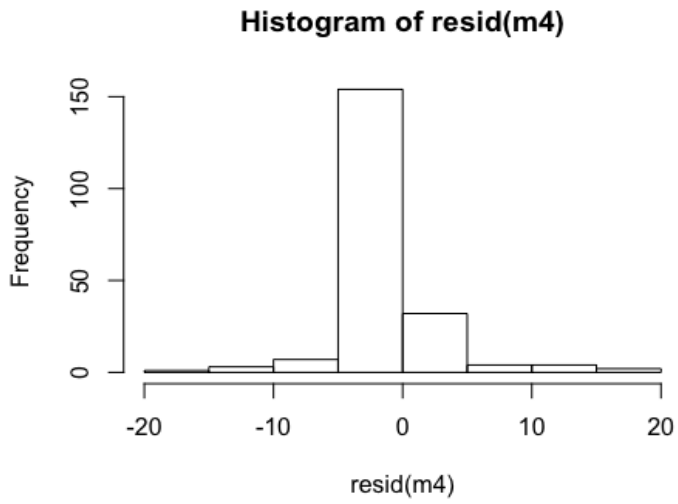
```
summary(m4)
```

```
## Generalized linear mixed model fit by maximum likelihood (Laplace
##   Approximation) [glmerMod]
##  Family: poisson  ( log )
## Formula: Particles ~ Plastic + Matrix + (1 | ID)
##    Data: na.omit(MusselData)
##
##      AIC      BIC   logLik deviance df.resid
##   3465.7   3489.0  -1725.8   3451.7      200
##
## Scaled residuals:
##     Min      1Q  Median      3Q     Max
## -10.859  -0.600  -0.128  -0.020  46.899
##
## Random effects:
##  Groups Name        Variance Std.Dev.
```

```
##  ID     (Intercept) 9.794    3.13
## Number of obs: 207, groups:  ID, 60
##
## Fixed effects:
##                    Estimate Std. Error z value Pr(>|z|)
## (Intercept)         -7.3862     0.9945  -7.427 1.11e-13 ***
## Plastic0.1           2.3913     1.1884   2.012   0.0442 *
## Plastic5             5.7662     1.1919   4.838 1.31e-06 ***
## MatrixGills          1.6021     0.1999   8.015 1.10e-15 ***
## MatrixIntestine      5.6769     0.1832  30.989  < 2e-16 ***
## MatrixPseudofeces    3.2181     0.1877  17.142  < 2e-16 ***
## ---
## Signif. codes:  0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1
##
## Correlation of Fixed Effects:
##             (Intr) Pls0.1 Plstc5 TssGll TssInt
## Plastic0.1  -0.702
## Plastic5    -0.788  0.580
## MatrixGills -0.168 -0.001  0.001
## TissuIntstn -0.184  0.000  0.000  0.910
## TissuPsdfcs -0.179  0.000  0.000  0.888  0.968
```

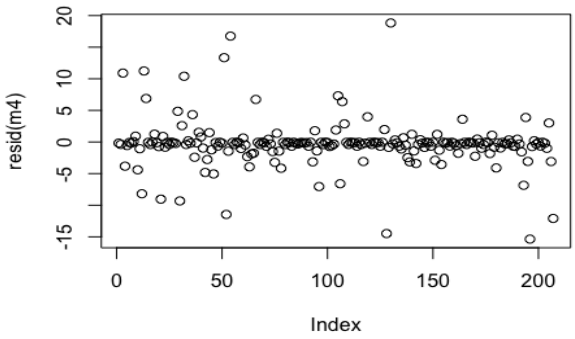
D.1.2 Model residuals of the best fit model

```
hist(resid(m4))
```



The histogram looks normally distributed, hence m4 is a model of good fit.

```
plot(resid(m4))
```

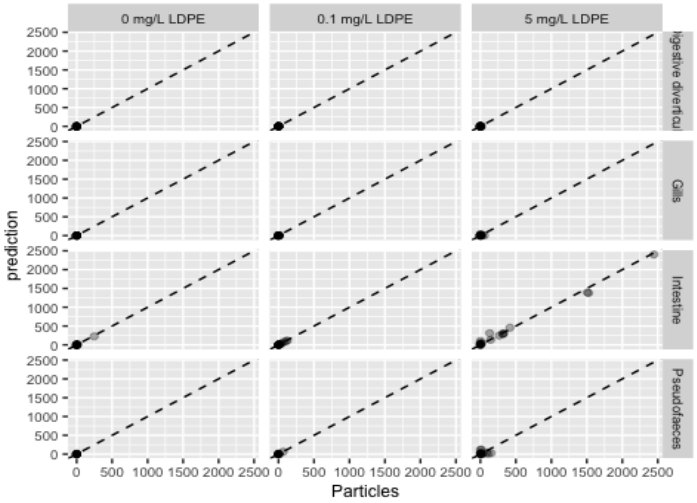


In a normal case, we would expect no patterns from the plot of residuals. In this case, the model predicts the zeroes. The negative values indicate that the predictions were too high. However, overall, the residual plot looks okay.

D.1.2 Prediction plots

```
MusselData = na.omit(MusselData)
fits = predict(m4, type = "response")
MusselData$prediction = fits

Plastic_labels <- c("0" = "0 mg/L LDPE", "0.1" = "0.1 mg/L LDPE", "5" = "5
mg/L LDPE")
MusselData %>%
  ggplot(aes(x = Particles, y = prediction)) +
  geom_point(alpha = 0.3) +
  facet_grid(Matrix~Plastic, labeller = labeller(Plastic = Plastic_labels))
+
  geom_abline(intercept = 0, slope = 1, linetype = "dashed")+
  theme(text=element_text(size=9))
```



This plot predicts the effect of plastic concentration and **Matrix** for different input values. This prediction works quite well. *However, I will try a generalized additive model with the mgcv package.*

D.2 mgcv package and a generalized additive model (gam)

The generalized additive model (gam) is a generalized linear model (glm), but with smoothing functions. Here, I have used a gam approach with a negative binomial family, which uses only parametric parameters, but only one smoothing functions- the random factor for the different mussel individuals (s(ID, bs="re", k=10)).

I have tested 4 different versions of gam-models.

D.2.1 Gam model 1

```
model.gam1 <- gam(Particles ~ Algae * Plastic +
                    Matrix + Exposure +
                    s(ID, bs="re"),
                  data = na.omit(MusselData),
                  family = poisson,
                  method = "REML", select = TRUE)
summary(model.gam1)

##
## Family: poisson
## Link function: log
##
## Formula:
## Particles ~ Algae * Plastic + Matrix + Exposure + s(ID, bs = "re")
##
## Parametric coefficients:
##                     Estimate Std. Error z value Pr(>|z|)
## (Intercept)          -6.7974     1.4254  -4.769 1.85e-06 ***
## Algae2                0.7750     1.8895   0.410  0.68168
## Plastic0.1            2.4187     1.7760   1.362  0.17325
## Plastic5              4.8155     1.7176   2.804  0.00505 **
## MatrixGills           1.6026     0.1999   8.017 1.08e-15 ***
## MatrixIntestine       5.6770     0.1832  30.989  < 2e-16 ***
## MatrixPseudofeces     3.2182     0.1877  17.142  < 2e-16 ***
## ExposureShort-term    0.3397     0.9699   0.350  0.72615
## Algae2:Plastic0.1    -1.1614     2.5190  -0.461  0.64474
## Algae2:Plastic5      -0.2366     2.4368  -0.097  0.92266
## ---
## Signif. codes:  0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1
##
## Approximate significance of smooth terms:
##         edf Ref.df Chi.sq p-value
## s(ID) 43.15     54   7772  <2e-16 ***
## ---
## Signif. codes:  0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1
##
## R-sq.(adj) =  0.982   Deviance explained = 94.2%
## -REML = 1724.3  Scale est. = 1         n = 207
```

From model.gam1, it seems the interaction between Algae and Plastic (Algae*Plastic) can be removed, as it is not significant.

D.2.2 Gam model 2

```
model.gam2 <- gam(Particles ~ Plastic + Algae +
                    Matrix + Exposure +
                    s(ID, bs="re"),
                  data = na.omit(MusselData),
                  family = poisson,
                  method = "REML", select = TRUE)

summary(model.gam2)

##
## Family: poisson
## Link function: log
##
## Formula:
## Particles ~ Plastic + Algae + Matrix + Exposure + s(ID, bs = "re")
##
## Parametric coefficients:
##                     Estimate Std. Error z value Pr(>|z|)
## (Intercept)         -6.5159     1.1301  -5.766 8.13e-09 ***
## Plastic0.1           1.8316     1.2177   1.504    0.133
## Plastic5             4.6741     1.1777   3.969 7.22e-05 ***
## Algae2               0.2862     0.9372   0.305    0.760
## MatrixGills          1.6027     0.1999   8.018 1.08e-15 ***
## MatrixIntestine      5.6769     0.1832  30.989  < 2e-16 ***
## MatrixPseudofeces    3.2183     0.1877  17.142  < 2e-16 ***
## ExposureShort-term   0.3275     0.9369   0.350    0.727
## ---
## Signif. codes:  0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1
##
## Approximate significance of smooth terms:
##         edf Ref.df Chi.sq p-value
## s(ID) 44.76     56   8164  <2e-16 ***
## ---
## Signif. codes:  0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1
##
## R-sq.(adj) =  0.982   Deviance explained = 94.2%
## -REML = 1727.9  Scale est. = 1         n = 207
```

Summary of model.gam2 shows Algae is the least significant (0.760), and is excluded from the next model.

D.2.3 Gam model 3

```
model.gam3 <- gam(Particles ~ Plastic +
                    Matrix + Exposure +
                    s(ID, bs="re"),
                  data = na.omit(MusselData),
                  family = poisson,
                  method = "REML", select = TRUE)
summary(model.gam3)
```

```
##
## Family: poisson
## Link function: log
##
## Formula:
## Particles ~ Plastic + Matrix + Exposure + s(ID, bs = "re")
##
## Parametric coefficients:
##                     Estimate Std. Error z value Pr(>|z|)
## (Intercept)          -6.3621     1.0217  -6.227 4.75e-10 ***
## Plastic0.1            1.8364     1.1967   1.535    0.125
## Plastic5              4.6663     1.1575   4.031 5.55e-05 ***
## MatrixGills           1.6027     0.1999   8.018 1.08e-15 ***
## MatrixIntestine       5.6769     0.1832  30.989  < 2e-16 ***
## MatrixPseudofeces     3.2183     0.1877  17.143  < 2e-16 ***
## ExposureShort-term    0.3302     0.9205   0.359    0.720
## ---
## Signif. codes:  0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1
##
## Approximate significance of smooth terms:
##         edf Ref.df Chi.sq p-value
## s(ID) 45.59     57   9489  <2e-16 ***
## ---
## Signif. codes:  0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1
##
## R-sq.(adj) =  0.982   Deviance explained = 94.2%
## -REML = 1728.8  Scale est. = 1         n = 207
```

Summary of model.gam3 shows that exposure is the least signficiant (0.720), and is excluded from the next model.

D.2.4 Gam model 4

```
model.gam4 <- gam(Particles ~ Plastic +
                    Matrix  +
                    s(ID, bs="re"),
                  data = na.omit(MusselData),
                  family = poisson,
                  method = "REML", select = TRUE)
summary(model.gam4)
```

```
##
## Family: poisson
## Link function: log
##
## Formula:
## Particles ~ Plastic + Matrix + s(ID, bs = "re")
##
## Parametric coefficients:
##                   Estimate Std. Error z value Pr(>|z|)
## (Intercept)        -6.1847     0.9051  -6.833 8.32e-12 ***
## Plastic0.1          1.8316     1.1796   1.553     0.12
## Plastic5            4.6598     1.1403   4.086 4.38e-05 ***
## MatrixGills         1.6027     0.1999   8.018 1.08e-15 ***
## MatrixIntestine     5.6769     0.1832  30.989  < 2e-16 ***
## MatrixPseudofeces   3.2183     0.1877  17.143  < 2e-16 ***
## ---
## Signif. codes:  0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1
##
## Approximate significance of smooth terms:
##         edf Ref.df Chi.sq p-value
## s(ID) 46.36     57   9496  <2e-16 ***
## ---
## Signif. codes:  0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1
##
## R-sq.(adj) =  0.982   Deviance explained = 94.2%
## -REML = 1729.6  Scale est. = 1         n = 207
```

D.2.5 Checking the AIC of the 4 different gam.models:

```
AICc(model.gam1,model.gam2,model.gam3,model.gam4)
```

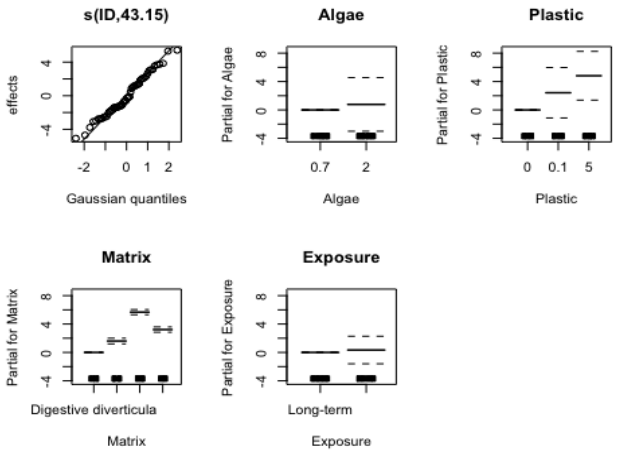
```
##                   df     AICc
## model.gam1 53.48100 3326.148
## model.gam2 53.07989 3325.246
## model.gam3 52.90984 3324.927
## model.gam4 52.67498 3324.345
```

The models have very similar AICs. The general rule of considering AIC is that if the difference between the AIC with the lowest and the second lowest model is less than 2, then all the models are quite equally well fitted. **I have therefore chosen the simplest model, model.gam4.**

D.2.6 Plots of partial effects

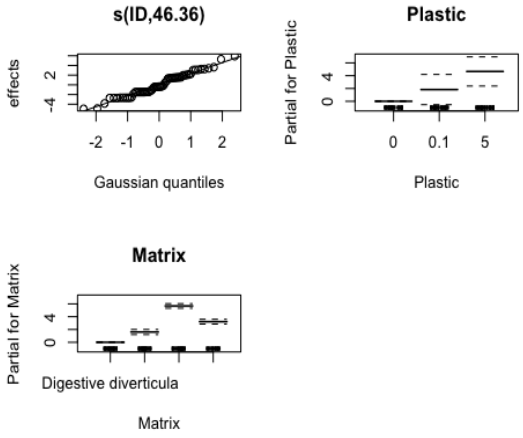
I will compare the plots of partial effects of the different variables to confirm model.gam4:

```
plot(model.gam1, all.terms=TRUE, pages = 1)
```



The plots of partial effects show how much each factor contributes to predicting the number of particles. The plots show little effect from Algae and Exposure. The dotted lines represent confidence intervals. The confidence intervals of 2 mg/L Algae includes the predictions for 0.7 mg/L. This supports the decision to choose model.gam4.

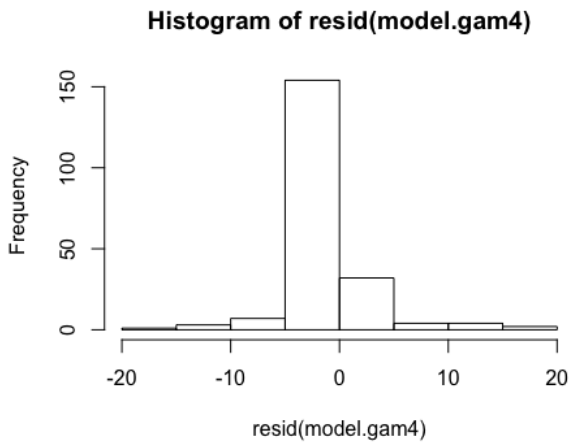
```
plot(model.gam4, all.terms=TRUE, pages = 1)
```



The partials plots for Plastic and Matrix show a larger effect on the number of particles. All the partials for the different Matrixes are different from each other. Partials for 0.1 mg/L and 5 mg/L plastic concentrations are not completely different, but different enough.

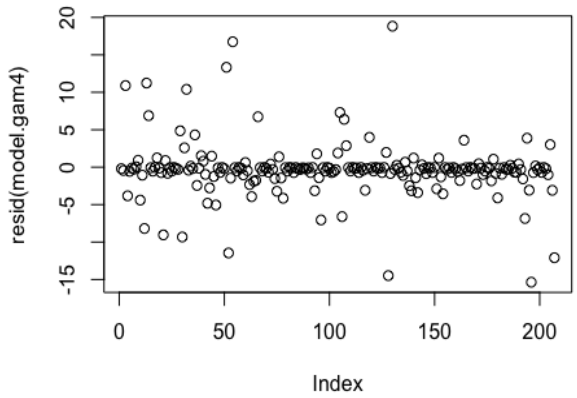
D.2.7 Model residuals of the simplest model

```
hist(resid(model.gam4))
```



The histogram looks normally distributed, hence model.gam4 is a model of good fit.

```
plot(resid(model.gam4))
```



Overall, the residual plot looks okay.

D.2.8 Random effects

Random effects are used as the data includes Matrices from the same individual mussels.

```
gam.vcomp(model.gam4)
```

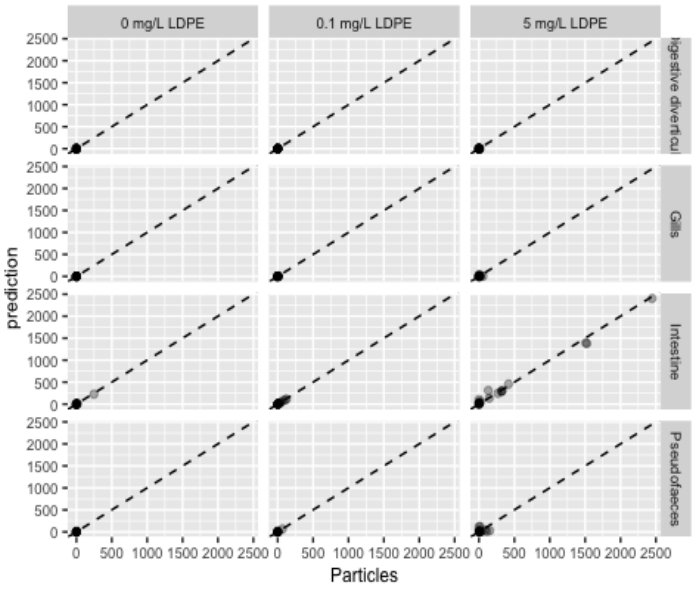
```
##
## Standard deviations and 0.95 confidence intervals:
##
##         std.dev    lower    upper
## s(ID) 3.171366 2.409529 4.174078
##
## Rank: 1/1
```

The confidence interval does not include 0, hence the individuals seem to vary from each other.

D.2.9 Prediction plots

```
MusselData = na.omit(MusselData)
fits = predict(model.gam1, type = "response", se = TRUE)
MusselData$prediction = fits$fit
MusselData$se = fits$se.fit

Plastic_labels <- c("0" = "0 mg/L LDPE", "0.1" = "0.1 mg/L LDPE", "5" = "5
mg/L LDPE")
MusselData %>%
  ggplot(aes(x = Particles, y = prediction)) +
  geom_point(alpha = 0.3) +
  facet_grid(Matrix~Plastic, labeller = labeller(Plastic = Plastic_labels))
+
  geom_abline(intercept = 0, slope = 1, linetype = "dashed")+
  theme(text=element_text(size=9))
```



This plot predicts the effect of the various variables for different input values. This prediction works well.

Which model to use- gmler or gam?

As the gmler model gives an error warning for some of the different variants of the model, I have decided to use the second approach- gam, with gam model 4.