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Environmental Sciences and Natural Resource Management

Effect of nanoplastics in the marine organism *Tisbe Battagliai*

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Environment and natural resources

Preface

This master's thesis was written at the faculty for Environmental Sciences and Natural Resource Management (MINA), Norwegian University of Life Sciences (NMBU) in 2018/2019 and was a part of a project at Norwegian Institute for Water Research (NIVA) called "Plastics- does size matter?". All experimental work was carried out in NIVA´s lab in Oslo between August and March 2018-2019. Main supervisor at NMBU and NIVA was Knut Erik Tollefsen, and co-supervisor was Tânia Gomes (NIVA).

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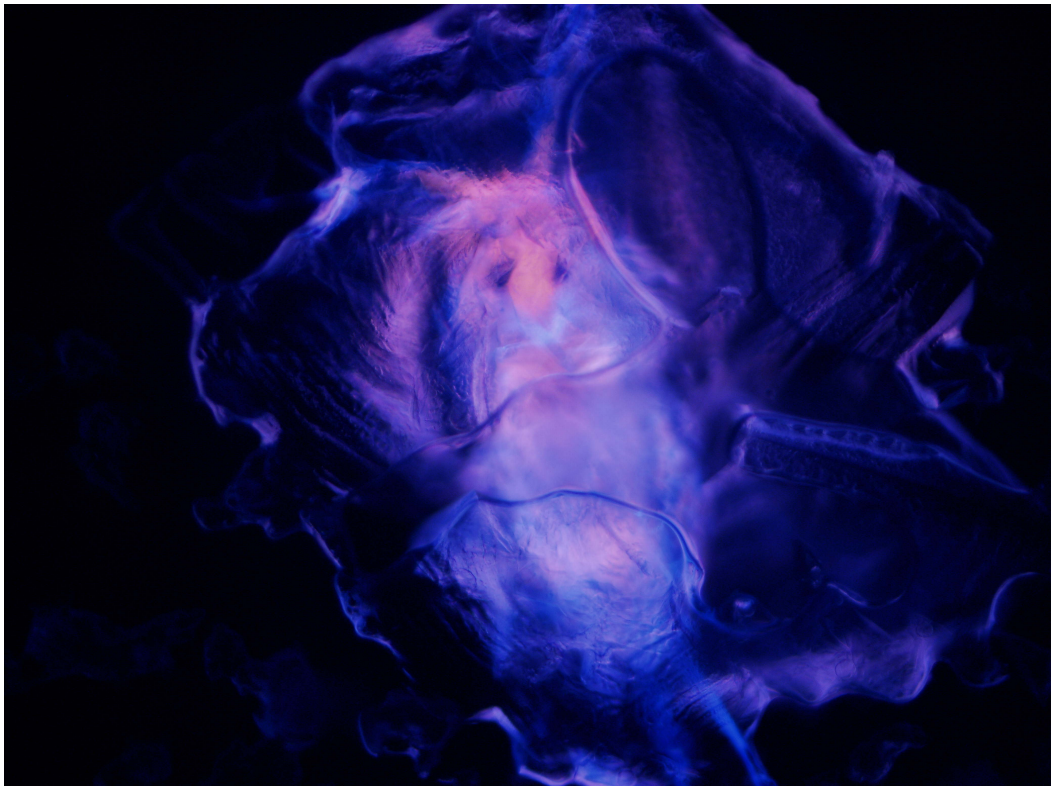

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Abstract

Plastic is a material made out of polymer chains derived from oil and gas, and together with additives these polymers have numerous applications. Plastic is used in daily life as wrapping around food, in cosmetics and in clothes, and is inevitable nowadays. However, of all the 322 million metric tons of plastic that are produced annually, between 4.8 and 12.7 million metric tons are estimated to enter the oceans. When in the oceans, plastic debris can be degraded by physical and chemical processes to micro- and nanoplastics (MP and NP). These small particles (<5 mm) may be a threat to organisms in the marine environment, as they can affect their development, reproduction and survival. Microcrustaceans are widespread in the oceans with both benthic and pelagic species, which may be affected by plastic particles in the oceans. Many of the microcrustacean species are filter feeders and do not select their food intake. These animals have a high risk of ingesting NP particles (<100 nm), and can thus be suitable species to test and evaluate for ecotoxicological effects of NPs. In this thesis, the toxicity of the NP particles polystyrene (PS) and poly (methyl methacrylate) (PMMA), both non-functionalised and with functional carboxylic (PS-COOH and PMMA-COOH) and aminated (PS-NH_2) groups were studied in the copepod *Tisbe battagliai* to assess potential adverse effects of these particles. The NP particles were characterised by dynamic light scattering (DLS), where size (Z-average), surface charge and polydispersity were measured in Milli-Q water (MQW) and natural seawater (NSW), to describe their properties. Acute mortality and oxidative stress were determined for *T. battagliai* when exposed to the same NP particles. The NPs PS-NH_2 and PMMA-COOH were found to not aggregate in NSW (Z-average < 140nm), while the other particles formed agglomerates (Z-average > 1800nm). Whereas most particles displayed a negative surface charge, PS-NH_2 was positively charged in both medias. To test acute mortality, copepods were exposed to different concentrations of NPs (0, 0.5, 1, 5, 10, 25, 50 and 100 μg/mL) in NSW for 48 hours. PS-NH_2 was shown to be the most toxic particle, with an EC_{50} value of 7.8 μg/mL. This particle was therefore chosen for further studies to determine reactive oxygen species (ROS) formation and lipid peroxidation as markers for potential oxidative stress and damage. To detect ROS formation and lipid peroxidation within the copepods, *in vivo* tests using fluorescent probes were conducted. Copepods were exposed to PS-NH_2 in several concentrations and three different fluorescent detecting probes (DHR123, H_2DCFDA and C11-BODIPY) were added. All three probes showed a significant rise in fluorescence compared to the control for the highest concentration tested (100 μg/mL), but the methods were not successfully optimised and need to be further developed to get more precise results. Suggestions

for improvements in the method and future research are proposed to get an enhanced ecotoxicologial assessment of micro- and nanoplastics. Overall, results suggest that the NPs PMMA-COOH and PS-NH_2, which do not agglomerate, have the greatest potential of entering the copepods through ingestion because of their small size. Results also suggest that the positive surface charge of PS-NH_2 increased the toxicity of this polymer. Given the results from the acute test where PS-NH_2 was the most lethal particle tested, and from the significant level of fluorescence measured in the probe test, it is possible that the mortality of copepods exposed to PS-NH_2 is caused by oxidative stress formed inside the animals.

Sammendrag

Plast er et materiale bestående av lange polymerkjeder som er laget av olje og gass, og sammen med tilleggsstoffer som tilsettes under produksjonen får polymerene flerfoldige bruksområder. Plast brukes i dagliglivet til innpakning av matvarer, i kosmetikk og i klær, og er uunngåelig nå til dags. 322 millioner tonn plast produseres hvert år, og det anslås at mellom 4,8 og 12,7 millioner tonn av den plasten havner i havet. I havet kan plast brytes ned på grunn av fysiske og kjemiske prosesser, og bli til mikro- og nanoplast (MP og NP). Disse små partiklene (<5 mm) kan være en trussel for organismer i havet, fordi partiklene kan påvirke organismenes utvikling, reproduksjon og overlevelse. Det finnes mange små krepsdyr i havet, både bentiske og pelagiske arter, som kan påvirkes av plastpartikler i det marine miljøet. Mange av disse små krepsdyrartene filtrerer maten sin og velger dermed ikke selv hva de spiser. Disse dyrene får en økt risiko for inntak av NP-partikler (<100 nm) og kan derfor være egnede arter til å teste og evaluere økotoksikologiske effekter av NP. I denne oppgaven ble toksisiteten av NP-partiklene polystyren (PS) og poly(metylmetakrylat) (PMMA), både ikke-funksjonaliserte og med funksjonelle karboksyl- (PS-COOH og PMMA-COOH) og aminerte (PS-NH_2) grupper, forsket på i hoppekrepsen *Tisbe battagliai* for å vurdere potensielle bivirkninger disse partiklene kan føre med seg. NP-partiklene ble karakterisert gjennom dynamisk lysspredning (DLS), hvor størrelse (Z-gjennomsnitt), overflateladning og polydispersitet ble målt i Milli-Q-vann (MQW) og naturlig sjøvann (NSW) for å beskrive partiklenes egenskaper. Akutt dødelighet og oksidativt stress ble bestemt for *T. battagliai* eksponert for de overnevnte NP-partiklene. NP-partiklene PS-NH_2 og PMMA-COOH aggregerte ikke i NSW (størrelse <140 nm) mens de andre partiklene dannet mikroaggregater (størrelse >1800 nm). Mens de fleste partiklene hadde en negativ overflateladning, var PS-NH_2 positivt ladd i begge de testede mediene. For å teste akutt dødelighet ble krepsdyrene eksponert for forskjellige konsentrasjoner av NP (0, 0,5, 1, 5, 10, 25, 50 og 100 μg/ml) i NSW i 48 timer. PS-NH_2 viste seg å være den mest giftige partikkelen, med en EC_{50}-verdi på 7,8 μg/ml. Denne plastpolymeren ble derfor valgt til videre testing for å bestemme dannelse av reaktive oksygenforbindelser (ROS) som markør for potensielt oksidativt stress og ødeleggelse i organismene. For å oppdage oksidativt stress (ROS-dannelse og lipidperoksidasjon) i krepsdyrene ble en *in vivo*-metode med fluorescerende prober gjennomført. Krepsdyr ble eksponert for PS-NH_2-partikler i flere konsentrasjoner og tre forskjellige fluorescerende prober (DHR123, H_2DCFDA og C11-BODIPY) ble tilsatt. Alle de tre probene viste en signifikant økning i fluorescens, sammenlignet med kontrollen, for den høyeste konsentrasjonen som ble testet (100 μg/mL), men metoden ble ikke helt optimalisert,

og testen må videreutvikles for å oppnå mer pålitelige resultater. Karakteriseringen av partiklene viste at PMMA-COOH og PS-NH_2, de polymerene som ikke agglomererer, har størst potensiale til å komme inn i krepsdyrene gjennom inntak på grunn av den lille størrelsen. Det foreslås også at det er den positive overflateladningen til PS-NH_2 som øker polymerens toksisitet. Gitt resultatene fra den akutte testen hvor PS-NH_2 var den mest dødelige partikkelen som ble testet, og fra det betydelige nivået av fluorescens målt i probetesten, er det mulig at krepsdyr utsatt for PS-NH_2 dør på grunn av oksidativt stress dannet inne i organismene.

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1. Introduction

The modern plastic was developed as early as in 1907, and after the mass production of these polymers started in the 1930s, their production only continued to expand (Mattsson et al., 2015; Ore & Stori, 2018). In 2015, 322 million metric tons of plastic were produced on a world-wide basis, and from 1975 to 2012, plastic production has had an increase of 620% (Jambeck et al., 2015; Lusher, 2017). Plastics are made of long chains of different monomers such as ethylene, styrene and propylene, which are linked together through polymerisation of polymers (Bolgar et al., 2007; Ore & Stori, 2018). These monomers are extracted from mostly oil and gas, and about 4 % of the oil and gas used today is used in plastic production (Hopewell et al., 2009). Substances are added to the polymers during their manufacturing to give them different properties. These substances are called additives. Additives are intended to improve the performance and increase the usefulness of the plastic polymers, by making them heat resistant, more flexible or resistant against oxidation or UV-radiation (Al-Malaika et al., 2017; Bolgar et al., 2007). Additives make plastic a very useful material, with numerous applications not only in day to day products, as for example packing of consumer goods, but also in technology and in the medicine industry (Lusher, 2017). There is also plastic in a lot of clothes as synthetic workout clothing and fleece jackets, and food is covered in plastic to retain quality for a longer time. We have a great benefit of this modern material and due to easy manufacturing, low costs, and numerous properties such as light weight and durability, plastic materials have become a necessity in most homes and industries (da Costa et al., 2016). Even though plastics are considered as inevitable, they also cause problems, mainly as plastic debris that affect organisms in the marine environment.

1.1. Plastic pollution in the marine environment

Approximately 50% of the plastics that are produced annually are incorporated in items that have a single-use only purpose and are discarded after one time use, like wrappings around different products (Galloway et al., 2017). Only around 5% of all the plastic products that are produced are recycled on a global basis, and 10% of all the plastic that is not recycled is expected to end up in the oceans (Cole et al., 2011; Lusher, 2017). This is somewhere between 4.8 and 12.7 million metric tons annually (Booth et al., 2017). Nowadays, plastic debris are found all over the marine environment, from the sea bottom to the sea surface, from coastlines to Arctic sea ice, and this pollution is affecting the marine environment (Jambeck et al., 2015).

In a report from SINTEF, Booth et al. (2017) stated that the largest quantity of microplastics is found in benthic organisms, along beaches and shorelines, and in coastal sediments. There is documentation that over 180 marine species have ingested plastic debris, and at least 267 different species have been affected by this threat in the oceans, like zooplankton, sea turtles, fish, marine birds and mammals. This global problem is therefore something that needs to be investigated further, as well as prevented to achieve an environmental sustainable situation (Wang et al., 2016).

1.1.1. Definitions, sources, abundance and distribution

Surveys on the presence of plastic litter in the environment have identified different sized particles that depending on their size range can be separated in macro- meso- micro- and nanoplastic (da Costa et al., 2016). There is not a clear definition of how big or small these plastic particles have to be to be categorised into the different sizes, but several suggestions for categorisation do exist. da Costa et al. (2016) made a schematic overview on different plastic size definitions collected from literature. The authors have somewhat different opinions on the size ranges, as the EU commission operates with a smaller size range for nanoplastics than Browne et al. (2007), and Hartmann et al. (2015) as shown in Figure 1 from da Costa et al. (2016).

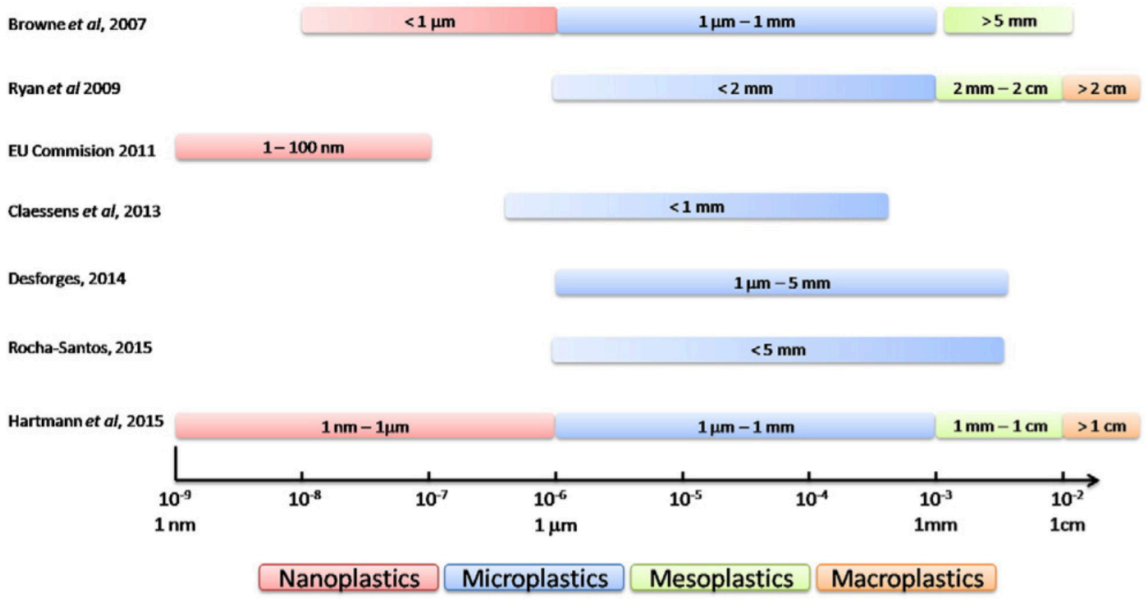

Figure 1: Definition of sizes of plastic particles from different authors. da Costa et al. (2016)

Given the different size definitions presented in literature, in this thesis the size definitions used will be in accordance to Bråte et al. (2017):

- Macroplastics are defined as large items of plastic that are >5mm.
- Microplastics are defined as plastic particles that are between 0.1 μm and 5000 μm in their longest dimension. This is equivalent to 0.0001 – 5.0 mm.
- Nanoplastics are defined as plastic particles that are between 1 nm and 100 nm. This is equivalent to 0.001 – 0.1 μm.

The size range of mesoplastics is not defined by Bråte et al. (2017), and will not be discussed further in this thesis.

Most of the plastic that enters the environment is made of macroplastic particles. Almost 80% of the plastic pollution in the ocean originates from land, while the last 20% is debris from sea industry, like lost fishing nets and equipment from aquaculture (Andrady, 2011; Jambeck et al., 2015; Miljødirektoratet, 2019). The marine plastic litter that originates from land is nowadays often a result of plastic waste from countries with a lot of plastic production and poor management of plastic debris (Gourmelon, 2015). This is the case of, for instance China, where there are more accidental inputs, illegal dumping and insufficient treatment capacity compared to western countries (Hopewell et al., 2009; Jambeck et al., 2015). Since plastics are not fully degradable in our timescale, a lot of the plastic debris that is already in the oceans may originate from several years ago, when there were inadequate litter treatment systems everywhere, and debris was dumped directly in the nature (Hopewell et al., 2009).

When plastic is exposed to physical forces such as sunlight, the material will start to degrade due to photodegradation. Other mechanisms that may break down plastics into smaller pieces are hydrolysis, mechanical degradation, thermal degradation and biodegradation. As for plastic particles in the marine environment, abrasion and mechanical degradation because of external forces such as sand and wave forces, together with photodegradation are the main causes for degradation (Booth et al., 2017). When macroplastics are degraded into pieces as small as 5 mm in diameter, the plastic particles are called microplastics (MPs).

The definition for microplastics can be divided into two subgroups, primary and secondary MPs. Primary MPs are the particles that are produced as pristine microbeads or other MP particle types, and then added to other products (Bråte et al., 2018). Toiletries and cosmetics often contains these primary MPs, as for example facial scrubs, exfoliators and toothpaste (Boucher & Friot, 2017). Primary MPs can also be used in medicine as vectors for drugs and in air-blasting technologies (Patel et al., 2009). Another type of primary microplastics is tyre wear from vehicular traffic and textile fibres from washing machines. MPs incorporated in consumer products and textile fibres will normally find their way into the marine environment, mostly

through the sewage system (Boucher & Friot, 2017). On the other hand, secondary MPs are microsized particles that result from the degradation of larger plastic particles (such as macroplastics) in nature due to natural weathering, UV radiation, mechanical stress and/or other mechanisms (Boucher & Friot, 2017; Cole et al., 2011). Secondary MPs can also degrade into smaller pieces, and become nanoplastics. Of all the MPs found in the ocean, about 99% are secondary MPs (Cole et al., 2011). MPs are often reported to enter the digestive system of organisms. There are, however, several findings of MP particles that have entered other biological tissues, like gills, ovaries, and digestive glands (Snell & Hicks, 2011; Wang et al., 2016).

Nanoplastics (NPs, <100 nm) are the smallest plastic particles that are originated mostly from degradation of MPs. The NPs are either manufactured as primary NPs and are present in their native form in products such as paints, medical products and electronics, or secondary NPs caused by fragmentation and degradation of bigger particles such as MPs (Koelmans et al., 2015). From the marine litter composition, NPs are least characterised, being potentially the most hazardous, and thus often considered the largest threat to marine organisms (Lambert & Wagner, 2016). NPs are so small that the particles can enter cellular membranes, and potentially be more harmful than larger plastic particles (Bergami et al., 2017). The NPs, especially with functional groups on the surface, have a molecular structure that is similar to proteins, and would more easily pass through cell membranes than bigger particles (Rossi et al., 2013). Since the NP particles have a bigger surface to volume ratio than larger plastic particles, it is possible that more toxic compounds are bound to the surface of the smallest particles, which may increase the toxicity of the NPs when entering the cells of organisms (Bergami et al., 2017). Nanoplastic particles will be the focus of this thesis.

1.1.2. Plastic polymers

Plastic polymers are synthetic materials that are extracted from oil or gas, and their backbone is made of hydrocarbons, and structured so electrons are evenly spread over the hydrocarbon (Cole et al., 2011; Ore & Stori, 2009). Polymers do not have a positive and a negative side but are nonpolar and hydrophobic, which implies that they do not dissolve in water (Pedersen, 2018). Since hydrocarbon polymers will not dissolve when entering the water phase, plastic particles will not vanish when entering the oceans as marine litter. NP particles consist of a core of different polymers and variable functional groups. It is the functional groups that determine their chemical reactivity and surface charge (Bergami et al., 2016). The most common plastic polymers in use are shown in Figure 2. Of the 280 million tonnes of plastic that were produced

in 2012, 90% were composed of polyethylene (PE), polypropylene (PP), polyvinyl chloride (PVC), polystyrene (PS) and polyethylene terephthalate (PET) (Mattsson et al., 2015).

Figure 2: The most common plastic polymers in use (Thompson, 2018).

The parameters that affect the properties of plastic particles are what type of polymer core the particles are made of, the different additives that alter the characteristics of the particles and the functional groups present on the particle surface (Lusher, 2017). In this thesis, it is the polymer type that will be mostly emphasised. From the most common plastic polymers in use, polystyrene (PS) and poly(methyl methacrylate) (PMMA), with different functional groups were chosen for this thesis.

1.1.2.1. Polystyrene

Polystyrene (PS) $(C_8H_8)_n$ is a polymer made of styrene, which was one of the first synthetic thermoplastics to be on the market (Helseth & Ore, 2018b). PS can be both solid, hard plastic or expanded PS (EPS), which is a foam called styrofoam (Booth et al., 2017). PS is used for disposable utensils, cups and covers, while EPS is mostly used for building isolation and packaging (Booth et al., 2017; Helseth & Ore, 2018b). Of the European plastic demand in 2015,

PS and EPS accounted for approximately 6.9% of the most commonly used plastic polymers, that is about 3.4 million tonnes, which makes PS one of the three most used plastic types in the world (Booth et al., 2017). The monomer styrene has properties that can be carcinogenic and also disrupt the endocrine system. Because of these toxic properties and because of the persistence of the polymer made from styrene, polystyrene might be a serious hazard to the marine environment (Bergami et al., 2016). Regular PS has a density of 1.05 g cm^{-3} which is higher than low-density polyethylene (LDPE) and polypropylene (PP). PS will therefore be more likely to sink than the other two most used plastic types (Mattsson et al., 2015; Wang et al., 2016).

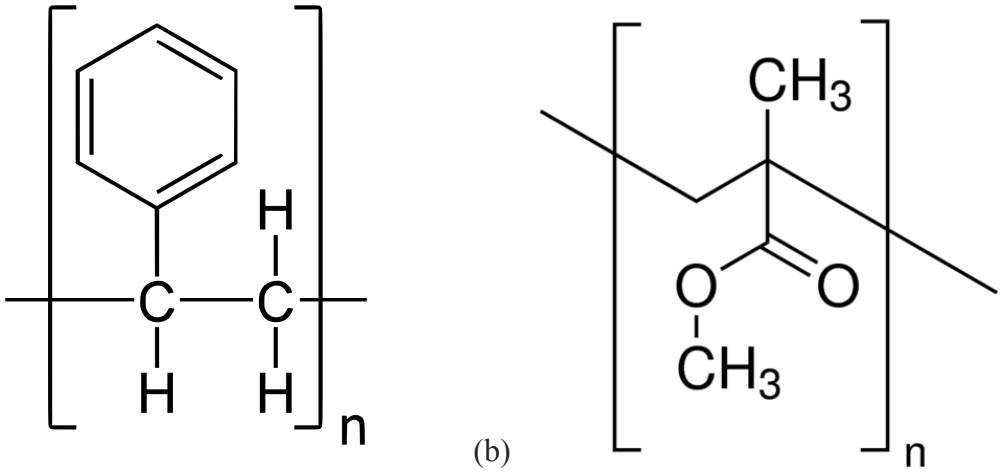

Figure 3: Chemical structures to (a) PS and (b) PMMA (Booth et al., 2016; Yikrazuul, 2008)

1.1.2.2. Poly (methyl methacrylate)

Poly (methyl methacrylate) (PMMA) $(C_5O_2H_8)_n$ is a polymer composed of methyl methacrylate and was manufactured for the first time in the 1930s (Helseth, 2018). PMMA has a high density (1.18 g cm^{-3}), and is therefore likely to sink when entering the water body (Lusher et al., 2017a). As a hard plastic, PMMA is mostly used as plexiglass in replacement of regular glass windows, but PMMA is also known as acrylic, and can be used in textiles and paints, as well as acrylic nails (Helseth, 2018; Lusher et al., 2017a). PMMA is also known to be an important material in human transplants and prosthetics, especially in eye surgery (*Poly(methyl methacrylate)*, 2018). PMMA is not as commonly used as PS, but it has been found in mussels, Atlantic cod and sewage sludge collected from different places along the Norwegian coast (Lusher et al., 2017a; Lusher et al., 2017b).

1.1.2.3. Behaviour and implications

Most of the plastic particles that enter the marine environment are affected by ocean currents. Plastics debris with a low density will be carried with the surface currents, while polymers with higher density can be transported with underlying currents (Wang et al., 2016). A lot of the plastic debris in the ocean accumulates in gyres or patches, because of the pattern of ocean currents, wind patterns and the Coriolis effect that transports the debris (Chen et al., 2017; Rossi et al., 2013; Wang et al., 2016). High density plastic debris that sink quickly to lower water depths can also be transported with ocean currents. This is because water gets higher density with depth, so a lot of high density plastics would remain suspended in deeper waters for a longer time than initially assumed (Wang et al., 2016).

There are several ways to sample plastic debris to see what kind of polymer composition the debris is made of, such as beach combing, marine trawls with meshes and sediment sampling (Booth et al., 2017; Mattsson et al., 2015). However, these methods are not suitable for smaller particles such as small MP and NP particles (Mattsson et al., 2015). Therefore, it is not known how much NPs exists in the oceans, and what kind of polymer composition these NPs are made of. It is hypothesized that the places and depths in the ocean where MP particles have been detected will also contain NP particles. By looking at the density of the different polymers, one can also estimate where to find NPs composed of the different polymers, even though the buoyancy may change when the particles become smaller and weigh less, and therefore are more affected by ocean currents (da Costa et al., 2016).

1.1.2.4. Hazards associated with plastic composition

There are several ways in which plastic particles can be hazardous for marine organisms. One of the most common ways is related to ingestion of big plastic particles (as macroplastics). Large plastic pieces, like old plastic bags that have ended up in the water surface, may resemble food and be digested by fish and marine mammals. If these plastic particles are not excreted, they can clog parts of the digestive system and lead to reduced nutrition uptake. In the worst case scenario these animals may starve and ultimately die, as seen for the Cuvier´s beaked whale that was found dead at Sotra in Norway some years ago (Aandahl & Valgermo, 2017). The same principle is applicable for smaller organisms and MPs. Since their digestive system is smaller, MPs may clog the system, and micro particles may also aggregate in the digestive system (Bergami et al., 2016; Cole et al., 2011). These smaller organisms often belong to a low trophic level, and are not able to distinguish between plastic particles and food, as the case of zooplankton (Lusher & Pettersen, 2017).

Plastic particles may also be harmful to organisms because of the presence of additives that are included in the fabrication of plastic polymers. Additives are added to plastic polymers to modify their properties and features, so desired properties are improved, and negative properties are eliminated (Murphy, 2001). When the unique kinds of polymers are used together with different additives, each plastic type gets dissimilar characteristics that make them useful in all sorts of way. Some of the substances are added to make the plastic material softer and increase their plasticity (Heudorf et al., 2007). These additives are also called plasticizers, and phthalates are an example of this type of substance (Wilkinson et al., 2017). Other substances that alter the function of plastic materials are polybrominated-diphenyl ethers, nonyphenol and triclosan, which make plastics more resistant to heat, oxidative damage and biodegradation, and extend their longevity (Cole et al., 2011). These additives may leach out of the plastic materials into the environment because of natural breakdown of plastic particles in marine waters, or in organisms when they are being ingested because of gastrointestinal gut fluids (Koelmans et al., 2014). The chemicals in the additives may change biological processes in the organisms, like the endocrine system. This can disrupt the development, reproduction or mobility of the organisms and cause toxicity (Cole et al., 2011). Some additives, like phthalates are known to have carcinogenic effects (Murphy, 2001).

Another potential hazard of plastic particles is related to the sorption of pollutants into the plastic surface. Plastic polymers are lipophilic because of their hydrophobic character, and other lipophilic pollutants will have a high affinity to these plastic particles (Gschwend & Wu, 1985). Some organic pollutants, like polychlorinated biphenyls (PCB) and polycyclic aromatic hydrocarbons (PAH), are lipophilic and have a high affinity to other lipophilic substances. Since some of these organic pollutants have a higher affinity to plastic particles than to sediments, it is possible that these pollutants will enter the food chain since they adhere to plastic particles in the benthic zone (Teuten et al., 2009). MPs can therefore be regarded as a potential vector both for transport of contaminants in the environment and for transfer of chemicals to organisms that ingest them (Koelmans et al., 2013). Booth et al. (2017) stated that the largest amounts of small plastic particles are found in benthic organisms among coastal sediments. Benthic organisms may therefore be particularly vulnerable for pollutants adhered to plastic pieces and will be a key species to study the uptake of MPs in aquatic organisms.

When plastic particles first enter the marine environment, their surface is smooth with no net charge. After a while, when the particles start to degrade, the surface will become rougher, and can be coated with proteins and biomolecules like carboxylated functional groups.

This mechanism would also make it easier for other lipophilic compounds such as PCB to adhere to the plastic particles (Galloway et al., 2017).

Particle surface chemistry has been suggested as relevant for the toxic potential of particles. For this reason, it becomes important to test plastic particles with different functional surface groups, as carboxylated and aminated functional groups, in comparison with plain, non-functionalised particles (Booth et al., 2016). The NP particles used in this thesis are composed of PS and PMMA (Figure 3 a and b), with and without functionalisation, i.e., PS and PMMA with functional carboxylic group (COOH) and PS with functional amino groups (NH_2). Plastic particles are produced with different functional surface groups to give them different functionalities or to alter their surface charge in a special way. For PS NPs, carboxylated particles will have a negative surface charge while aminated particles will have a positive surface charge (Della Torre et al., 2014). Polymers are synthesised with functional groups to give them many functions to be used in different applications, like photonics, biosensors, drug delivery tools and nanocomposites (Bergami et al., 2017). The surface charge is known to be important for the toxicity of NP particles. In the studies done by Bergami et al. (2017) and Della Torre et al. (2014) the authors concluded that PS-NH_2 is more damaging for the cells of the tested organisms (brine shrimp larvae and sea urchin embryos) than PS-COOH, suggesting that it is the positive surface charge of the particles that increases cell death.

1.2. Interactions between plastics and marine crustaceans

Since there is an absence of studies explaining exposure to and environmental relevant concentrations of NPs, studies about MPs interference with crustaceans have to be evaluated as well. It is likely that areas where MP particles are present will also contain NP particles, as the nanoparticles are more weathered and degraded MP particles. Bottom grazers and filter feeders are expected to be exposed to NPs, and these organism types include crustaceans such as copepods, lobsters, mussels and sessile barnacles (Bergami et al., 2016; Nerland et al., 2014). Copepods are found both in benthic and pelagic habitats, and it can be assumed that some of these copepodic species can potentially be exposed to NP particles. Filter feeders are especially at risk as they filter water and ingest all types of particles present in the water, including the NPs (Bråte et al., 2017). Microcrustaceans will therefore be more likely to ingest nano-sized debris through dietary uptake as they are exposed directly to the surrounding water (Bergami et al., 2016).

1.2.1. Uptake, accumulation and trophic transfer

There have been some studies exploring uptake, accumulation and trophic transfer of NPs to crustaceans. Chae et al. (2018) reported a study on the trophic transfer of polystyrene NPs in a freshwater food chain, that showed that NPs in high doses are easily transferred through the food chain. Trophic transfer of NPs have been confirmed by several authors and is expected to be one of the main routes of exposure for organisms at higher trophic levels (Chae & An, 2017). Mattsson et al. (2014) found negative effects on feeding, behaviour and metabolism in fish related to a food chain transfer from NP contaminated algae. This study shows that NPs can affect organisms on several trophic levels due to uptake in producer organisms and trophic transfer to consumers. Bergami et al. (2016) did a study on effects of NPs in a brine shrimp larvae where it was discovered that accumulation did not affect mortality of the test species, although it caused other sub-lethal effects. The threat of accumulation of NP particles needs to be further studied to unravel the risks connected to NPs.

1.2.2. Ecotoxicological effects

The toxicity of plastic particles is controlled by a number of factors, like size of the particles and surface charge (Bergami et al., 2016). Toxicity can cause cell death and/or death of the organism, but there are also sub-lethal endpoints when measuring toxicity of a harmful substance. Experiments with ingestion of microplastic particles rarely show lethal endpoints, and the EC_{50} values for these types of experiments are normally absent (Walker et al., 2012). Since NPs are so small that they may pass biological barriers, it is possible that NP particles may cause a bigger risk for the organisms than MP particles (Bergami et al., 2017). Sub-lethal effects may work as a stressor to the organisms that will affect reproduction, behaviour or growth of organisms without causing mortality (Ford et al., 2003). Sub-lethal endpoints are important to study, to get a total understanding of the complexity of nanosized plastic particles. Examples of sub-lethal endpoints are effects development and reproduction, and the formation of cellular oxidative stress subsequent oxidative damage.

1.2.2.1. Development, reproduction and mortality

Mortality is a commonly studied toxicity endpoint, which will be one of the endpoints used in this thesis. Concentrations of NPs that are too low to cause mortality, may affect organisms in a sub-lethal way. Development and reproduction are important sub-lethal endpoints to study, as these processes can be affected at non-lethal plastic concentrations (Galloway et al., 2017).

Ingestion of MP particles may limit food uptake, and therefore affect growth and development in organisms, as explained in section 1.2.3.5. In a study by Bergami et al. (2016), several sub-lethal effects were reported for brine shrimp exposed to NP particles, as behavioural, physiological and biochemical effects on the larvae stadium. Other studies have shown that NPs affect the development of embryos and reproduction of the freshwater crustacean *Daphnia galeata*, the feeding rate of the blue mussel *Mytilus edulis* and population growth of *Daphnia magna* (Besseling et al., 2014; Cui et al., 2017; Wegner et al., 2012). These studies show that NPs have several sub-lethal endpoints that needs to be studied further to understand the full risk of NPs.

1.2.2.2. Oxidative stress

Reactive oxygen species (ROS) are unstable molecules with oxygen that can easily react with other molecules within a cell, and generate more ROS (Kiani-Esfahani et al., 2012). ROS are often called free radicals, because both terms are molecules with an unpaired electron; however, free radicals do not need to contain oxygen, yet they often do (Nordberg & Arnér, 2001). Examples of ROS are superoxide radical ($O2^{\bullet-}$), hydrogen peroxide (H_2O_2), hydroxyl radical ($HO2^{\bullet-}$) and singlet oxygen ($^1O_2$) (Gomes et al., 2005). Since ROS are easily reactive with other molecules, a newly formed radical can start a chain reaction of formation of free radicals, which may lead to oxidative damage to different parts of the cell, like proteins, mitochondria, DNA and lipids (Apel & Hirt, 2004; Nordberg & Arnér, 2001). Antioxidants, like different vitamins and enzymes, can protect cells from reactive oxygen species. When an organism is exposed to a stressor, ROS are formed in the cells. With too much ROS produced, there will occur an imbalance between the amount of ROS and antioxidants in the cells. This process is called oxidative stress (Aarnes, 2012). When oxidative stress occurs, different parts of the cell, like the mitochondria, may be destroyed, which can lead to apoptosis (Kannan & Jain, 2000). One of the most common forms of oxidative damage caused by the increase of ROS is the degradation of membrane lipids, a process called lipid peroxidation. Free radicals take oxygen from the lipids in cell membranes, causing the membrane lipids to be destroyed (Mylonas & Kouretas, 1999).

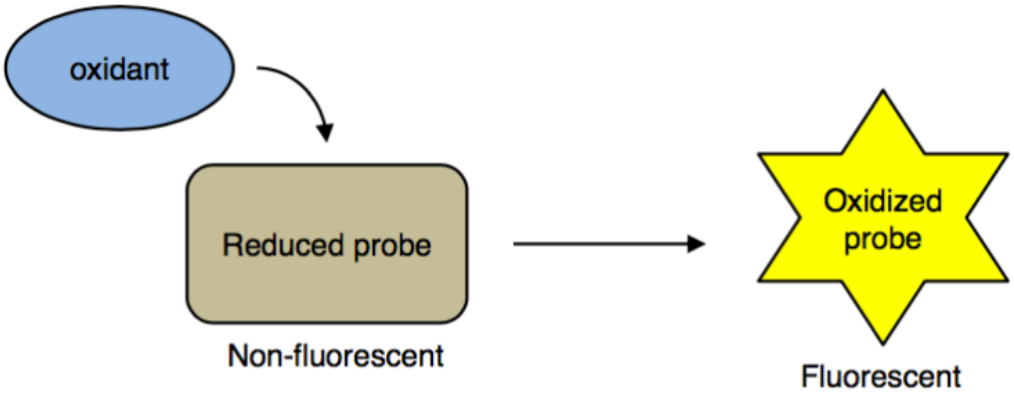

Figure 4: Shows how an oxidant will react with ROS and oxidise to a fluorescent compound (Winterbourn, 2014).

1.3. Test species: *Tisbe battagliai*

1.3.1. Ecological role and life cycle

Tisbe battagliai (<250 µm in diameter) is a crustacean from the subclass Copepoda, order Harpacticoida, family Tisbe (Walter & Boxshall, 2018). This epibenthic copepod lives in the marine environment just above or on bottom sediments (Hines & Ogburn). Microcrustaceans from the genus Tisbe are ecologically very important because harpacticoids are one of the largest meiobenthic groups in the ocean and have a widespread ecological distribution, being therefore representative of large areas of the ocean (Hines & Ogburn; Hutchinson et al., 1999). Since *T. battagliai* are filter feeders, it is assumed that the organisms ingest all particles present in their surrounding water, including NP particles (Bråte et al., 2017). *T. battagliai* are also a food source for other animals, like fish and larger invertebrates, and are important intermediate steps in the food web structure (Hutchinson et al., 1999).

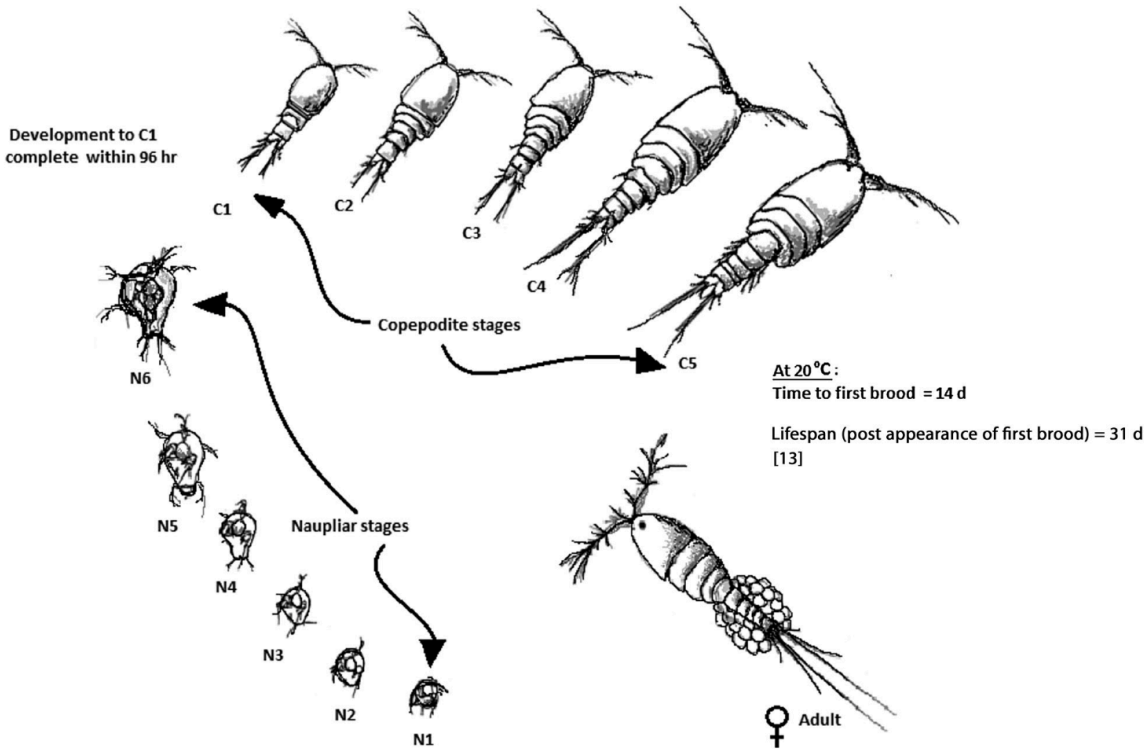

Figure 5: Life stages of Tisbe battagliai (Macken et al., 2015).

1.3.2. Use of *T. battagliai* as a model species

Animals of the genus Tisbe are especially suitable for environmental risk assessment and are recommended by the International Organization for Standardization (ISO) (1999) for acute testing, ISO Guideline 14669 "Water quality- determination of acute lethal toxicity to marine copepods (*Copepoda, Crustacea*)" (Diz et al., 2009). These copepods have a short life cycle, as shown in Figure 5, with 6 naupliar and 5 copepodic stages over a period of 31 days (Macken et al., 2015). The first copepodite stage is reached after 4 days, and they reach their reproductive state after approximately 14 days (Williams, 1992). Populations of *T. battagliai* can be harvested any time of the year in all the different life stages. It is easy to find individuals for several types of bioassays, like copepodic stage for acute testing or nauplii that are less than 18 hours old for developmental tests (Diz et al., 2009). Laboratory conditions are suitable for these copepods, being easy and fast to maintain. This together with the fact that they are very small and require small space and little equipment, makes these organisms suitable for toxicity studies in the laboratory (Williams, 1992).

1.4. Aims of the study

The main objectives of this study was to characterise the properties of different plastic polymers, and to see how these polymers cause mortality and oxidative stress in the copepod *T. battagliai*. The plastic polymers chosen include PMMA, carboxylated PMMA, PS, carboxylated PS and aminated PS, and it is assumed that these particles can potentially be consumed by these filter-feeders. These studies may indicate if NPs pose a potential threat to copepods in the marine environment.

The ultimate aims of the work were to:

- Distinguish NPs behaviour in different media through characterisation of their properties
- Find the lethal concentrations of the different NPs through acute mortality testing
- Detect if NP induced oxidative stress in copepods by using fluorescent probes for the detection of ROS formation
- Detect if NP induced oxidative stress in cell membranes of the copepods by using a lipid peroxidation test with a fluorescent probe

2. Material and methods

All experiments were performed in the laboratory at NIVA in Oslo during the period June 2018-March 2019. This work focused on lethal and sublethal endpoints in copepods of the species *T. battagliai* when exposed to NPs, shown in Figure 6. Acute lethality was tested using all particle types explained in section 1.1.3.2, as well as characterisation of the particles through dynamic light scattering. Sub-lethal tests to check for ROS formation and lipid peroxidation with fluorescent probes were only performed for the NP PS-NH_2.

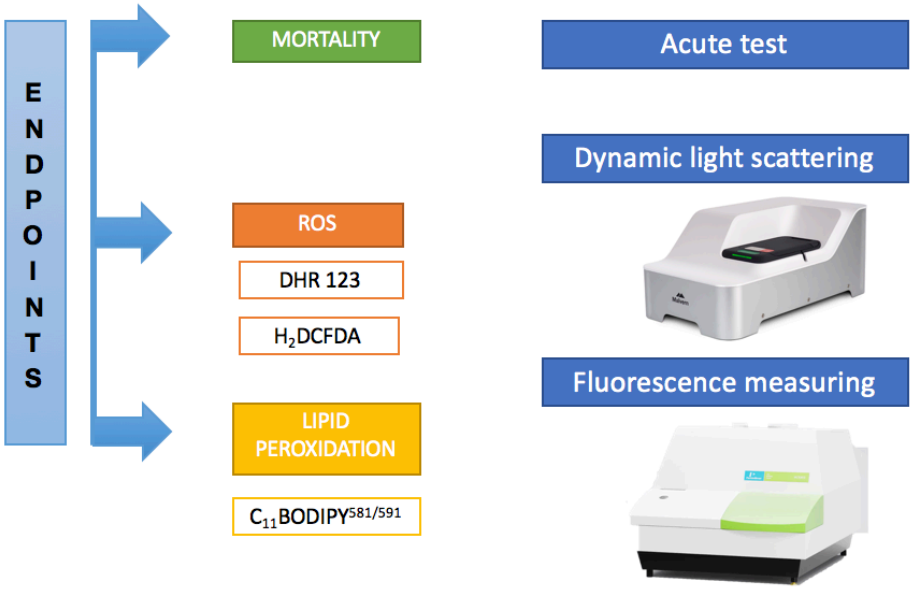

Figure 6: Shows the different endpoints studied in this thesis with the methods used.

2.1. Nanoplastics

The NP particles used in these experiments were polystyrene (PS) and poly(methyl methacrylate) (PMMA), as described in section 1.1.3.2. The nanoplastic particles were first characterised before the tests were conducted.

2.1.1. Nanoplastics preparation

Plain and negatively charged carboxylated poly(methyl methacrylate) and polystyrene nanoplastics with diameter of 50 nm were purchased from Phosphorex Inc. (Table 1). The particles were supplied dispersed in deionized water with 0.1% Tween 20 and 2 mM of NaN_3 as preservative agents. According to the manufacturer, the mean diameter of each particle was 50 nm, and the particles met the official primary particle standards from National institute of Standards and Technology (www.nist.gov), as seen in Table 1. Preliminary tests were

conducted to evaluate the effects of preservatives (Tween 20 and NaN_3) in PMMA and PS NPLs suspensions obtained from Phosphorex but the results are reported herein as they are outside the scope of this thesis. No significant effects were detected (data not shown), so the presence of preservatives in NPs suspensions was considered negligible to the observed toxicity. Positively charged amino-modified polystyrene particles were purchased from Sigma-Aldrich (Table 1), with an advertised target mean of 50 nm in diameter. All particles were stored at $4^{O}C$.

Table 1: Specifics about the plastic polymers provided by the suppliers.

	PMMA	**PMMA–COOH**	**PS**	**PS–COOH**	**PS-NH_2**
POLYMER	PMMA	PMMA	Polystyrene	Polystyrene	Polystyrene
PRODUCER	Phosphorex	Phosphorex	Phosphorex	Phosphorex	Sigma-Aldrich
SURFACE	None	COOH	None	COOH	NH_2
TARGET MEAN DIAMETER (µm)	0.05	0.05	0.05	0.05	0.05
ACTUAL MEAN DIAMETER (µm)	0.060	0.055	0.041	0.052	0.045-0.055
STANDARD DEVIATION (µm)	0.013	0.009	0.009	0.014	Not provided
DENSITY (g/cm^3)	1.19	1.19	1.05	1.05	1.04 – 1.06
CONCEN-TRATION (mg/mL)	10	10	10	10	25
N SPHERES PER mL	$7.53x10^{13}$	$9.65x10^{13}$	$2.64x10^{14}$	$1.28x10^{14}$	$3.60x10^{14}$
ANTI-MICROBIAL AGENT	2 mM NaN_3	2 mM NaN_3	2 mM NaN_3	2 mM NaN_3	None
MEDIA	0.1% Tween 20 in DI water	0.1% Tween 20 in DI water	0.1% Tween 20 in DI water	0.1% Tween 20 in DI water	No preservatives. Suspended in water.
SOLID CONTENT	1%	1%	1%	1%	2.5%

2.1.2. Nanoplastics characterisation

Primary characterization of NPs (plain, COOH and NH_2 functionalised) in milli-Q water (MQW) and natural seawater (0.22 μm filtered NSW, T= 20 °C, salinity 36‰, pH 8.0) was performed using Dynamic Light Scattering (DLS, Malvern instruments), combined with the Zetasizer Nano Series software, version 7.02 (Particular Sciences, UK). The NP stock solutions were prepared in MQW and NSW, and contained 50 μg/mL of NPs (plain and functionalised). Z-average (nm), Polydispersity Index (PDI, dimensionless) and Zeta (ζ-) potential (mV) were measured as key parameters describing potential behaviour in complex environmental medias. Measurements were carried out in triplicate, each containing 16 runs of 10 s for size parameters and for zeta-potential. The zeta-potential measures the surface charge of the particles, and will state if the particles have anionic (negative) or cationic (positive) surface charge. It is important to analyse the zeta-potential, as the surface charge may affect the behaviour of the NPs, such as stability and aggregation (Bergami et al., 2017). The PDI is a measure on the dispersion of polymer particles in a medium (Rane & Choi, 2005). A high PDI would indicate that the polymer solution contains many different sizes of the polymer and therefore polymer colloids which weigh differently from each other, while a low PDI gives a monodisperse mixture with a very uniform polymer sample (Helseth & Ore, 2018a; Rane & Choi, 2005).

2.2. Experimental set-up

2.2.1. Preparation of stock solutions

For all NPs, a stock solution of 100 μg/mL was prepared directly from the suspensions provided by the suppliers. This stock solution was serially diluted in natural seawater (NSW) to obtain working solutions with a range of 0.5-100 μg/mL. NPs final suspensions in NSW were prepared from the working solutions and quickly vortexed prior to use, but not sonicated. This method of dispersion has been suggested as the most environmentally realistic for NPs in the marine environment (Bergami et al., 2017; Singh et al., 2010). Concentrations used in the exposure experiments are reported as μg/mL, and their corresponding concentrations in terms of particle number are reported in Table 2 (more detailed concentration calculations are found in Appendix A).

Table 2: Concentration of nanoplastics used in the exposures in terms of weight and number of particles per ml.

Concentration (µg/mL)	PS NPs/mL	PS-COOH NPs/mL	PS-NH_2 NPLs/mL	PMMA NPs/mL	PMMA-COOH NPs/mL
0.5	$1.32x10^{10}$	$6.40x10^{9}$	$7.20x10^{9}$	$3.77x10^{9}$	$4.83x10^{9}$
1	$2.64x10^{10}$	$1.28x10^{10}$	$1.44x10^{10}$	$7.53x10^{9}$	$9.65x10^{9}$
5	$1.32x10^{11}$	$6.40x10^{10}$	$7.20x10^{10}$	$3.77x10^{10}$	$4.83x10^{10}$
10	$2.64x10^{11}$	$1.28x10^{11}$	$1.44x10^{11}$	$7.53x10^{10}$	$9.65x10^{10}$
25	$6.60x10^{11}$	$3.20x10^{11}$	$3.60x10^{11}$	$1.88x10^{11}$	$2.41x10^{11}$
50	$1.32x10^{12}$	$6.40x10^{11}$	$7.20x10^{11}$	$3.76x10^{11}$	$4.83x10^{11}$
100	$2.64x10^{12}$	$1.28x10^{12}$	$1.44x10^{12}$	$7.53x10^{11}$	$9.65x10^{11}$

2.2.2. Water quality parameter preparation

Exposure experiments were performed following the ISO Guideline 14669 for "Water quality-determination of acute lethal toxicity to marine copepods (*Copepoda, Crustacea*)" (Standardization, 1999). Natural seawater (NSW) collected at 60 m depth at Solbergstrand in the outer Oslofjord was used as exposure media in these experiments. This seawater was filtered through a 0.22 µm filter prior to use, to make sure that other organisms and particles would not interfere with the tests. The salinity of the NSW should be between 30-36‰, so the final salinity of the seawater batch used was adjusted if necessary. Dissolved oxygen (DO), pH and salinity were checked prior to the start and at the end of the experiments. According to the ISO Guideline 14669, the pH should be between 7.7 and 8.3 at the initiation of the experiments for valid results. The pH was measured using the pH meter Orion Star A211 (Beverly, USA). In addition, the DO of the test media (NSW) should be less than 70% of air saturation, hence above 4 µg/mL, to be valid for the experiments. The DO was measured during the experiments with an InoLab oxi7310 DO meter (Weilheim, Germany). Salinity was also checked both at the initiation and at the end of the experiments using a refractometer (Hard refractometer Biolab). The temperature in the laboratory should be between 18 and $22^{O}C$ for optimal test results, so temperature was also monitored during the exposure period. Variations within this temperature interval will not disturb the living standard of the copepods, as these are normal conditions for this species.

2.2.3. Acute test

The acute test was done to see at which concentrations the NPs used are lethal for the organisms during a period of 48 hours. This test only checks for mortality, and not for other sub-lethal endpoints. The acute experiments were conducted over several weeks, where all the different particles were tested three times each. The triplication of the experiments gave 12 sets of data per concentration for all the particles.

2.2.3.1. Experimental organisms

The *T. battagliai* used in experiments are from in house cultures at NIVA, cultured in the same conditions as those used for the tests. The cultures are fed with a mixture of the microalgae *Rhodomona baltica* and *Isochrysis galbana* every week and separated by size with renewal of filtered NSW every two weeks. At the start of each test, *T. battagliai* were separated from the in house cultures using a series of mesh filters to select animals 6 ± 2 days of age (size range of 100 and 150 µm). Animals were then gently washed from the meshes with filtered seawater into a petri dish until being used in exposures.

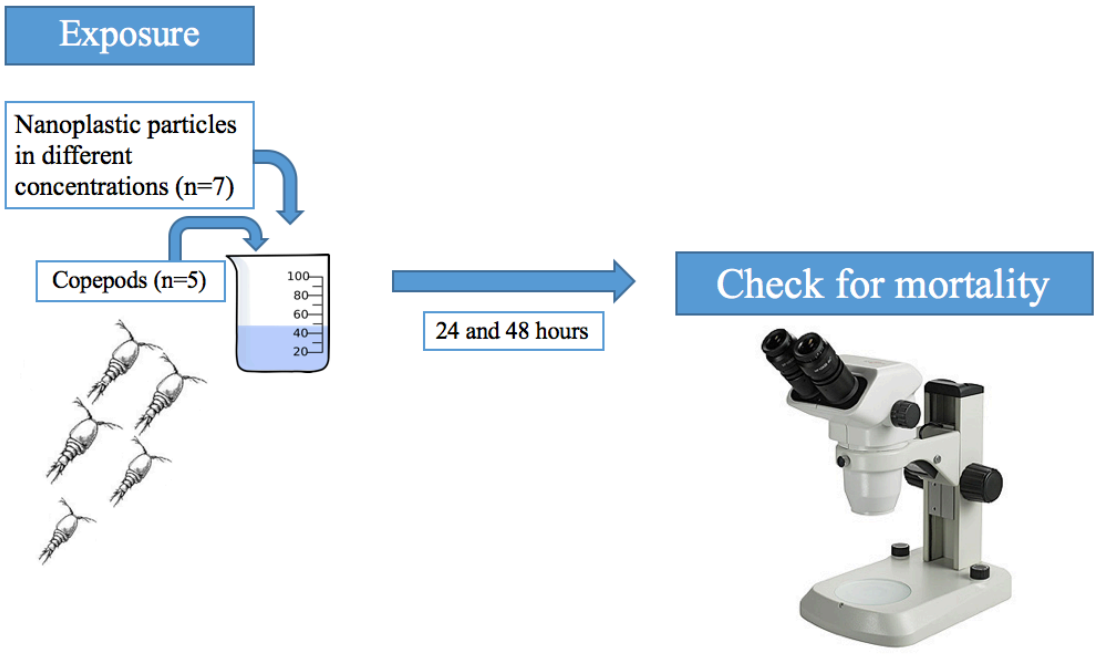

Figure 7: Simplified exposure setup for the acute test. Copepods are added to each NP concentration with the aid of a microscope, and mortality was checked after 24 and 48 hours' exposure.

2.2.3.2. Acute exposure

Small beakers (~10 mL maximum volume) that were washed and marked with NPs concentration and replicate number were used as exposure vessels. Four replicate beakers from

each concentration and control, each containing five copepods were used, making a total of 28 beakers for each NP type. Each replicate beaker was filled with 4.5 mL of each test concentration. Animals were first transferred to a loading well using a glass Pasteur pipette, to minimize the transfer of additional volume of NSW into each treatment. Five living animals were then added to each replicate beaker with the aid of a stereo microscope with light from below (Nikon SMZ 745 T with an Infinity 1 Camera attached (Lumenera, Tokyo, Japan). A simplified schematic over the experimental setup is shown in Figure 7.

The acute test was started after addition of animals, and exposure vessels placed in a climate-controlled room with a photoperiod of 16:8 light:dark cycle, for a period of 48 hours. The animals were not fed during the exposure period. The animals were counted after 24 and 48 hours, and the number of survivors noted. The animals were considered dead if they did not move within 20 seconds. Air was blown at copepods with a pipette to confirm if they were actually dead, as they can lie still for more than 20 seconds without moving even when alive. As mentioned previously, pH, DO and salinity were checked at the initiation of the test and after 48 hours. Tests were repeated three times for all the particles to get a robust data set.

2.2.4. Oxidative stress determination

Fluorescent probe tests were performed to establish if oxidative stress could be the cause for the mortality of copepods seen in the acute testing with PS-NH_2 particles. An *in vivo* method that measures ROS formation and/or lipid peroxidation in living organisms was used and was conducted to understand the mechanisms behind the toxicity of these NP particles. The final setup for the method used is shown in Figure 8. ROS formation and lipid peroxidation are endpoints when studying oxidative stress (Cheloni & Slaveykova, 2013). To detect ROS formation and lipid peroxidation in living cells, it is possible to use fluorescent probes. When adding a probe to the media with an exposed animal, the probe will react with ROS and oxidise to a fluorescent compound, as shown in Figure 4, and the amount of fluorescence will be equivalent to the amount of ROS in the cells (Gomes et al., 2018). From the several fluorescent probes available, the two probes for ROS detection chosen for this experiment were dihydrorhodamine 123 (DHR123, Invitrogen, Molecular Probes Inc., Eugene, OR, USA) and *2'*,7'-dichlorodihydrofluorescein diacetate (H_2DCFDA, Invitrogen, Molecular Probes Inc., Eugene, OR, USA), and the probe for lipid peroxidation was 4,4-difluoro-5-(4-phenyl-1,3-butadienyl)-4-bora-3a,4a-diaza-s-indacene-3-undecanoicbacid (C11-BODIPY$^{581/591}$, Invitrogen, Molecular Probes Inc., Eugene, OR, USA) (Gomes et al., 2005).

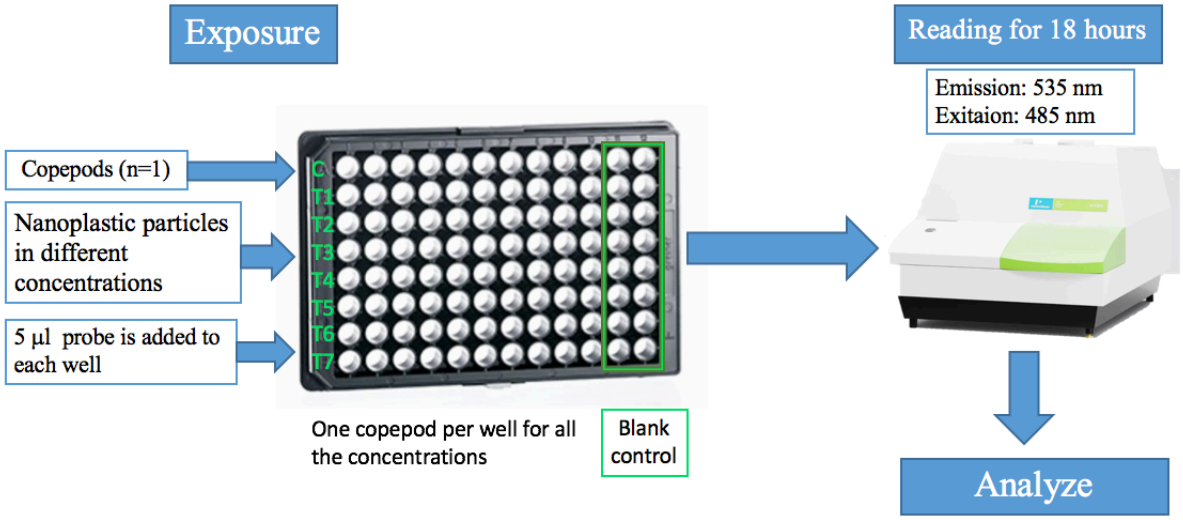

Figure 8: Schematics over the method used for the probe experiments.

2.2.4.1. Method development and optimisation

For the fluorescent probe tests, it is necessary to have copepods replication, 96 well black microplates, the probes and NP at different concentrations. The test was run in a fluorescent plate reader (1420 Multilabel Counter, Victor 3, Perkin Elmer), with excitation 485 nm and emission 535 nm. The different probes used in this test were the DHR 123 and H_2DCFDA for ROS formation and the probe C11-BODIPY for lipid peroxidation.

DHR123 is a probe that in the presence of ROS will oxidise to rhodamine 123 which is a fluorescent dye (as explained in Figure 4) (Kalyanaraman et al., 2012). The probe will diffuse into cells, where it in contact with ROS will transform to rhodamine 123 and move to the mitochondria where it is sequestered. Therefore, this probe is normally used as an indication of ROS formation in the mitochondria (Kiani-Esfahani et al., 2012). This probe was prepared in dimethyl sulfoxide (DMSO) liquid as a 50mM stock solution, and kept frozen until use.

H_2DCFDA also enters the cells passively, and when exposed to a variety of ROS the probe will oxidise to DCFH after being hydrolysed by cellular esterase. DCFH is a highly fluorescent final product that is localised in the cytosol (Kalyanaraman et al., 2012). The H_2DCFDA probe was prepared in DMSO liquid in a 20mM stock solution and stored at -20^{O}C prior to use. These probes are not ROS selective, so they are suitable to detect all oxidative activity within the cells, but it is not possible to differentiate which kind of ROS the probes were oxidised by (Soh, 2006).

C11-BODIPY$^{581/591}$, (or LPO in Figures in this thesis), is a fatty acid analogue, that can easily enter the cell membrane because of its lipophilic character (Cheloni & Slaveykova,

2013). Once inside cells, this probe can be oxidised by oxyl-radicals together with the endogenous fatty acids and shift its fluorescence from red to green (Pap et al., 1999). With a high amount of ROS being formed in the lipid cell membranes, there will be an increased potential for lipid peroxidation, and consequently higher measured fluorescence with use of this probe. Similar to the two other probes, a 2.5 mM stock solution of C11-BODIPY was prepared in DMSO and kept frozen. The stock solutions for all the probes were divided in aliquots of 50 µl and stored at -20^{O}C in the dark. A final concentration of 50 µM was used for all the probes in the exposure wells.

The methods with the fluorescent probes used in this thesis were adapted from similar methods using the freshwater planktonic crustacean *Daphnia magna* (Gomes et al., 2018). This crustacean is bigger than copepods of the species *T. battagliai*, so adjustments were made with regard to size of the animals and changes in test medium from freshwater to seawater. To implement these adaptions, several method development steps were performed. Several tests were run to see if the copepods exposed to the probes gave a high enough fluorescent signal to be detected using the fluorescent plate reader. First, tests were run without any stressor (only copepods and fluorescent probes) to see if the probes gave any fluorescence signal in the copepods compared to a blank control (no copepods). Copepods were transferred to a 96 well black microplate and the different probes were added to the wells (as described in Figure 8, only without stressor added). The plates were transferred to the fluorescent plate reader and read once every hour for a total of six hours. After the tests were done without stressors, additional tests were done with hydrogen peroxide (H_2O_2) as a positive control. The animals were exposed to different concentrations of H_2O_2 for 24 hours, and then they were transferred to a 96 well black microplate, after which the fluorescent probes were added. The optimisation without any stressor was not done for the probe H_2DCFDA, and with only one replication for the test with H_2O_2, as this probe was unavailable at the time these experiments were done. Further details and results from the probe method development are in Appendix B.

After optimisation, several tests were run with the copepods exposed to NP particles, as shown in Figure 8. The animals were exposed to the same concentrations as those used in the acute tests (see Table 2 in Appendix A). However, copepods were not incubated for 24 hours before fluorescence measuring was conducted (as was done for H_2O_2), but were co-exposed to NP particles and fluorescent probes simultaneously. The animals were separated from the cultures and transferred to a 96 well black microplate where they were exposed to the NP concentrations. The different probes were then added to each well and the readings in the

fluorescence machine were initiated immediately after exposure. Fluorescence from each well was measured every hour for 18 hours total (overnight) to check for the formation of ROS and lipid peroxidation.

After the fluorescent readings were completed, pictures of all copepods were taken using a fluorescent microscope (Olympus DP72, Olympus Optical Co., Ltd). Length was used for the normalisation of the data obtained for each fluorescent probe, and is presented in Appendix B. In addition to regular pictures, pictures from each concentration were taken to try to detect if fluorescence emitted from the probes was found within the body of the copepods or just in solution. The pictures were taken with a fluorescence microscope Olympus DP72 coupled with a XCite Series 120 PC fluorescence light source (Olympus Optical Co., Ltd).

2.3. Data analysis

Statistical analyses were performed using GraphPad Prism 8 (GraphPad Software Inc., La Jolla, CA, USA). Dose-response curves for all the plastic polymers used in the acute testing were made through none-linear regression analysis in GraphPad, after which the EC values were calculated. The dose-response curves show the actual response to different concentrations and should ideally have a Sigmoid form (S-curve) (Sebaugh & McCray, 2003). To summarise the lethality of NP plastic towards the copepod *T. battagliai*, EC_{50} values were calculated for each particle type. The EC_{50} values calculated represent the concentrations of NPs in which 50 % of the population was dead (Walker et al., 2012). In addition to EC_{50} concentrations, the EC_{10} values were also calculated, which represent the NP concentrations where 10 % of the population was dead.

The estimation of the highest concentration that shows no effect (NOEC) and the lowest concentration that shows a significant effect (LOEC) are additional techniques that can be used to summarise toxicity tests. These methods are normally included in biological statistics to amplify the EC values, even though their use has been considered outdated and questioned in recent years (Warne & van Dam, 2008). The NOEC and LOEC were calculated for each particle type using either One-way ANOVA or Kruskall-Wallis tests. One-way ANOVA was used if the data passed the assumptions of parametric testing, i.e. normality and homogeneity of variances. If the data were normally distributed, a Dunnett´s multiple comparisons test was performed to find the NOEC and LOEC values. If the data did not pass the assumptions of homogeneity and normality, a non-parametric Kruskal-Wallis test was performed, after which

the Dunn´s multiple comparisons test was used to compare every mean to the control. The significant level was set to $p<0.05$.

When finding the NOEC and LOEC values, the data for PS-NH_2 were normally distributed, and the data for PS-COOH after 48 hours were normally distributed after Log-transformation, so the parametric tests for multiple comparisons following a one-way ANOVA (Dunnett´s test) were used for these polymer types. All the other data were not normally distributed, so a Kruskal-Wallis test is performed with Dunn´s multiple comparisons test to find NOEC and LOEC values for rest of the plastic polymers, as described in 2.4.

For the results obtained for the fluorescent probe tests, statistical tests were also performed in Graphpad Prism 8 to check if the data from copepods exposed differed significantly from the control group. The significant level was set to $p<0.05$. The Mann-Whitney non-parametric test was used to look for differences between the control and the other concentrations, as none of the data were normally distributed and this test was considered as more sensitive than Dunn´s multiple comparisons test (McKnight & Najab, 2010). Microsoft Excel was used for visualisation of the graphical results obtained for the probe tests.

3. Results

The results are presented under three main sections, as the results obtained can be evaluated individually before looking at the relationships between them. The results are divided in: 1) Nanoplastics characterisation, 2) Results from the acute tests and 3) Oxidative stress determination using fluorescent probes. The results from the method development on the use of fluorescent probes can be found in Appendix B.

3.1. Nanoplastics characterisation

The five plastic particles displayed differences in size when measured by dynamic light scattering (DLS) while suspended in different media (Table 3). The sizes obtained for the particles in Milli-Q water (MQW) are similar to the particle sizes advertised by the suppliers. PMMA is the only particle that is bigger than what was confirmed by the supplier, with about 5 nm higher diameter (63.4 nm compared with 60 nm) when comparing the Z-average (Table 3) with the target mean diameter in Table 1. On the other hand, PS is about 5 nm smaller than what was confirmed in MQW (46.6 nm compared with 41 nm). In NSW, the particle sizes ranged from 57.9 nm for PMMA-COOH to over 3000 nm for PS-COOH, as shown in Table 3. The three particles that increased the most in Z-average, PMMA, PS and PS-COOH, all form micro aggregates in NSW, while PMMA-COOH and PS-NH_2 stay in a nanosized dimension.

The zeta-potential was negative for all of the particles in MQW, which indicates an anionic surface charge, except for PS-NH_2 which had a positive zeta-potential. The zeta-potential for the first four particles suspended in NSW are not presented in Table 3, as the measurements performed by DLS were not reliable. This was probably due to a stability problem related to the particles properties while suspended in NSW, that did not allow for a proper characterisation of the particles surface charge using DLS.

The polydispersion index (PDI) changed for all the particles when comparing the results for MQW with those for NSW, as all of the values increased at least 0.13. PMMA-COOH and PS-NH_2 were the particles most monodisperse in NSW, with an increase in PDI of 0.15 and 0.13, respectively. PMMA, PS and PS-COOH were more polydisperse in NSW, with increases in PDI values of 0.32, 0.42 and 0.36, respectively, as seen in Table 3.

Table 3: Properties to the NP particles in different media.. The data is presented as average ± standard deviation.

NPs (50 µg/mL)	MEDIA	Z-AVERAGE (nm)	ZETA-POTENTIAL (mV)	POLYDISPERSION INDEX
PMMA	MQW	63.4 ± 0.3	-35.3 ± 1.0	0.02 ± 0.01
	NSW	2272 ± 134.5		0.34 ± 0.03
PMMA-COOH	MQW	55.7 ± 0.1	-31.1 ± 1.5	0.08 ± 0.01
	NSW	57.9 ± 0.1		0.23 ± 0.01
PS	MQW	46.6 ± 0.3	-58.9 ± 0.6	0.05 ± 0.02
	NSW	1838 ± 451.2		0.47 ± 0.03
PS-COOH	MQW	49.7 ± 0.1	-36.9 ± 0.6	0.06 ± 0.01
	NSW	3652 ± 283.2		0.42 ± 0.1
PS-NH_2	MQW	57.8 ± 0.5	40.4 ± 3.2	0.12 ± 0.01
	NSW	132.9 ± 5.9	8.3 ± 0.5	0.25 ± 5.9

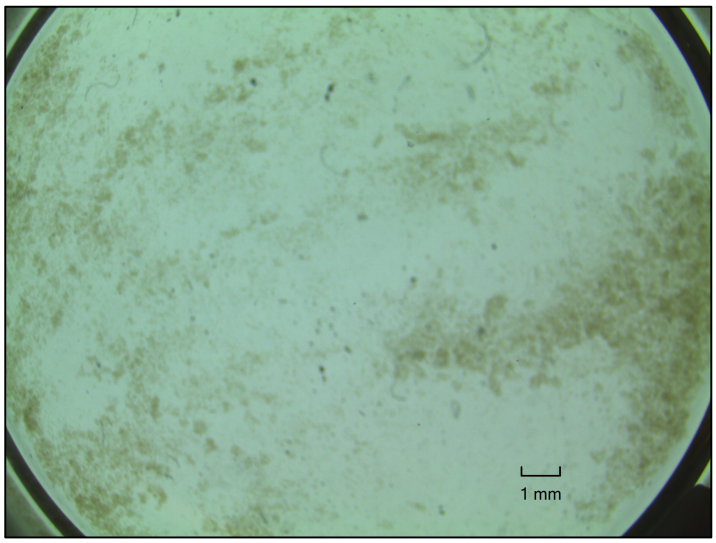

Figure 10: PMMA particles displayed in a test beaker after 48 hours.

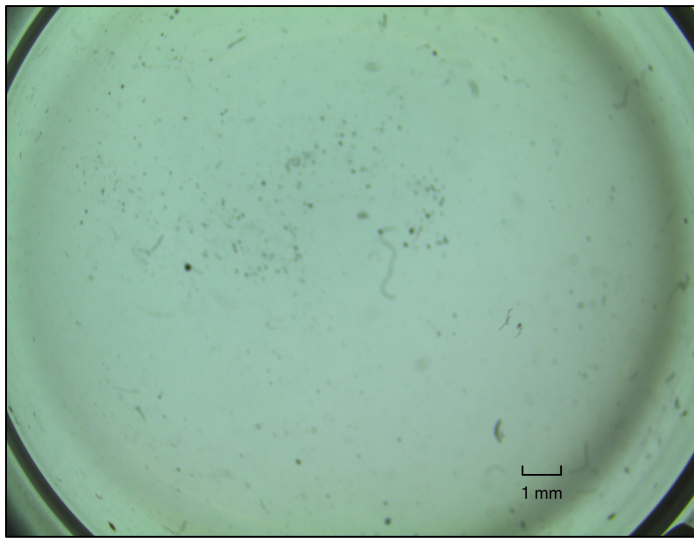

Figure 9: PMMA-COOH particles displayed in a test beaker after 48 hours.

The characterisation results show that PMMA agglomerated when suspended to seawater, while PMMA-COOH particles stayed in approximately the same size range. This difference in particle behaviour was also seen in the exposure beakers after the 48 hours' exposure (Figures 9 and 10). In Figure 9 it is shown that PMMA is clearly more aggregated than PMMA-COOH in Figure 10, as the plastic particles appear as brown agglomerates, while in Figure 10 there is a clearer media without visible particles. Similar aggregations were also seen for PS exposure vessels, as seen in Figure 11.

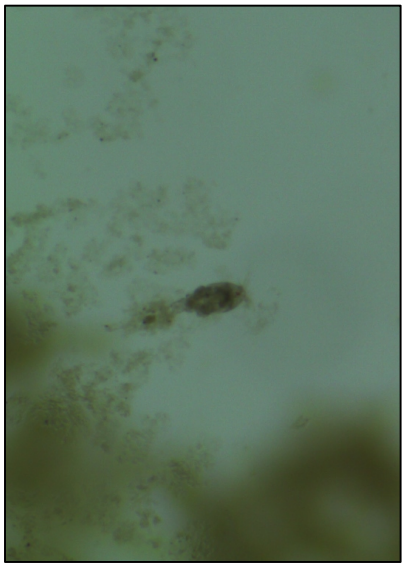

Figure 11: A copepod is visible with some plastic particles attached to its body. This picture was taken for the polymer PS.

3.2. Acute experiments

3.2.1. Water quality parameters

Table 4 shows the values for physico-chemical parameters that determine the water quality measured at the initiation and at the end of the experiments for all plastic particles and the NSW control (all data is presented in Appendix A). All the parameters met the criteria stated in the ISO 14669 guideline, which makes the experiments valid. The changes that occurs in pH, DO and salinity are within what is expected and match the measured parameters for the control group (NSW), and there were no outliers observed.

Table 4: Water quality parameters measured during the experiments.

PLASTIC TYPE	**TEMPERATURE ^{O}C**		**PH**		**DO µg/mL**		**SALINITY ‰**	
	Start	Finish	Start	Finish	Start	Finish	Start	Finish
NSW	19-21	19-21	7.9-8.12	8.13-8.29	6.97-6.99	7.08-7.11	35	36
PMMA	19-21	19-21	7.84-8.02	8.15-8.23	6.86-6.99	7.05-7.27	35	36-37
PMMA-COOH	19-21	19-21	7.76-7.85	8.03-8.27	6.89-6.98	7.07-7.29	35	36-37
PS	19-21	19-21	7.86-8.06	8.16-8.31	7.06-7.12	7.12-7.35	35	36-37
PS-COOH	19-21	19-21	7.94-8.07	8.03-8.24	6.95	7.01-7.26	35	35-37
PS-NH2	19-21	19-21	7.98-8.28	8.19-8.25	7.08	7.18-7.23	35	36-39

3.2.2. Acute exposure

The dose-response curves obtained for 24 and 48 hours´ exposure to each NP are shown in Figures 12 and 13. Most particles showed an increase in mortality with a higher concentration of NPs after 24 and 48 hours, as shown in Figures 12 and 13. PMMA showed almost no mortality in any of the concentrations, while PMMA-COOH had a slightly increase in mortality at the higher concentrations used (50 μg/mL and 100 μg/mL). PS had a significant increase in mortality only for the highest concentration after 48 hours, while PS-COOH and PS-NH_2 showed mortality on some lower concentrations as well, with a NOEC on 25 μg/mL and 1 μg/mL after 48 hours, respectively. The only particles that gave a high-quality concentration-dependent increase in mortality was PS-NH_2 for both 24 and 48 hours´ exposure. More detailed information on the mortality results recorded can be found in Appendix A.

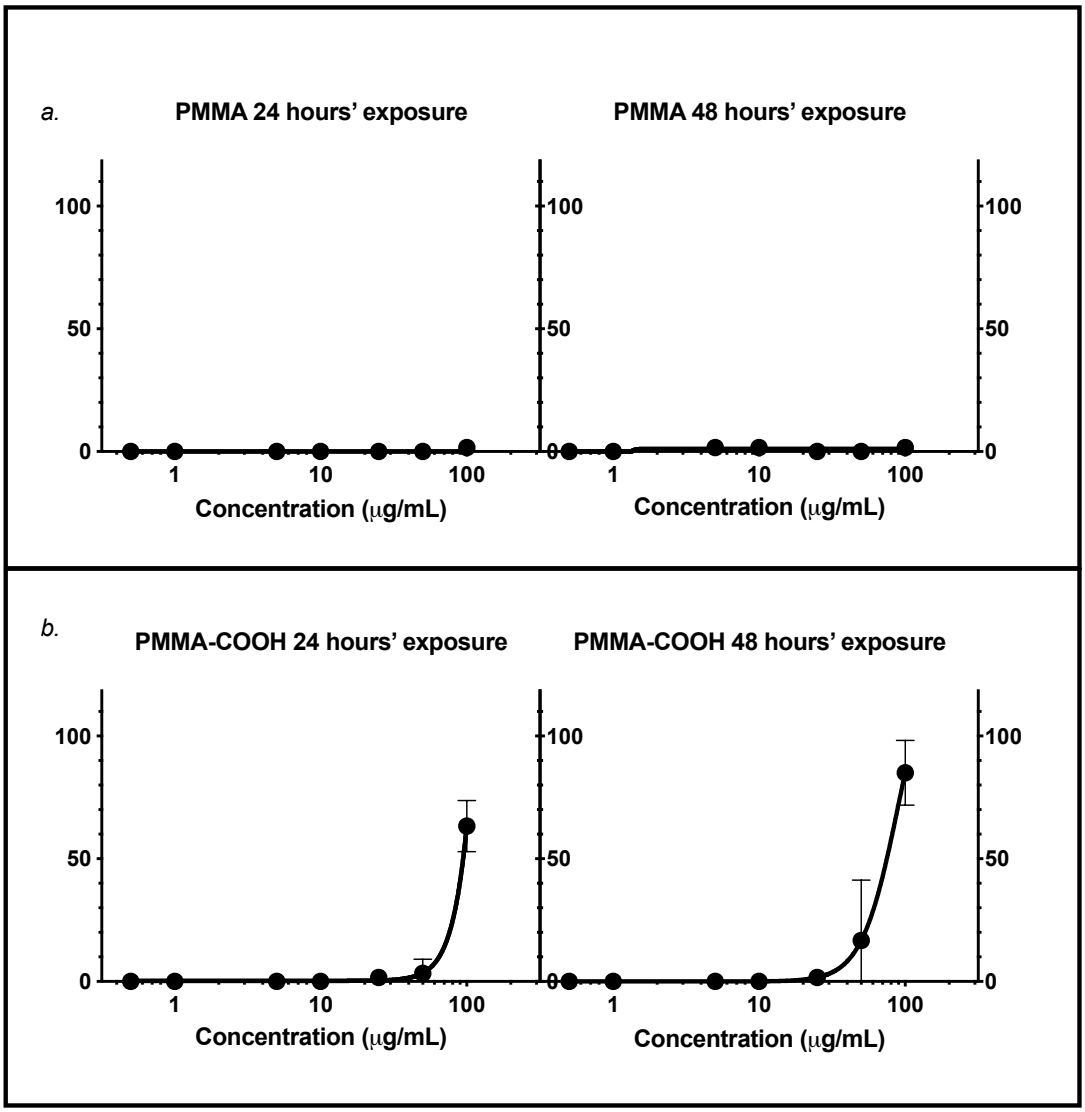

Figure 12: Concentration-response curves displaying average mortality of copepods exposed to the NP particles a) PMMA and b) PMMA-COOH in the concentrations 0, 0.5, 1, 5, 10, 25, 50 and 100 μg/mL(X-axis), after 24 and 48 hours´ exposure. Y-axis indicates percentage of mortality, where 100 implies that all the copepods are dead.

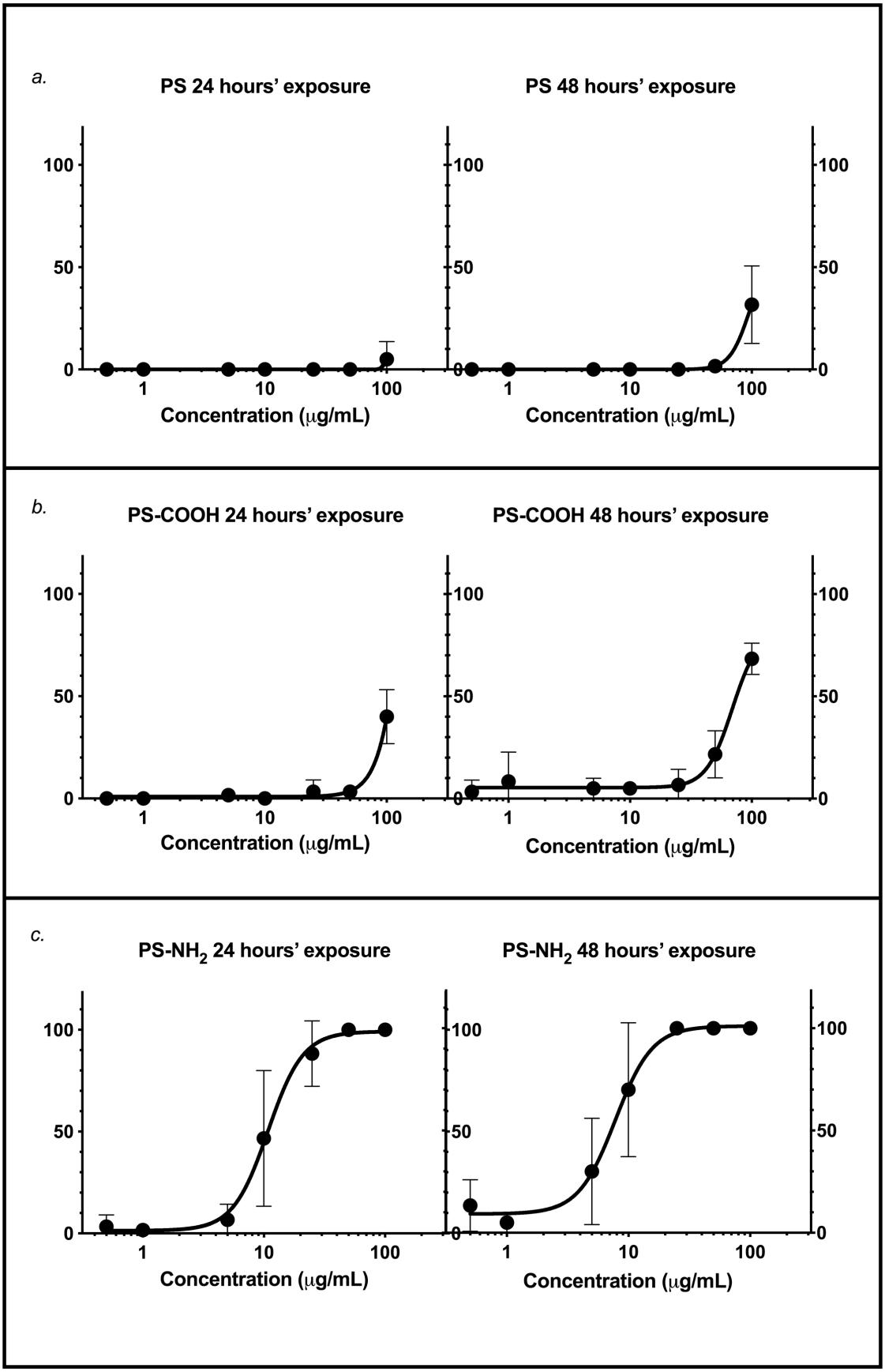

Figure 13: Dose-response curves displaying average mortality of copepods exposed to the NP particles a) PS, b) PS-COOH and c) PS-NH_2 in the concentrations 0, 0.5, 1, 5, 10, 25, 50 and 100 μg/mL (X-axis), after 24 and 48 hours' exposure. Y-axis indicates percentage of mortality, where 100 implies that all the copepods are dead.

Table 5: EC values, NOEC and LOEC values for 24 and 48 hours´ exposure, together with error estimates (top and bottom) for 48 hours´ exposure for all the NP particles. Concentrations in (µg/mL). NC –not calculated.

	PMMA	PMMA-COOH	PS	PS-COOH	PS-NH_2
EC_{10} 24 HOURS	88.3	>100	88.3	>100	5.2
EC_{50} 24 HOURS	98.6	>100	98.5	>100	10.8
EC_{10} 48 HOURS	>100	47.6	51.9	40.3	3.6
EC_{50} 48 HOURS	>100	89.5	95.6	69.3	7.8
NOEC 24 HOURS	>100	50	>100	50	5
LOEC 24 HOURS	>100	100	>100	100	10
NOEC 48 HOURS	50	50	50	25	1
LOEC 48 HOURS	100	100	100	50	5
95% CI TOP 48 HOURS	NC	73.3	NC	58.7	93.5 – 109.6
95% CI BOTTOM 48 HOURS	NC	-6.1	NC	0.13 – 9.97	0.4 – 17.9

Table 5 shows a summary of all the effective concentrations calculated after the acute tests performed for all NP particles. PS-NH_2 has the lowest EC values calculated, with EC_{50} values of 10.8 and 7.8 µg/mL after 24 and 48 hours´ exposure, respectively. PS-COOH was the second most lethal particle tested, with an EC_{50} of 69.3 µg/mL and a LOEC and NOEC of 50 and 25 µg/mL, respectively, after 48 hours´ exposure. PMMA, PMMA-COOH and PS all had higher EC_{50} values, and the highest NOEC of 50 µg/mL. The NOEC and LOEC values that are above 100 show that there was no significant variance between the control and the highest concentration, which applies to PMMA and PS after 24 hours´ exposure. A LOEC of 100 was also registered for PMMA, PMMA-COOH and PS at 48 hours´ exposure, reflecting the lower toxicity of these particles.

3.3. Oxidative stress

The *in vivo* tests for the determination of sub-lethal effects in the form of ROS formation and lipid peroxidation were only performed for PS-NH_2, as this particle was the only one that showed relevant acute lethality in copepods. The results obtained for optimisation of the fluorescent probe tests can be found in Appendix B. The raw fluorescence data measured with the different probes when the copepods were exposed to PS-NH_2 particles are the only data presented here, as the normalisation of data in relation to background fluorescence and length of organisms was challenging (see Appendix B for details). Since many of the copepods were dead after 24 hours´ exposure in the highest concentrations during the acute tests (as shown in Figure 13 c. and more thoroughly in Appendix A), the fluorescence presented here was measured during a lower time of exposure to the plastic particles, as described in the methods. The total time of exposure was 18 hours, after which one time-point for each probe was chosen to represent the data obtained (8 hours´ exposure). The full data set can be accessed in Appendix B. This time point was chosen since the copepods would have been exposed to the NPs for a long enough period of time to potentially ingest particles, the probes would have had time to react with any ROS formed, and the animals would have still been alive. Fluorescence was measured in arbitrary units (a.u), and fluorescent pictures taken from the experiments with the different probes can be found in Appendix B

3.3.1. ROS formation DHR123

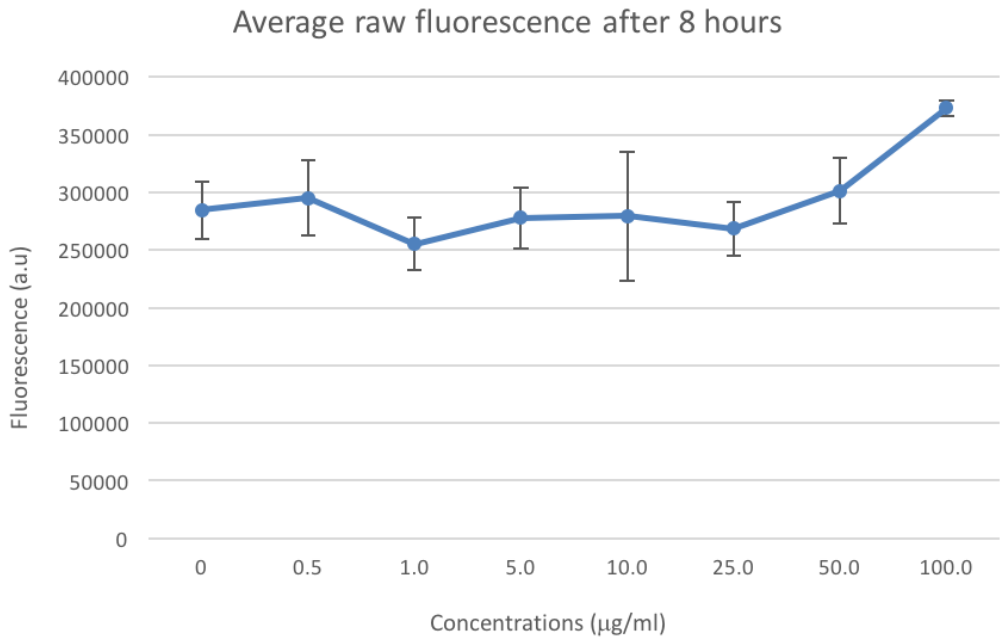

Figure 14: Increase of average fluorescence measured for the probe DHR123 after 8 hours' exposure of copepods to PS-NH_2 particles. Each point on the X-axis refers to the separate concentrations measured with standard deviation lines for the average of the standard deviation of all the measurements. Y-axis indicates the average fluorescence measured.

Figure 14 shows the average raw fluorescence that was measured after the copepods were exposed to PS-NH_2 and the probe DHR123 for 8 hours. This Figure shows that the concentrations between 1 µg/mL and 25 µg/mL of NPs had a lower fluorescence than the control group. On the other hand, the concentrations 0.5 µg/mL and 50 µg/mL has a slightly increase compared to the control, while the concentration 100 µg/mL was the only that showed a significant increase compared to the control.

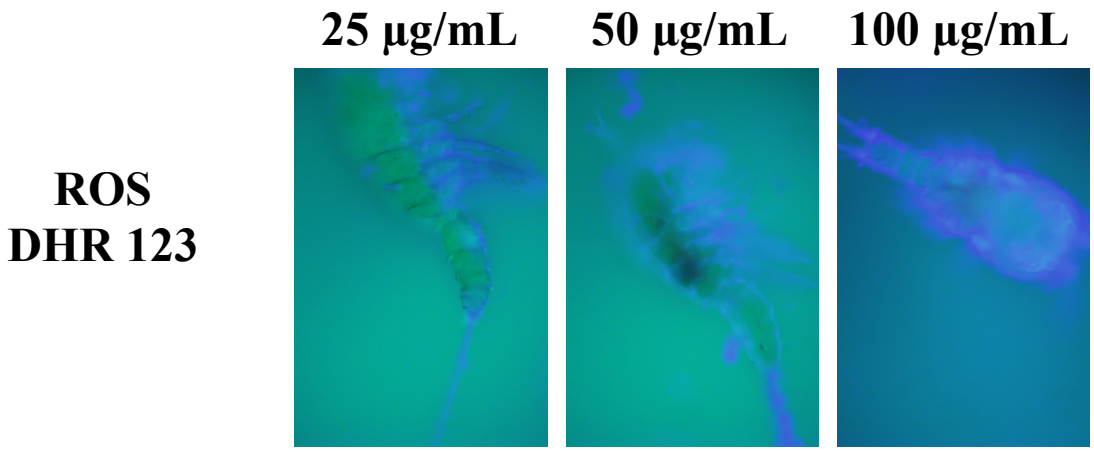

Figure 15: Fluorescence detected in copepods exposed to PS-NH_2 and the ROS detecting probe DHR 123, using a fluorescent microscope Olympus DP72.

Figure 15 shows pictures of three different copepods taken with a fluorescent camera after the 18 hours´ exposure was finished. The fluorescent intensity was different inside distinct areas of the organisms, possibly associated with an increase in ROS formation in these areas. Even though there is some evidence of fluorescence in the media surrounding the copepods, the copepods presented higher fluorescence, probably reflecting the presence of particles inside them due to ingestion. However, the ingestion of the particles could not be confirmed. Additional fluorescent pictures taken for all concentrations can be found in Appendix B.

3.3.2. ROS formation H_2DCFDA

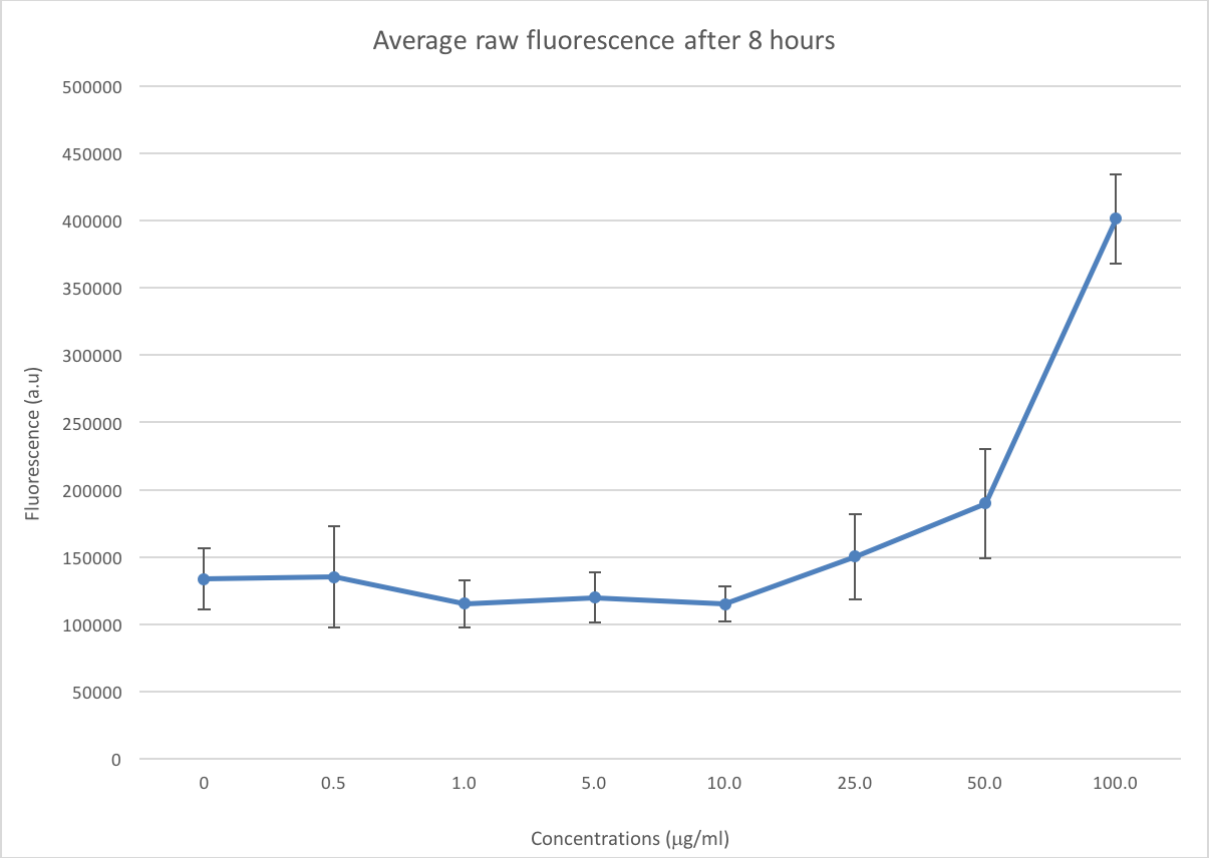

Figure 16: Increase of average fluorescence measured for the probe H_2DCFDA after 8 hours' exposure of copepods to PS-NH_2 particles. Each point on the X-axis refers to the separate concentrations measured with standard deviation lines for the average of the standard deviation of all the measurements. Y-axis indicates the average fluorescence measured.

Figure 16 shows the average raw fluorescence that was measured after the copepods had been exposed to PS-NH_2 and the probe H_2DCFDA for 8 hours. The concentrations between 1 µg/mL and 10 µg/mL of NPs show a lower fluorescence than the control. The concentrations 0.5 µg/mL and 25 µg/mL show higher fluorescence than the control, although this increase was not significant. Finally, copepods exposed to 50 µg/mL and 100 µg/mL of PS-NH_2 had a significant increase compared to the control when the fluorescence was measured after 8 hours.

3.3.3. Lipid peroxidation C11-BODIPY$^{581/591}$

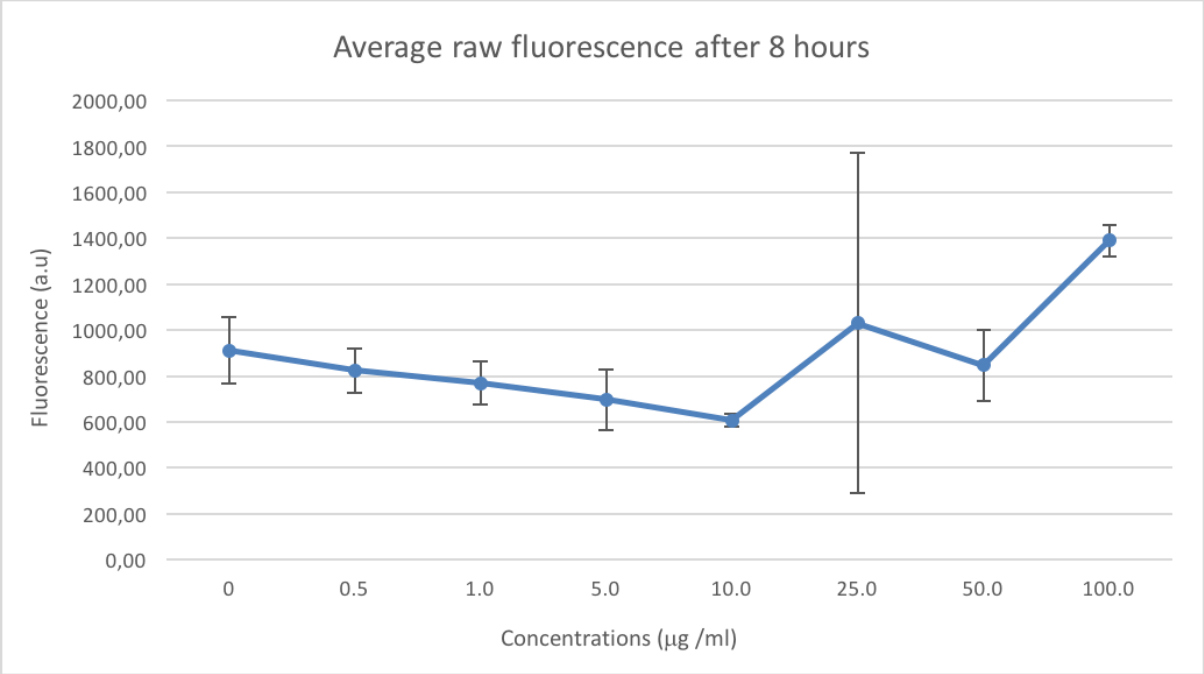

Figure 17: Increase of average fluorescence measured for the probe C11-BODIPY after 8 hours' exposure of copepods to PS-NH_2 particles. Each point on the X-axis refers to the separate concentrations measured with standard deviation lines for the average of the standard deviation of all the measurements. Y-axis indicates the average fluorescence measured.

Figure 17 shows the average raw fluorescence from each concentration that was measured after the copepods had been exposed to PS-NH_2 and the probe measuring lipid peroxidation for 8 hours. The concentrations from 0.5 µg/mL to 10 µg/mL and 50 µg/mL of NPs showed lower fluorescence than the fluorescence measured in the control. On the other hand, at 25 µg/mL a higher fluorescence was detected when compared with the control, although this increase was not significant (the standard deviation was extremely high). The highest concentration of PS-NH_2 (100 µg/mL) displayed a significant increase in fluorescence when the copepods were exposed for 8 hours.

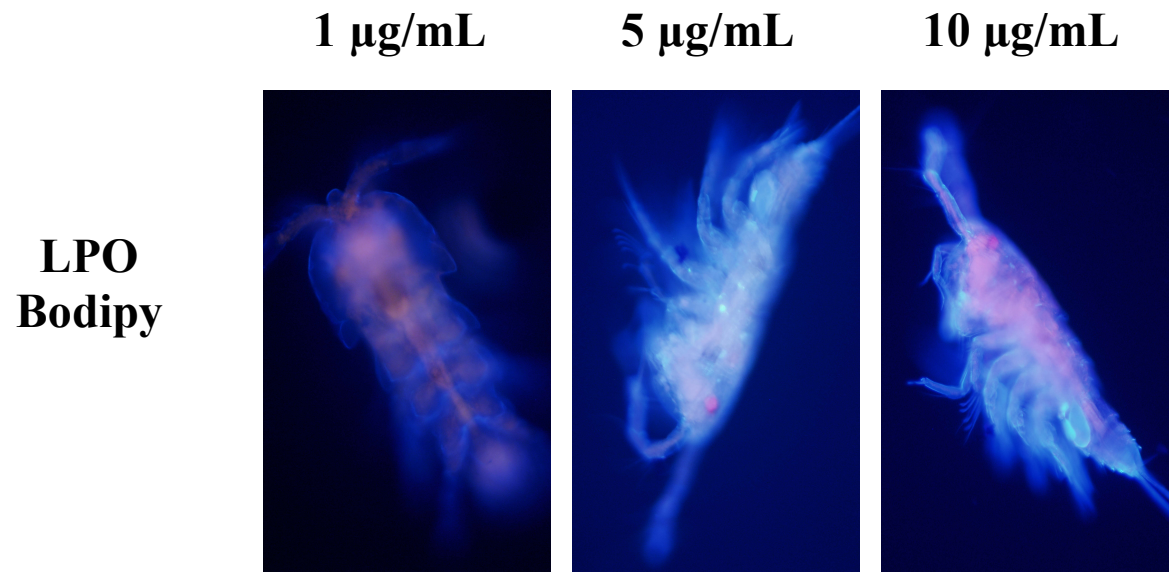

Figure 18: Fluorescence detected in copepods exposed to PS-NH_2 and probe for lipid peroxidation, using the fluorescent microscope Olympus DP72. There are some elevations in fluorescence detected in the copepods, visible for 5 and 10 µg/mL.

In Figure 18, the fluorescent pictures of three different copepods co-exposed to PS-NH_2 and C11-BODIPY$^{581/591}$ show an increased fluorescence in areas associated with formation of lipid peroxidation. This increase in fluorescence seem to be comparable with the increase in fluorescence seen in Figure 15 for the DHR 123 probe. The fluorescence obtained for C11-BODIPY$^{581/591}$ also seem to be mostly concentrated in the gut of the copepods, and may be caused by ingestion of particles. This possible ingestion of particles is most distinct for 5 µg/mL, and can be seen as light turquoise dots inside the copepod. The same dots can be seen for the 10 µg/mL exposure, only as larger areas in the rear region of the copepod. Additional fluorescent pictures taken for all concentrations can be found in Appendix B.

4. Discussion

The discussion is divided into 5 parts that cover 1) Characterisation of the NP particles, 2) Acute toxicity, 3) Oxidative stress, 4) Assessment of environmental relevance and 5) Evaluation of contribution to science and suggestions for future work.

4.1. Nanoplastic characterisation

As stated in literature, the toxicity of plastic particles may be influenced by their composition and properties, and will mainly depend on particle size, polymer type, surface charge and surface modifications (Besseling et al., 2014 (Bergami et al., 2016). For this reason, it is necessary to understand how NP particles characteristics change in different medias. The NP particle characterisation done through dynamic light scattering is shown in Table 3 in the results part 3.1, and shows that there are differences in how the particles behave in different media compositions. The NPs PMMA-COOH and PS-NH_2 stayed approximately the same size in NSW compared to MQW, with an increase on 2 nm for PMMA-COOH and 75 nm for PS-NH_2, respectively, as shown under Z-average in Table 3. This rather small increase in size for PS-NH_2 was also observed by Bergami et al. (2017) and Manfra et al. (2017). In these studies, the particles size increased 69 nm and 54 nm from MQW to NSW, respectively. The other three polymer types (PMMA, PS and PS-COOH) formed micro-aggregates (all above 1500 nm) when exposed to NSW. This increase in Z-average was also observed by Bergami et al. (2017) and Manfra et al. (2017) for the particle PS-COOH, in which an increase of 1010 nm and 940 nm from MQW to NSW was recorded. These differences in aggregation were also confirmed visually for PMMA and PMMA-COOH. The pictures taken after the completion of the acute test (Figure 9 and 10) clearly demonstrate aggregation of PMMA after 48 hours exposure in NSW, while no aggregation was clearly evident for PMMA-COOH. The lack of visible particles means that the particles are probably too small to be detected under the microscope after 48 hours. Unfortunately, Z-average values for PMMA, PMMA-COOH and PS cannot be not found in other studies, and thus it was not possible to compare these sizes with others in the literature.

The determination of surface charge of NPs is important to understand particle behaviour, as the particle charge is known to affect their behaviour in terms of stability and aggregation (Bergami et al., 2017). All the particles showed a negative zeta-potential, the measure of surface charge, in MQW except for PS-NH_2 which had a positive zeta-potential (Table 3). The NP PS-NH_2 was the only particle in this study that was confirmed as positively

charged, as the zeta-potential was not measured in NSW for the non-functionalized and carboxylated particles due to stability issues during the measurements. However, in the study by Bergami et al. (2016), the PS-COOH (40 nm) particles used maintained a negative charge while suspended in seawater. Chen et al. (2018) also measured the zeta-potential for PS, PS-COOH, PMMA and PMMA-COOH (25 nm) in artificial seawater (ASW), and discovered a negative surface charge for all the particles. It is likely to assume that the zeta-potential for the particles would be similar in NSW, though naturally occurring colloids may affect their charge in NSW (Bergami et al., 2016).

The polydispersity index (PDI) measures the distribution of molecular mass in a polymer sample (far right in Table 3) (Rane & Choi, 20). A similar increase from MQW to NSW for PS-COOH and PS-NH_2 (respectively an increase of 36 and 13) was found in other studies, and were proposed as being typical PDI values for these polymer types (Bergami et al., 2016; Manfra et al., 2017). Since the PDI measures distribution of molecular mass, it should correlate with Z-average. A more polydisperse sample (and therefore a high PDI) would reflect larger aggregates than a more monodisperse sample (Helseth & Ore, 2018a; Rane & Choi, 2005). There is a noticeable similar trend between the Z-average size and PDI measured for most of the polymer types. The polymer types PMMA-COOH and PS-NH_2 with the smallest measured sizes in NSW (57.9 and 132.9 nm), were the least aggregated particles in NSW, and also showed the lowest increase in polydispersion. The polymer types PMMA and PS-COOH also followed the same pattern as the NPs already discussed, where PMMA had smaller aggregates than PS-COOH in NSW, and also the lowest PDI (Table 3). The NP polymer that did not follow this pattern was PS, which formed smaller aggregates than both PMMA and PS-COOH in NSW, but had the highest PDI value of all polymer types. This polymer type had a lower zeta-potential than all the other particles (-58.9 mV), which may have affected its PDI value. It is also possible that this polymer type behaved differently from the others because of the lack of functional groups. The PS has a higher zeta-potential than PS-COOH when measured in artificial seawater (ASW) (-20 and -21 mV, respectively), which can indicate that natural organic matter may have interfered and altered the properties of these NPs (Bergami et al., 2016; Chen et al., 2018).

The difference in sizes of aggregates observed in Table 3 may occur because of differences in stability for each of the polymer types. NSW has a high ionic strength, and may screen the particle surface charges of the polymers, causing the visible aggregation (Bergami et al., 2016). The NP PS-COOH was negatively surface charged, while PS-NH_2 had a positive surface charge, which may answer why agglomeration was observed for only PS-COOH

(Bergami et al., 2017). An interesting observation concerning the agglomeration, is that one can observe that the polymer type must have something to say for the agglomeration of particles, as well as the presence of functional groups. This is evident when comparing the polymer types PMMA-COOH and PS-COOH, which are made of different polymer cores, but have the same functional groups. The NP PMMA-COOH stayed approximately the same size in NSW as in MQW, while PS-COOH originated micro-aggregates in NSW (Table 3). The NPs PMMA-COOH and PS-COOH have similar zeta-potentials in MQW, but since the zeta-potential was unfortunately not measured in NSW, it is uncertain if the surface charge of the particles was causing aggregation of one polymer type while the other stayed monodispersed. Booth et al. (2016) suggested that the visible agglomeration for all particle agglomerating NP polymers may occur because of heteroaggregation with other particles in the natural seawater. Even though the water used in this thesis was filtered at 0.22μm, it is possible that other parameters than the ones measured may alter NPs characteristics, like natural organic matter (NOM), proteins and ionic salts, that are not present in MQW. Because of the complexity of the aquatic environment, there are many factors that may trigger the observed aggregation, but it is not easy to say exactly what the cause is.

4.2. Acute test

Very few NPs have been properly tested for toxicity, and of the existing studies very few show lethal endpoints and corresponding EC_{50} values (Walker et al., 2012). There are no studies that report the role of particle type and functionalisation on toxicity to the marine copepod *T. battagliai* in literature, being this study the first one to look at the effects of plain PS and PMMA in comparison to carboxylated and aminated PMMA and PS. The results obtained showed large variances in toxicity for all particles, from EC_{50} values of about 7.8 μg/mL for PS-NH_2 to above 100 μg/mL for PMMA. These results clearly demonstrated that polymer type, size, functional groups and surface charge of the particles affected their toxicity. It was for example seen that PMMA-COOH (EC_{50} = 89.5 μg/mL) and PS-COOH (EC_{50} ≈ 69.3 μg/mL) were apparently more toxic than the plain polymers PMMA (EC_{50} >100 μg/mL) and PS (EC_{50} ≈ 95.6 μg/mL). A reason why polymers with functional groups seem more lethal than plain polymers, is possibly because the functional groups make the molecular structure of the plastic particles similar to the structure of proteins. Such a structure will make these particles cross easier over cell membranes than the similar particles without functional groups (Bergami et al., 2017).

In another study, microcrustaceans (including *D. magna* and *Corophium volutator*, a marine crustacean) were exposed to non-carboxylated PMMA (125 nm) (Booth et al. (2016). With an EC_{50} value of >1000 μg/mL for *D. magna* and >500 μg/mL for *C. volutator*, this particle type was found not to cause acute toxicity in these crustaceans at reasonable concentrations (Booth et al., 2016). The findings made by Booth et al. (2016) cohere with the results obtained in this thesis, where an EC_{50} value >100 μg/mL was found for PMMA. Studies on PMMA-COOH toxicity do not exist yet, so direct comparison between effect values could not be conducted. In this thesis, there was a slightly higher mortality for the carboxylic polymer PMMA-COOH than for the non-functionalised PMMA, (Figure 12 a, b). When reviewing the z-average sizes of these particles, it is seen that PMMA agglomerated in NSW while PMMA-COOH stayed approximately the same size. Since PMMA-COOH remained the same size over a longer period of time, the plastic particle was possibly more toxic to the copepods, due to larger potential for ingestion and interaction with cells and intracellular biological targets (Bergami et al., 2017). When studying the results for the PMMA particles, it is likely to believe that it is the functional –COOH group that made the polymer stable in NSW and not agglomerate. These results further confirm that the functional group also makes the particle more toxic, as is more similar to proteins, as discussed above (Bergami et al., 2017).

Bergami et al. (2016) tested the acute toxicity of the particle PS-COOH and PS-NH_2, but with another life stage of another marine crustacean, the larvae stadium of the brine shrimp. The results authors got cohere with the results obtained in this thesis, as well as the results showed by Della Torre et al. (2014) with sea urchins embryos, where the PS-NH_2 appears to be more lethal than the PS-COOH particles. The results for PS-COOH and PS-NH_2 found in the two abovementioned articles coheres with the findings in this thesis, as the results here show EC_{50} values of about 69.3 and 7.8 μg/mL, respectively. Della Torre et al. (2014) did not find an EC_{50} for PS-COOH, as this particle did not show any relevant effects on embryo development of sea urchins, while the EC_{50} for PS-NH_2 obtained was 3.8 μg/mL (Della Torre et al., 2014). It must be emphasised that the EC_{50} values found by these authors cannot be directly compared with the lethal concentrations found in this thesis, as their EC_{50} values show the concentration that affects embryo development, not the concentration that kills them. Embryo development and adult mortality are fundamentally different endpoints and may be caused by dissimilar toxic mechanisms. However, the results found from Della Torre et al. (2014) showed the same trend as the EC_{50} values found in this thesis, and verified that PS-NH_2 was more toxic than PS-COOH.

The NP PS without any functional groups had a smaller size in NSW than PS-COOH, (Table 3), but displayed higher mortality ($EC_{50} \approx 69.2$ μg/mL) than non-functionalised PS ($EC_{50} \approx 95.6$ μg/mL) (Table 5). This contrasted with what was observed for PMMA and PMMA-COOH, where the smallest particle size displayed the largest toxic potency in terms of mortality. These variances may indicate that the functional groups are the main reason for why the copepods react differently when being exposed to the particles, potentially due to their difference in surface charge (Bergami et al., 2017; Della Torre et al., 2014). The most lethal polymer type observed in this study was without doubt PS-NH_2 ($EC_{50} \approx 7.8$ μg/mL). The NP PS-NH_2 was the only plastic particle that was lethal for the crustaceans showing a clear concentration-dependent increase in mortality. This was also the only particle with a positive surface charge, as seen in Table 3. Della Torre et al. (2014) stated that both PS-COOH and PS-NH_2 entered the cells of the tested organisms, although it was only PS-NH_2 that caused lipid peroxidation and ROS formation. The authors suggest that it is the positive surface charge that makes the PS-NH_2 particles so toxic.

4.3. Oxidative stress determination

Several studies have suggested that particle surface chemistry is relevant for the toxic potential of NPs, not only for cell death and/or death of the organism, but also for sub-lethal toxicity (Bergami et al., 2017). The results obtained in the acute testing showed that PS-NH_2 was the only plastic polymer that was considered toxic from the polymers tested, and this acute toxicity seem to be associated with the type of particle functionalisation, as well as it surface charge. With mortality seen at low NP concentrations, this was the polymer type tested with the largest toxic potential, and it is interesting to try to understand how this polymer affected the organisms on a sub-lethal level.

The formation of oxidative stress is one example of a sub-lethal endpoint that can be determined in aquatic organisms, and it has been indicated as one of the possible effects of NP particles (Della Torre et al., 2014; Jeong et al., 2018). In the present study, for the two ROS-detecting probes, DHR123 and H_2DCFDA, it seems like H_2DCFDA was the most suitable to determine ROS production after exposure to PS-NH_2. With significant fluorescence for the two highest concentrations (50 μg/mL and 100 μg/mL) (Figure 16), this probe seems the most applicable when detecting ROS formation in copepods. This probe was also the only that showed a significant fluorescence after the normalisation of data for 100 μg/mL (Figure B19), which indicates the formation of ROS, although this method needs to be further optimised to get better results. Regular ROS formation after exposure to NPs was also detected by Jeong et al. (2018) using the fluorescent probe H_2DCFDA. In this study, rotifers (which are about the same size as copepods) were exposed to non-functionalised PS, and significant differences were found at concentrations as low as 0.1 μg/mL in the tested animals. However, in the study by Jeong et al. (2018) another method was used for the detection of ROS; about 2000 rotifers were exposed to the NPs for 24 hours, washed and then homogenized. The supernatant was used in this case for the probe test to detect ROS formation and lipid peroxidation instead of an *in vivo* method like the one used in this thesis. Artificial seawater was also used by Jeong et al. (2018) instead of natural seawater, as in this study. Even though the natural seawater used in this thesis is filtered at 0.22 μm, it is possible that the water contained other substrates (like NOM) that may have affected the fluorescent probes or the NPs, and be the reason for the elevated fluorescence seen for the blank controls.

One of the most common examples of physiological damages associated with oxidative stress is the formation of lipid peroxidation (Mylonas & Kouretas, 1999). The study from Jeong et al. (2018) imply that NP particles cause harm to membranes in chemical or physical ways.

In this thesis, the fluorescent probe C11-BODIPY was used to see if NP cause lipid peroxidation in *T. battagliai*. Cheloni and Slaveykova (2013) found the probe C11-BODIPY suitable for *in vivo* measurements of lipid peroxidation within green alga in MQW, and this is the first time this probe was used in this copepod species. Lipid peroxidation was one of the causes of membrane damage in rotifers exposed to PS NPs, and was detected by increased levels of malondialdehyde (MDA) which is the final product of lipid peroxidation (Jeong et al., 2018). In this study, even though the results obtained for C11-BODIPY after normalisation (Appendix B, Figure B17) showed no significant increase in fluorescence, and therefore no lipid peroxidation, the probe gave an increase in signal at the highest concentration tested (Figure 16), and should be considered in further studies after additional method development.

For both lipid peroxidation and ROS formation, Jeong et al. (2018) observed a decrease in fluorescence for the highest concentration tested (20 μg/mL). This finding does not correlate with what is seen in this thesis, where only the highest concentration had an increase in fluorescence. This could occur because of differences in the composition of water, properties of the NPs in the water, use of different species or life stage differences. These differences in results can also occur because of the dissimilarities in the methods used, as the current study used *in vivo* methods and the study of Jeong et al. (2018) used another method that is already described. Even though the results are not completely comparative, both studies see a significant increase in fluorescence on some levels, indicating that NP particles seem to induce oxidative stress in the organisms.

Overall, the significant increase in both ROS-formation and lipid peroxidation seen for the highest concentration (100 μg/mL) seem to indicate that the PS-NH_2 particles cause oxidative stress in the cells of the copepods. Jeong et al. (2018) confirmed this hypothesis, as authors saw that rotifers exposed to PS had a concentration-dependent increase in oxidative stress that was connected with the presence of the PS beads in the digestive tract of rotifers (Jeong et al. (2018). In an experiment by Snell and Hicks (2011), they also noticed that nanosized PS were dispersed in the entire body of the used rotifer organisms, while the bigger PS particles that were categorised as microplastic only stayed in the digestive trait. The same was observed by Jeong et al. (2018), who concluded that these smaller particles would have a longer retention time in the organisms than larger microplastic particles. Even though no concrete conclusions can be reached with the fluorescent pictures taken from the copepods exposed to PS-NH_2 and the fluorescent probes (Figures 15 and 18), there seems to be evidence of visible differences in fluorescent intensity in some areas inside the copepods. It looks like there is an increase of fluorescence inside the copepods, which can originate from interactions

with the aminated NPs, and consequently oxidative stress. The fluorescent pictures are similar to the findings from Booth et al. (2016) and Bergami et al. (2017), where NP particles were detected in the gut of the organisms. Some of the fluorescence in Figure 18 may however indicate that the particles are not in the gut, as they don't seem to follow the digestive tract. It may be difficult to distinguish without further studies if the observed particles actually are in the guts of the animals, if they are stuck to the outside, or if they have been dispersed in other cells of the body, as observed in the study of Snell and Hicks (2011).

4.4. Ecological relevance

Since there are no good sampling methods for measuring NP levels in the oceans, as there are for macro- and microplastics, it is difficult to estimate the amounts of NPs in the marine environment, and to find ecologically relevant concentrations of the different NP polymers (Della Torre et al., 2014; Mattsson et al., 2015). The amount of NP pollution in the surface layers in the oceans is mostly based on findings of larger plastic particles (Ter Halle et al., 2017). Of the large plastic particles found, most have a low density, like PP and PE.

Along the Swedish coast, MP particles are found with an abundance extending up to 102 000 particles per square meter, with an average on 7000 to 10 000 MP particles per square meter (Lönnstedt & Eklöv, 2016). However, it is hard to estimate how much of these particles are expected to degrade into nanoparticles within a relatively short time. These numbers from Lönnstedt and Eklöv (2016) were found through harvesting with mesh sizes down to 10 μm, and these environmentally relevant concentrations of MPs were found to affect the organisms tested in a negative way. Ter Halle et al. (2017) tried to estimate the amount of each polymer found in the North Atlantic gyre. The authors found that 2% of the small MPs collected with size <25μm were composed of PS. In comparison, none of the bigger MPs found (<300μm) were made out of this polymer core, and the concentrations found for small MPs were much bigger than for the larger MP. Further studies are needed to understand why smaller particles are more buoyant than the larger particles.

As for the particles used in this study, their concentration in the marine environment is currently non-existent and the information available in literature in based on assumptions on the quantification of bigger sized particles. In a study conducted by Booth et al. (2016), PMMA nanoplastic concentrations up to 1.0 μg/mL were considered environmental realistic. However, the crustaceans tested were not affected either by environmentally relevant concentrations of 1μg/mL and or by high concentrations (1000 μg/mL). The study by Booth et al. (2016) did not test PMMA-COOH, and environmentally realistic concentrations of this polymer are unknown, nor has it been used in other exposure studies with crustaceans. Ecologically relevant amounts of PS are also not currently known, although Bergami et al. (2016) states that because of the large production of this polymer annually, PS must be considered a threat to the marine environment. It is not certain how much of the PS that is found in the marine environment contains functional groups, although anionic PS (PS-COOH) has been suggested as more widespread in the oceans than the cationic PS-NH_2 (Bergami et al., 2016).

With a NOEC on 50 μg/mL in this thesis and ecological relevant concentrations on about 1 μg/mL, it could be assumed that PMMA would not pose a threat to microcrustaceans such as *T. Battagliai* in the marine environment (Booth et al., 2016). Since relevant concentrations are not available for PMMA-COOH, PS or functionalised PS, it is not certain if these particles will be a threat to marine organisms. However, with PS being a widely used NP, it must be assumed that environmental relevant concentrations can be higher than for PMMA. Based on the results obtained in this thesis, where a NOEC of 1 μg/mL was calculated for PS-NH_2, it can be hypothesised that this particle potentially poses a threat to the marine environment. Della Torre et al. (2014) suggests that PS-NH_2 causes oxidative stress, cytotoxicity and cell death in organisms. This toxicity could also apply to PS-COOH due to the presence of the COOH functional group, although to a lesser extent, so even small concentrations of the functionalised PS particles are able to harm organisms in the aquatic environment. A partially linkage between acute mortality and oxidative stress was obtained in this thesis, as significant increases in fluorescence were seen for the highest concentration tested (100 μg/mL). It can be hypothesised that this particle may harm organisms on a sub-lethal level.

Agglomeration was found for most of the used particles and seemed to affect the survival rate of small organisms. When the particles keep their initial size in the nanoscale (50 nm), it is easier for microcrustaceans to filter them as they filter the surrounding water. For example, *I. galbana* (algae used as food for copepods) has a size range of 4-6 μm in diameter, which is the size copepods normally ingests (Boussiba et al., 1988). When the particles aggregate, they increase in size. The copepods used in these experiments are 100-150 μm in the smallest dimension, and would not be able to ingest aggregates approaching their own size. A positive side with aggregation of the particles is that the particles may become so big and heavy, that they sink to the ocean bottom and become less available for ingestion from pelagic organisms. The particles can, however, be more available for benthic organisms. A negative side about the agglomeration of particles is that the bigger particles may stick to the copepods. This was clearly visible in the acute test after 48 hours (Figure 11). Even though agglomeration did not affect the mortality seen in the laboratory experiments performed in this thesis, particles may stick to the organisms and make them less mobile, and therefore harder to eat, breed and develop.

Even though not all particles caused mortality in copepods in the concentrations tested in this experiment, it is possible that environmental relevant concentrations of the same particles may act as stressors for organisms in the oceans. These are stressors that the organisms are not

used to, and may not affect them severely in laboratory experiments where all the water quality parameters are optimal. However, the copepods may notice a larger effect in their natural habitat where other stressors as predators, temperature changes and salinity changes can be present. The presence of multiple stressors may have a synergetic effect in organisms, that may increase the outcome of one of the stressors in comparison to the effect posed by only one stressor affecting them. It has been shown that several stressors, like temperature fluctuations and pH changes together with a non-natural compound like PCB and heavy metals may affect organisms more than without the natural changes (Benedetti et al., 2016; Vieira & Guilhermino, 2012). Nanoplastics were also shown to enhance the toxicity of other toxic compounds when exposed to organisms (Jeong et al., 2018). These types of synergetic effects need to be studied further to understand the complexity and risk of NP particles in the aquatic environment.

4.5. Strengths, weaknesses and needs for additional studies

The studies done in this thesis demonstrated that a combination of particle characterisation, acute toxicity and oxidative stress determination expanded the knowledge of NP impact in marine crustaceans such as *T. battagliai*. The NP properties were successfully characterised in NSW compared to MQW, and the differences in agglomeration and surface charge were in conjunction with other literature (for PS-COOH and PS-NH_2). Standardised toxicity studies testing acute mortality were able to rank the NP particles according to their toxicity potential in *T. battagliai*, with PS-NH_2 being the particle that shows the highest risk.

When working with living animals there are numerous factors that may alter their living standard and affect the performance of experiments, as mentioned in material and methods. In this thesis, two different batches of water were used for the experiments, collected at different time points from the Oslofjord. Even though the water was filtered at 0.22 μm before use, the copepods needed time to adjust to the new water and their reproduction declined, and unfortunately some of the tests planned had to be postponed. For this reason, enough time must always be set aside to do these type of experiments to ensure the repetition of exposure tests and enough replication in terms of number of organisms.

To investigate if agglomeration of particles affect how copepods eat, breed and develop, it is possible to do other types of tests over a longer time span and with different life cycles, as for example tests looking at effects in development and reproduction (Besseling et al., 2014). Most literature studying micro- and nanoplastics looks at effects from ingestion of particles and do not consider effects at the sub-lethal level like reproduction and development (Cole et al.,

2015). For this reason, it would be interesting to see how the copepods are reacting to agglomeration of particles over a longer time (e.g. chronic studies), since this was observed at nearly all concentrations for some of the particles, even some at lower concentrations. By using aggregating particles (i.e. PMMA-COOH) it would be possible to see if the agglomeration of particles affects the animals considering reproduction and development. From the comparison of results from acute tests with results from development and reproduction tests, it would be possible to evaluate where in the life cycle of the copepods the NP particles make the most harm, and to see what life stages are most vulnerable if exposed to NP particles.

Acute experiments only test for mortality, and the reasons that cause the mortality seen for some of the polymers, like PS-NH_2, are not known (Appendix A). To find out more about what causes the mortality seen for the acute tests, it is necessary to further check for ROS formation and lipid peroxidation, after the methods are fully optimised based on the suggestions in Appendix B, in addition to other endpoints related to oxidative stress as damage to proteins or DNA. In addition, the uptake and ingestion of the particles should also be studied in detail to see if they are the reason for the formation of oxidative stress in the exposed copepods.

Since there are a lot of plastic polymer types that are potentially present in the marine environment (as displayed in Figure 2), future research should focus on acute mortality and particle characterisation for more NP polymer types, as for example PP and PE that are two of the most common plastic types in the environment, as to see how the most abundant particles may affect organisms. Further studies should also look at other species that might be more vulnerable to the presence of NP particles (as for example benthic species), as well as different life stages (for example larval or embryonic stages). Additional toxicity mechanisms and sub-lethal endpoints of NPs should also be assessed, like cytotoxicity, DNA damage, inflammation, etc., to understand how these particles can negatively affect organisms and potentially lead to their death (Gomes et al., 2018).

5. Conclusion

The present study showed that the particles PMMA, PS, and PS-COOH formed microaggregates when exposed to NSW, while PMMA-COOH and PS-NH_2 stayed in a nanosized dimension. The plastic polymers clearly displayed different toxicity potentials, as showed by the acute standardised toxicity tests with the copepod *T. battagliai*, in which PS-NH_2 was the most toxic particle studied. Further studies looking at sub-lethal effects of the most toxic NP, showed that PS-NH_2 could cause ROS formation and lipid peroxidation, although challenges in the method development limited the ability to establish a fully reliable analysis method and coherent results. Suggestions for improvements in the method and future research were proposed to get an enhanced ecotoxicological assessment of micro- and nanoplastics. A basic risk assessment revealed that it is possible that the mortality seen for copepods exposed to PS-NH_2 is caused by oxidative stress formed inside the animals when they are exposed to these nanoplastic particles.

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Appendix A
Acute mortality

1. Concentration calculations

For all NPs, a stock solution of 100 µg/mL was prepared directly from the suspensions provided by the suppliers. This stock solution was serially diluted in natural seawater (NSW) to obtain working solutions with a range of 0.5-100 µg/mL, as seen in Table A1 and A2. The reason for why there are different calculations for PS-NH_2 compared to the other particles is that the concentration of these particles in the original solution is higher than for the other particles, so less volume of the original solution is needed to prepare the concentrations. These concentration calculations were also used for making the NP solutions used in the test to determine oxidative stress.

Table A1: Concentrations calculations for the NPs PMMA, PMMA-COOH, PS and PS-COOH

	C1 (µg/ml)	V1 (ml)	C2 (µg/ml)	V2 (ml)	
Stock	10000	0.500	**100**	50	**T7**
T1	**100**	0.125	0.5	25	
T2	**100**	0.250	1.0	25	
T3	**100**	1.250	5.0	25	
T4	**100**	2.50	10	25	
T5	**100**	6.250	25	25	
T6	**100**	12.50	50	25	
	TOTAL	22.88			

Table A2: Concentration calculations for the NP PS-NH_2

	C1 (µg/ml)	V1 (ml)	C2 (µg/ml)	V2 (ml)	
Stock	25000	0.200	**100**	50	**T7**
T1	250	0.05	0.5	25	
T2	250	0.1	1.0	25	
T3	250	0.5	5.0	25	
T4	250	1.	10	25	
T5	250	2.5	25	25	
T6	250	5.0	50	25	
	TOTAL	9.15			

2. Measured mortality

The percentage of mortality for the different concentrations was calculated for each polymer type based on data from three replicate experiments. The percentage in each grid (in Table A3-A12) was calculated from 5 initial living organisms in each beaker for all the concentrations. 0% indicates that all the organisms were alive at the end of exposure. 100% indicates no living copepods at the end of exposure. The concentration-response curves in Figure 12 and 13 are made from the average value of the three replicate exposure tests that are shown in the Tables A3-A12. There were four replicate vessels for each concentration as emphasised in the Tables.

Table A3: Measured mortality for 24 hours´ exposure of the NP PMMA

CONTROL	0	0	0	0	20	0	0	0	0	20	0	0
T1	0	0	0	0	0	0	0	0	0	0	0	0
T2	0	0	0	0	0	0	0	0	0	0	0	0
T3	0	0	0	0	0	0	0	0	0	0	0	0
T4	0	0	0	0	0	0	0	0	0	0	0	0
T5	0	0	0	0	0	0	0	0	0	0	0	0
T6	0	0	0	0	0	0	0	0	0	0	0	0
T7	0	0	20	0	0	0	0	0	0	0	0	0

Table A4: Measured mortality for 48 hours´ exposure of the NP PMMA

CONTROL	0	0	0	0	20	0	0	0	0	20	0	0
T1	0	0	0	0	0	0	0	0	0	0	0	0
T2	0	0	0	0	0	0	0	0	0	0	0	0
T3	0	0	0	0	0	0	20	0	0	0	0	0
T4	0	0	0	0	0	0	0	20	0	0	0	0
T5	0	0	0	0	0	0	0	0	0	0	0	0
T6	0	0	0	0	0	0	0	0	0	0	0	0
T7	0	0	20	0	0	0	0	0	0	0	0	0

Table A5: Measured mortality for 24 hours´ exposure of the NP PMMA-COOH

CONROL	0	0	0	0	0	0	0	0	0	0	0	0
T1	0	0	0	0	0	0	0	0	0	0	0	0
T2	0	0	0	0	0	0	0	0	0	0	0	0
T3	0	0	0	0	0	0	0	0	0	0	0	0
T4	0	0	0	0	0	0	0	0	0	0	0	0
T5	0	0	0	0	0	0	20	0	0	0	0	0
T6	0	0	0	0	0	0	20	20	0	0	0	0
T7	60	60	60	40	80	60	80	80	40	60	40	100

Table A6: Measured mortality for 48 hours´ exposure of the NP PMMA-COOH

CONTROL	0	0	0	0	20	0	0	0	0	20	0	0
T1	0	0	0	0	0	0	0	0	0	0	0	0
T2	0	0	0	0	0	0	0	0	0	0	0	0
T3	0	0	0	0	0	0	0	0	0	0	0	0
T4	0	0	0	0	0	0	0	0	0	0	0	0
T5	0	0	0	0	0	0	20	0	0	0	0	0
T6	0	0	0	0	40	40	40	60	20	0	0	0
T7	100	100	100	60	100	100	100	100	80	60	60	100

Table A7: Measured mortality for 24 hours´ exposure of the NP PS

CONTROL	0	0	0	0	0	0	0	0	0	0	0	0
T1	0	0	0	0	0	0	0	0	0	0	0	0
T2	0	0	0	0	0	0	0	0	0	0	0	0
T3	0	0	0	0	0	0	0	0	0	0	0	0
T4	0	0	0	0	0	0	0	0	0	0	0	0
T5	0	0	0	0	0	0	0	0	0	0	0	0
T6	0	0	0	0	0	0	0	0	0	0	0	0
T7	20	20	0	20	0	0	0	0	0	0	0	0

Table A8: Measured mortality for 48 hours´ exposure of the NP PS

CONTROL	0	0	0	0	20	0	0	0	0	20	0	0
T1	0	0	0	0	0	0	0	0	0	0	0	0
T2	0	0	0	0	0	0	0	0	0	0	0	0
T3	0	0	0	0	0	0	0	0	0	0	0	0
T4	0	0	0	0	0	0	0	0	0	0	0	0
T5	0	0	0	0	0	0	0	0	0	0	0	0
T6	0	0	0	0	0	0	0	20	0	0	0	0
T7	60	60	40	20	20	40	40	60	0	40	0	0

Table A9: Measured mortality for 24 hours´ exposure of the NP PS-COOH

CONTROL	0	0	0	0	0	0	0	0	0	0	0	0
T1	0	0	0	0	0	0	0	0	0	0	0	0
T2	0	0	0	0	0	0	0	0	0	0	0	0
T3	0	0	0	20	0	0	0	0	0	0	0	0
T4	0	0	0	0	0	0	0	0	0	0	0	0
T5	0	0	0	40	0	0	0	0	0	0	0	0
T6	0	0	0	0	0	0	20	0	0	0	20	0
T7	20	20	60	0	40	60	40	40	60	20	40	40

Table 10: Measured mortality for 48 hours´ exposure of the NP PS-COOH

CONTROL	0	0	0	0	20	0	0	0	0	20	0	0
T1	0	0	0	0	20	20	0	0	0	0	0	0
T2	20	0	60	20	0	0	0	0	0	0	0	0
T3	0	0	0	40	0	0	0	20	0	0	0	0
T4	20	0	0	0	0	0	20	0	0	0	0	20
T5	20	0	0	40	20	0	0	0	0	0	0	0
T6	0	0	20	40	40	20	60	20	40	0	20	0
T7	80	60	60	80	60	80	60	100	80	40	60	60

Table A11: Measured mortality for 24 hours´ exposure of the NP PS-NH_2

CONTROL	0	0	0	0	0	0	0	0	0	0	0	0
T1	20	20	0	0	0	0	0	0	0	0	0	0
T2	0	0	20	0	0	0	0	0	0	0	0	0
T3	20	40	0	0	0	0	0	20	0	0	0	0
T4	80	60	80	80	100	80	0	40	0	0	40	0
T5	100	100	100	100	100	80	100	100	60	80	40	100
T6	100	100	100	100	100	100	100	100	100	100	100	100
T7	100	100	100	100	100	100	100	100	100	100	100	100

Table A12: Measured mortality for 48 hours´ exposure of the NP PS-NH_2

CONTROL	0	0	0	0	20	0	0	0	0	20	0	0
T1	20	40	0	0	80	0	0	20	0	0	0	0
T2	0	0	20	0	20	0	20	0	0	0	0	0
T3	20	80	60	20	0	60	60	60	0	0	0	0
T4	100	100	100	100	100	100	0	100	60	40	40	0
T5	100	100	100	100	100	100	100	100	100	100	100	100
T6	100	100	100	100	100	100	100	100	100	100	100	100
T7	100	100	100	100	100	100	100	100	100	100	100	100

3. Water quality parameters

The raw data from the water quality parameters tested are presented here.

pH

PMMA-COOH I		
Conc.	Start pH	pH after 48 h
T1	7.76	8.16
T2	7.76	8.17
T3	7.76	8.17
T4	7.76	8.19
T5	7.76	8.16
T6	7.76	8.13
T7	7.76	8.12

PMMA-COOH II		
Conc.	Start pH	pH after 48h
T1	7.83	8.24
T2	7.83	8.27
T3	7.83	8.27
T4	7.83	8.26
T5	7.83	8.24
T6	7.83	8.2
T7	7.83	8.14

PMMA-COOH III		
Conc.	Start pH	pH after 48h
T1	7.85	8.12
T2	7.85	8.15
T3	7.85	8.15
T4	7.85	8.12
T5	7.85	8.14
T6	7.85	8.12
T7	7.85	8.03

PMMA I		
Conc.	Start pH	End pH
T1	7.84	8.19
T2	7.84	8.17
T3	7.84	8.17
T4	7.84	8.17
T5	7.84	8.17
T6	7.84	8.18
T7	7.84	8.17

PMMA II		
Conc.	Start pH	pH after 48h
T1	7.91	8.2
T2	7.91	8.23
T3	7.91	8.19
T4	7.91	8.22
T5	7.91	8.21
T6	7.91	8.2
T7	7.91	8.19

PMMA III		
Conc.	Start pH	pH after 48h
T1	8.02	8.16
T2	8.02	8.16
T3	8.02	8.17
T4	8.02	8.17
T5	8.02	8.15
T6	8.02	8.16
T7	8.02	8.15

PS I		
Conc.	Start pH	End pH
T1	7.86	8.16
T2	7.86	8.17
T3	7.86	8.16
T4	7.86	8.17
T5	7.86	8.16
T6	7.86	8.16
T7	7.86	8.18

PS II		
Conc.	Start pH	pH after 48h
T1	7.87	8.31
T2	7.87	8.31
T3	7.87	8.3
T4	7.87	8.3
T5	7.87	8.29
T6	7.87	8.24
T7	7.87	8.23

PS III		
Conc.	Start pH	pH after 48h
T1	8.06	8.28
T2	8.06	8.29
T3	8.06	8.28
T4	8.06	8.27
T5	8.06	8.28
T6	8.06	8.28
T7	8.06	8.25

PS-COOH I		
Conc.	Start pH	pH after 48h
T1	7.94	8.15
T2	7.94	8.13
T3	7.94	8.17
T4	7.94	8.18
T5	7.94	8.18
T6	7.94	8.1
T7	7.94	8.03

PS-COOH II		
Conc.	Start pH	pH after 48h
T1	7.94	8.13
T2	7.94	8.2
T3	7.94	8.17
T4	7.94	8.17
T5	7.94	8.17
T6	7.94	8.09
T7	7.94	8.06

PS-COOH III		
Conc.	Start pH	pH after 48h
T1	8.07	8.20
T2	8.07	8.24
T3	8.07	8.23
T4	8.07	8.23
T5	8.07	8.22
T6	8.07	8.20
T7	8.07	8.20

PS-NH_2 I		
Conc.	Start pH	pH after 48h
T1	8.02	8.25
T2	8.02	8.24
T3	8.02	8.25
T4	8.02	8.24
T5	8.02	8.24
T6	8.02	8.22
T7	8.02	8.23

PS-NH_2 II		
Conc.	Start pH	pH after 48h
T1	7.98	8.23
T2	7.98	8.24
T3	7.98	8.24
T4	7.98	8.23
T5	7.98	8.23
T6	7.98	8.24
T7	7.98	8.24

PS-NH_2 III		
Conc.	Start pH	pH after 48h
T1	8.28	8.19
T2	8.28	8.20
T3	8.28	8.23
T4	8.28	8.20
T5	8.28	8.22
T6	8.28	8.19
T7	8.28	8.23

Dissolved oxygen

The DO meter was broken for a large period, so DO was not measured for all of the repeated experiments.

PMMA-COOH

Conc.	Start DO	End DO
T1	6.98	7.22
T2	6.98	7.27
T3	6.98	7.21
T4	6.98	7.18
T5	6.98	7.17
T6	6.98	7.07
T7	6.98	NA

PMMA-COOH

Conc.	Start DO	End DO
T1	6.89	7.22
T2	6.89	7.25
T3	6.89	7.27
T4	6.89	7.29
T5	6.89	7.18
T6	6.89	7.16
T7	6.89	7.09

PMMA

Conc.	Start DO	End DO
T1	6.99	7.22
T2	6.99	7.27
T3	6.99	7.27
T4	6.99	7.26
T5	6.99	7.24
T6	6.99	7.25
T7	6.99	NA

PMMA

Conc.	Start DO	End DO
T1	6.86	7.09
T2	6.86	7.13
T3	6.86	7.08
T4	6.86	7.1
T5	6.86	7.03
T6	6.86	7.09
T7	6.86	7.05

PS

Conc.	Start DO	End DO
T1	7.12	7.19
T2	7.12	7.29
T3	7.12	7.27
T4	7.12	7.26
T5	7.12	7.26
T6	7.12	7.19
T7	7.12	

PS

Conc.	Start DO	End DO
T1	7.06	7.12
T2	7.06	7.21
T3	7.06	7.21
T4	7.06	7.29
T5	7.06	7.35
T6	7.06	7.33
T7	7.06	7.27

PS-COOH

Conc.	Start DO	End DO
T1	6.95	7.07
T2	6.95	7.01
T3	6.95	7.03
T4	6.95	7.13
T5	6.95	7.17
T6	6.95	7.26
T7	6.95	7.21

PS-NH_2

Conc.	Start DO	End DO
T1	7.08	7.22
T2	7.08	7.22
T3	7.08	7.23
T4	7.08	NA
T5	7.08	7.18
T6	7.08	7.21
T7	7.08	7.23

Salinity

	Start ‰	End ‰
PMMA	35	36

	Start ‰	End ‰
PMMA	35	36

	Start ‰	End ‰
PMMA	35.5	37

	Start ‰	End ‰
PMMA-COOH	35	36

	Start ‰	End ‰
PMMA-COOH	35	36

	Start ‰	End ‰
PMMA-COOH	35.5	37

	Start ‰	End ‰
PS	35	36

	Start ‰	End ‰
PS	35	36

	Start ‰	End ‰
PS	35.5	37

	Start ‰	End ‰
PS-COOH	35	35

	Start ‰	End ‰
PS-COOH	35	36

	Start ‰	End ‰
PS-COOH	35.5	37

	Start ‰	End ‰
$PS-NH_2$	35	36

	Start ‰	End ‰
$PS-NH_2$	35	36

	Start ‰	End ‰
$PS-NH_2$	35.5	39

Appendix B

Oxidative stress detection

1. Method development on the use of fluorescent probes

Because of differences in organism species and media, the method had to be further developed to achieve decent results, as explained in 2.2.4.1. From the results obtained in the method development, two time points (3 and 4 hours) were chosen to detect possible oxidative stress. The fluorescent values in the method development were calculated as fold induction compared to the control, where fluorescent values were divided on by average of the control. Figure B1 shows the entire method optimisation, while Figure 8 shows the plate setup for the probe tests run with the PS-NH_2 particles.

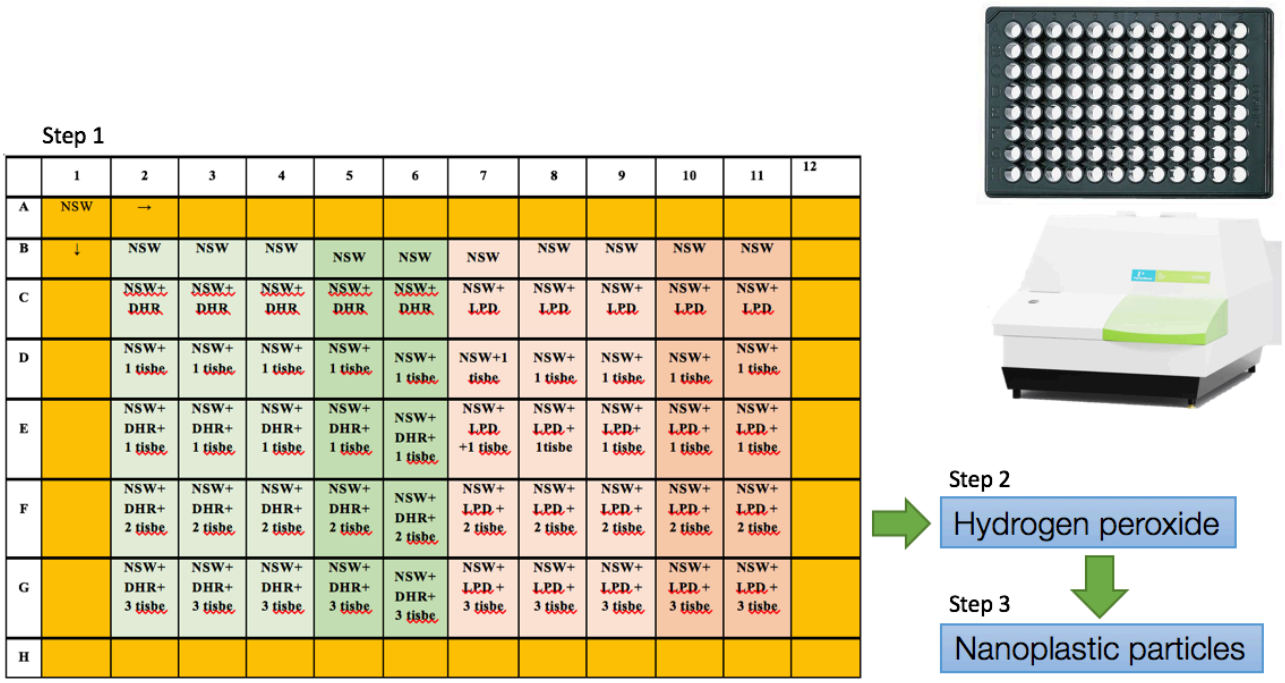

Figure B1: Schematics showing all the steps in the optimisation from 1) Plate setup for probe test without stressor to the same test run with stressors (steps 2 and 3). The plate setup differed somewhat for the three steps.

1.1.1. Probe test without stressor

The first test was initiated with the different probes without any stressor, to see if there was possible to detect a significant fluorescence signal in the copepods compared to control. For the probe DHR after performing a Mann-Whitney U test, it is shown that all the three test wells containing copepods differed significantly from the control group (Figure B2). This means that there is a significantly larger signal in fluorescence when copepods are present than without copepods. The signal is somewhat bigger for the wells containing three copepods than those containing only one or two copepods, but since the signal from the other wells are also significantly larger than the control, it was decided that it was enough to use one copepod per well in the following tests.

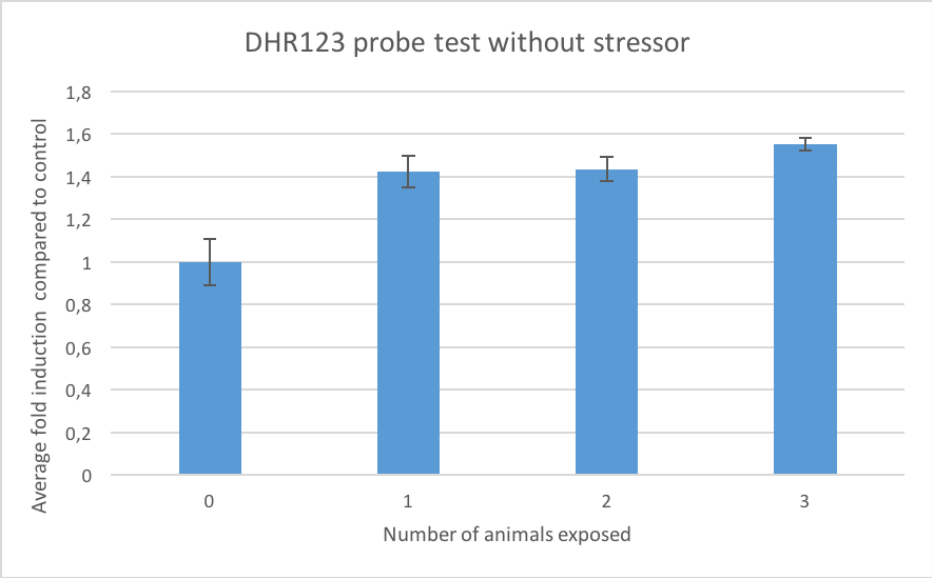

Figure B2: DHR probe with no stressor after 4 hours´ reading. X-axis indicates number of animals exposed to the probe, while the Y-axis shows the fluorescence in average fold induction compared to the control.

The LPO probe gave no significant results without a stressor, as all of the wells with copepods showed a lower fluorescence signal than the control wells (Figure B3). This could mean that there is no lipid peroxidation detected in the animals whatsoever, as this is a specific type of damage on the cell membranes, and there is no stressor added that would potentially cause this kind of oxidative stress (Pap et al., 1999). Another reason for why this specific LPO probe does not work, is that it may not be suitable for marine copepods, like for other species, as for example the microalgae species *Chlamydomonas reinhardtii* that responded well to this probe

(Cheloni & Slaveykova, 2013). This probe is more specific than the DHR probe, so one possibility is that there is no lipid peroxidation, but general ROS formation in the animals. Another possibility is that the animals are too small to give a significant effect. Since there is a chance that the probe is not working because there is no lipid peroxidation, the probe was further tested with H_2O_2, as showed in the next step of the optimising part.

The test without stressor was not performed for the H_2DCFDA probe as this probe was unavailable when the tests were conducted.

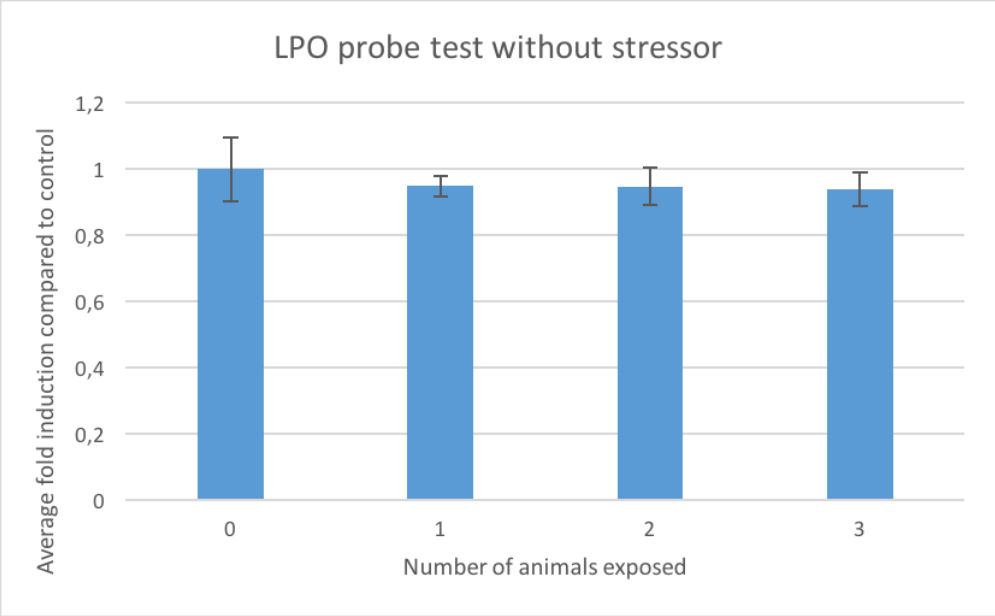

Figure B3: LPO probe with no stressor after 4 hours´ reading. X-axis indicates number of animals exposed to the probe, while the Y-axis shows the fluorescence in average fold induction compared to the control.

1.1.2. Probe test with H_2O_2 as stressor

All the probes were tested with hydrogen peroxide (H_2O_2) as a stressor, which has been used as a positive control for ROS formation in other species, as *Daphnia magna* (Gomes et al., 2018). H_2O_2 is an oxidant and is one of the most well-known reactive oxygen species. When animals are exposed to this stressor, oxyl-radical formation is triggered and there will potentially be more ROS in the cells of the animals that is measurable when performing an *in vivo* fluorescence test (Cheloni & Slaveykova, 2013). All concentrations were measured in µg/mL.

The objective of this test was to see if there was any ROS formation in response to this stressor comparatively with the control, even if it was not significant. The first time the probe test with the stressor H_2O_2 was conducted (repeat 1 in Figure B4 and B5), it gave promising results for the probe DHR123, even though with a high variance between replicas. All the fluorescence values for the concentrations tested were higher than the control, although the statistical significance of this increase it is not calculated. The second time the test was conducted, the fluorescence obtained was not as high as the first time (repeat 2).

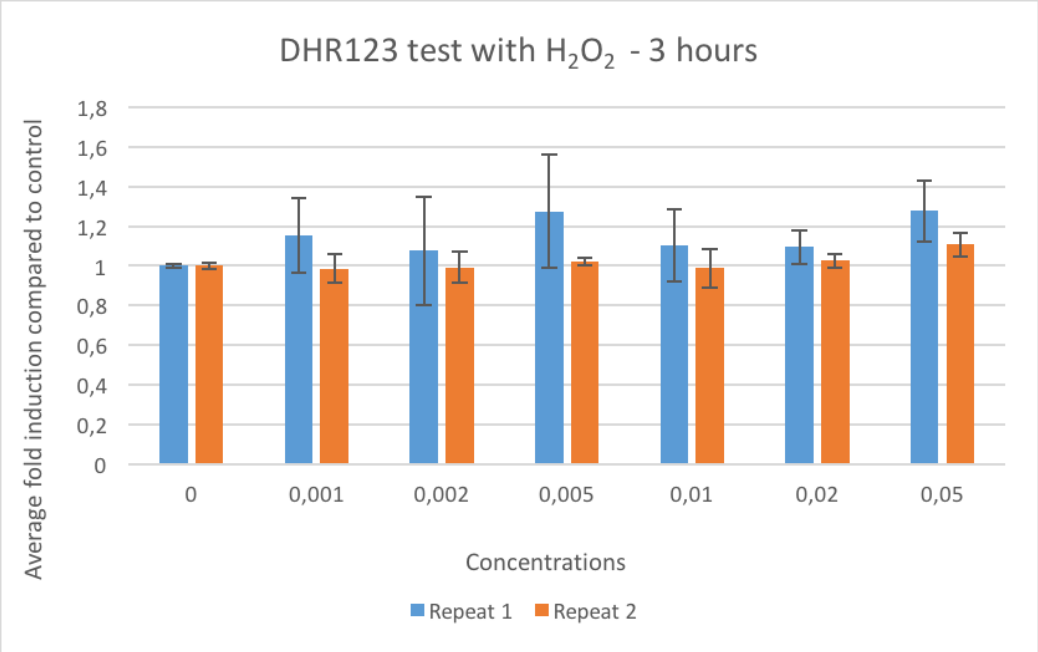

Figure B4: DHR123 probe with H_2O_2 as stressor after 3 hours´ reading. X-axis indicates the concentrations used for two repeated experiments, while the Y-axis shows the fluorescence in average fold induction compared to the control.

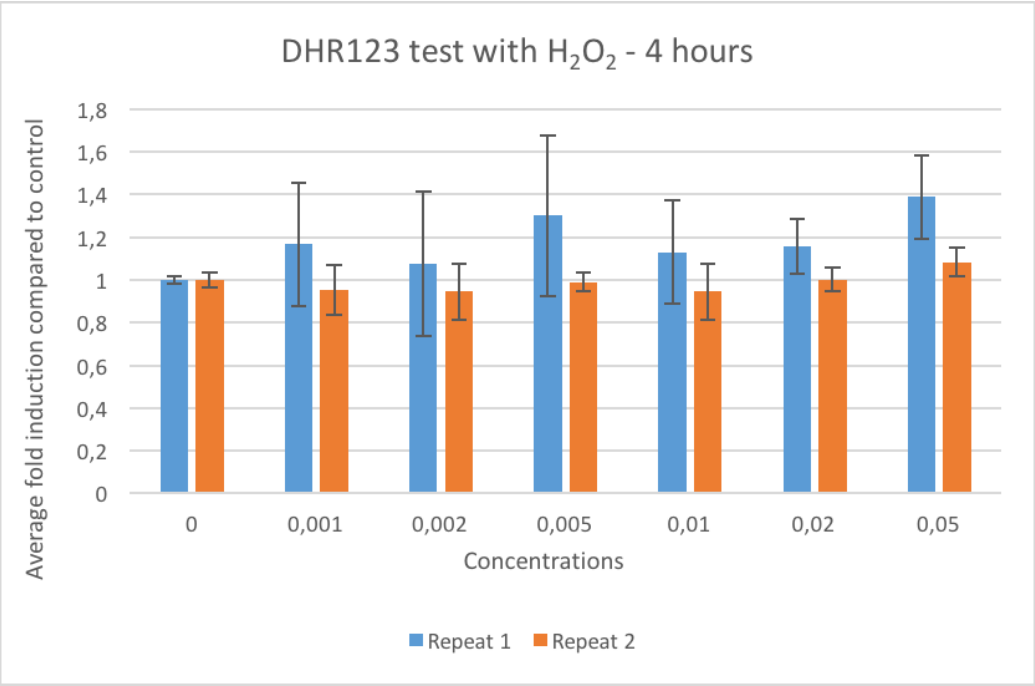

Figure B5: DHR123 probe with H_2O_2 as stressor after 4 hours' reading. X-axis indicates the concentrations used for two repeated experiments, while the Y-axis shows the fluorescence in average fold induction compared to the control.

For the probe C11-Bodipy, no fluorescent values differed clearly from the control, although some of the fluorescent values were slightly higher than the control in repeat 2 (figure B6 and B7).

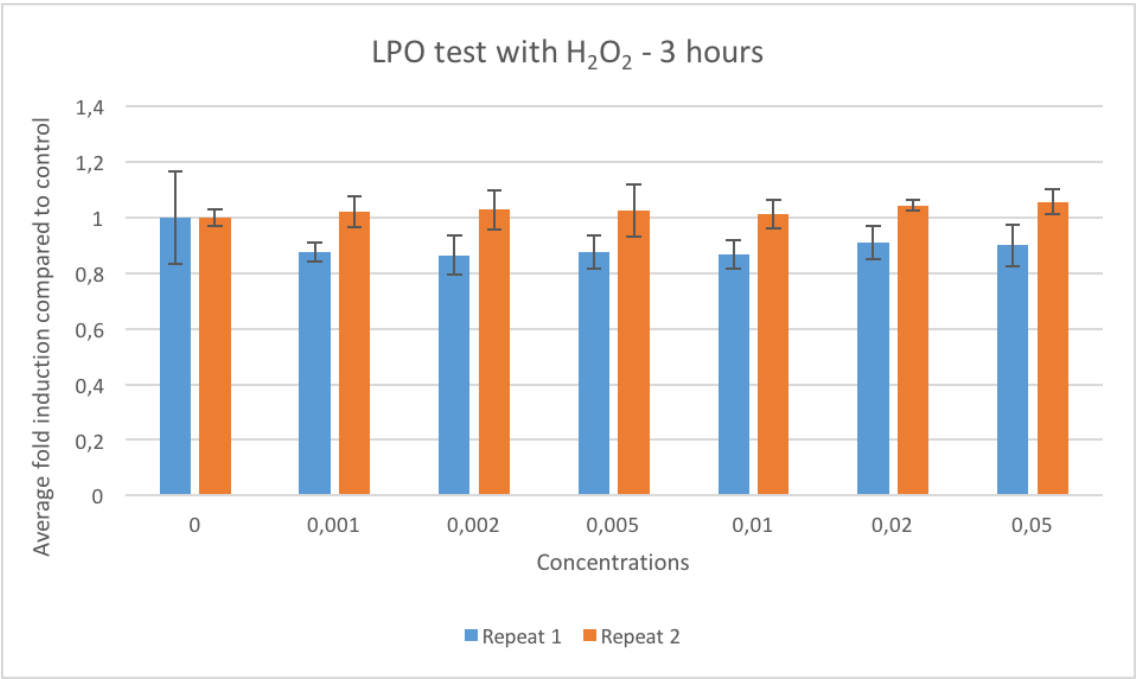

Figure B6: LPO probe with H_2O_2 as stressor after 3 hours' reading. X-axis indicates the concentrations used for two repeated experiments, while the Y-axis shows the fluorescence in average fold induction compared to the control.

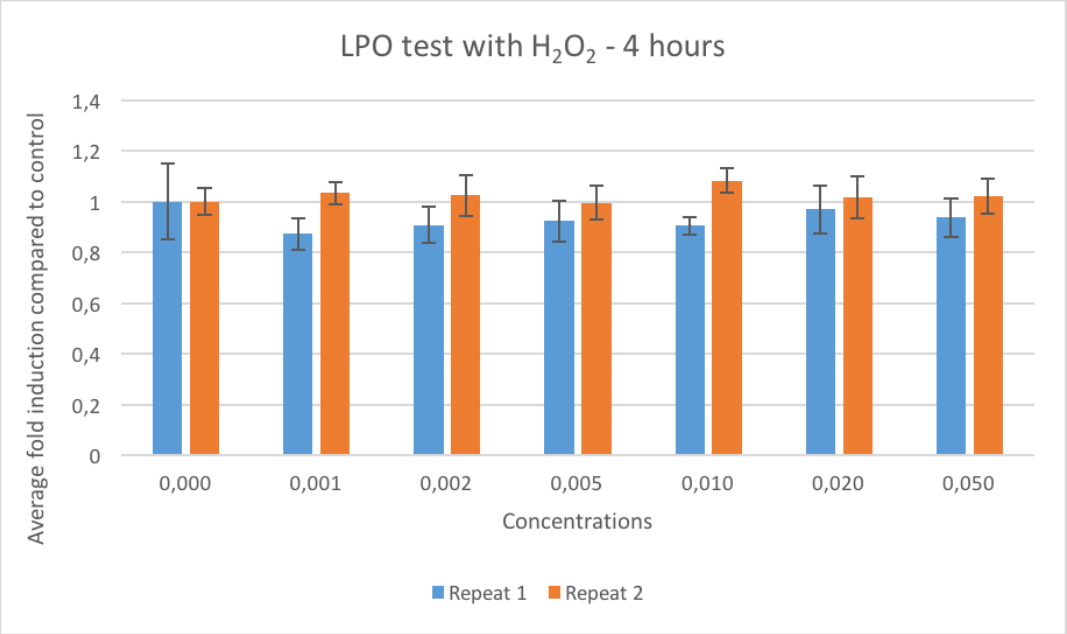

Figure B7: LPO probe with H2O2 as stressor after 4 hours´ reading. X-axis indicates the concentrations used for two repeated experiments, while the Y-axis shows the fluorescence in average fold induction compared to the control.

This test was conducted once for the newly received H_2DFCDA probe, with only the four highest H_2O_2 concentrations, to see if the H_2DFCDA probe will get a better signal than the two other probes. The H_2DCFDA probe did not give promising results either, as seen in Figure B8. What is clear here, is that the highest H_2O_2 concentration (0.05 µg/mL) got increasingly promising results the longer time after the initiation of the fluorescence reading, so it would be interesting to see how these results would have evolved after a longer exposure period.

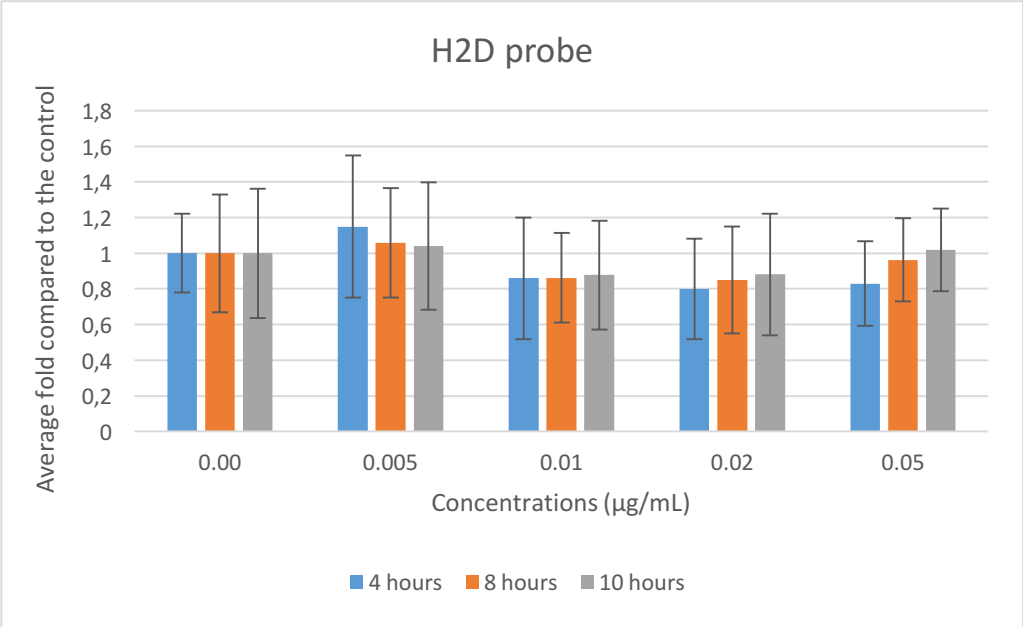

Figure B8: Three selected time points for the probe H_2DCFDA after exposure to H_2O_2. X-axis indicates the concentrations used for two repeated experiments, while the Y-axis shows the fluorescence in average fold induction compared to the control.

1.1.3. H_2O_2 shorter exposure

Additional tests were performed with the probes DHR123 and C11 Bodipy, in which the copepods were exposed to H_2O_2 for only six hours, to see if shorter exposure times could provide a clear response in terms of oxidative stress. A shorter exposure did not result in an increase in either ROS formation or lipid peroxidation, even though a smaller variation between replicates was detected.

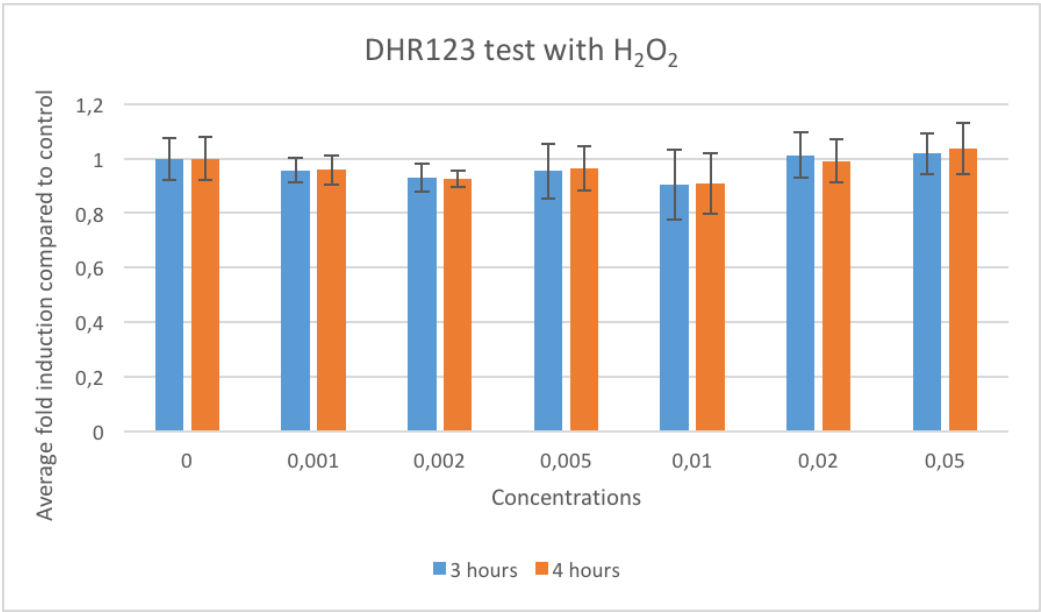

Figure B9: DHR123 probe with H_2O_2 exposure after 3 and 4 hours' reading. X-axis indicates the concentrations used for two repeated experiments, while the Y-axis shows the fluorescence in average fold induction compared to the control.

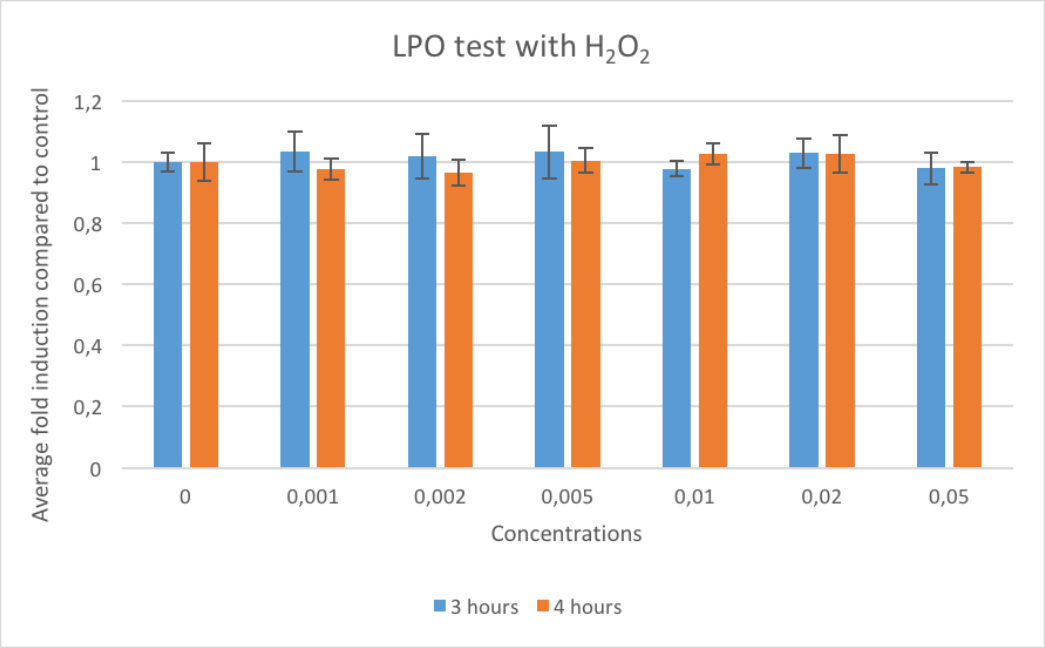

Figure B10: C11-BODIPY probe with H_2O_2 exposure after 3 and 4 hours´ reading. X-axis indicates the concentrations used for two repeated experiments, while the Y-axis shows the fluorescence in average fold induction compared to the control.

Since the probe test with H_2O_2 did not give promising results with only one copepod for all the probes tested, it was discussed if it would be better to use three copepods in each well when testing with nanoplastic particles, as this seemed to give a better signal when testing without any stressor (Figures B2 and B3). The method using three copepods was dismissed as it would have taken a lot more time, and additional water due to the pipetting of three animals into each well would have caused some uncertainty related to the dilution of the probes in the wells. In addition, this process would be hard to do with the available equipment without stressing the animals too much, and the results would not be fully trustworthy. However, with more biomass in each well it would be possible to detect more ROS production in the animals, so if doing this test again, more animals in each well would be a good way to start the optimisation. Additional pipetting techniques would need to be assessed, e.g. a syringe or pipette with a slim needle, to ensure that copepods do not get sucked back into the pipette when removing assess water before adding the exposure solution into the wells.

2. Nanoplastic exposure normalisation

After the copepods had been exposed to NPs with the different probes, the data had to be interpreted. In this process, the data was normalised considering the blank controls for each concentration and the lengths of the animals. The lengths were measured for each of the copepods by taking a picture of the animal and using a measuring tool in the programme (Cell^D Imaging software, Olympus). The length of the animals was not measured for the LPO probe. This is because the data was evaluated before the pictures were taken, and it was clear that the blanks for each concentration without copepods had higher fluorescence values than the control group and the wells for each concentration with copepods. In this section the raw average fluorescence recorded for the fluorescent probes exposures with PS-NH_2 is presented (Figures B11, B12 and B13), from where the 8 hours reading used in the results section was taken to show ROS formation and lipid peroxidation.

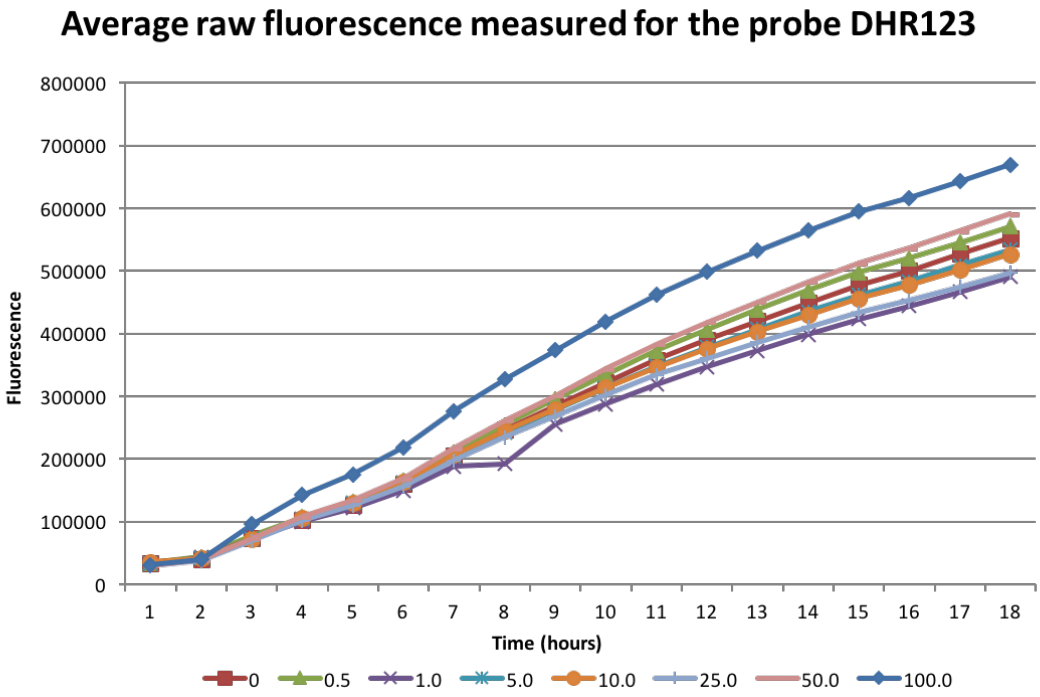

Figure B11: Average raw fluorescence measured for the probe DHR123 without normalisation. X-axis indicates time in hours, Y-axis indicates average raw fluorescence measured in a.u. The seven tested concentrations together with the control are displayed with its own colour which is described in the explanation under the graph.

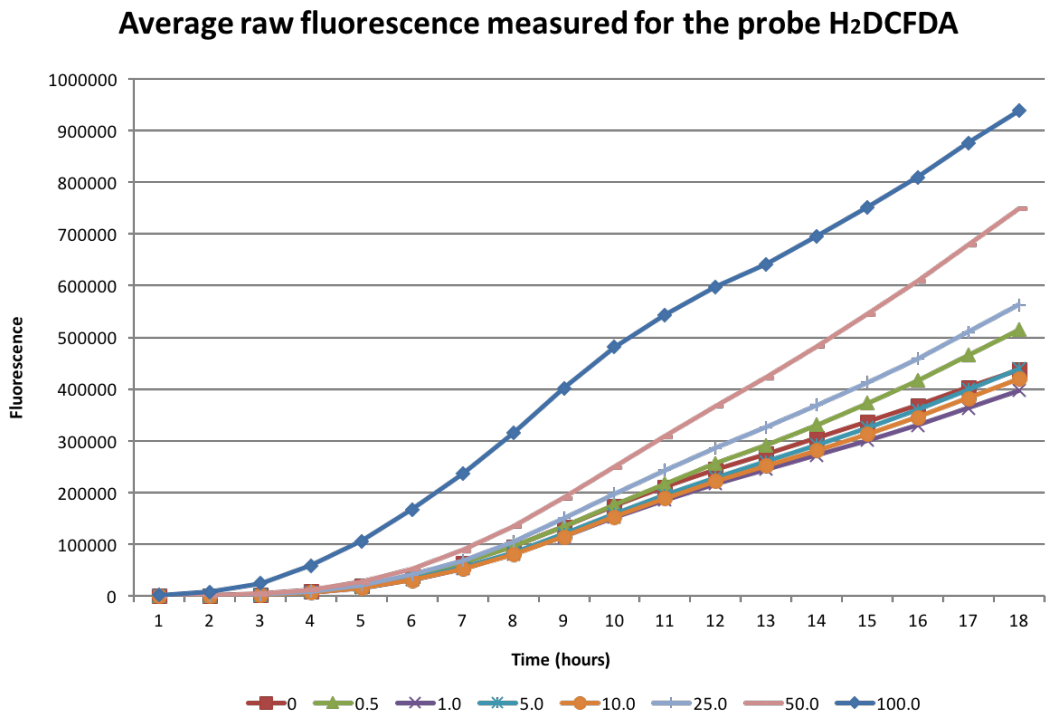

Figure B12: Average raw fluorescence measured for the probe H_2DCFDA without normalisation. X-axis indicates time in hours, Y-axis indicates average raw fluorescence measured in a.u. The seven tested concentrations together with the control are displayed with its own colour which is described in the explanation under the graph.

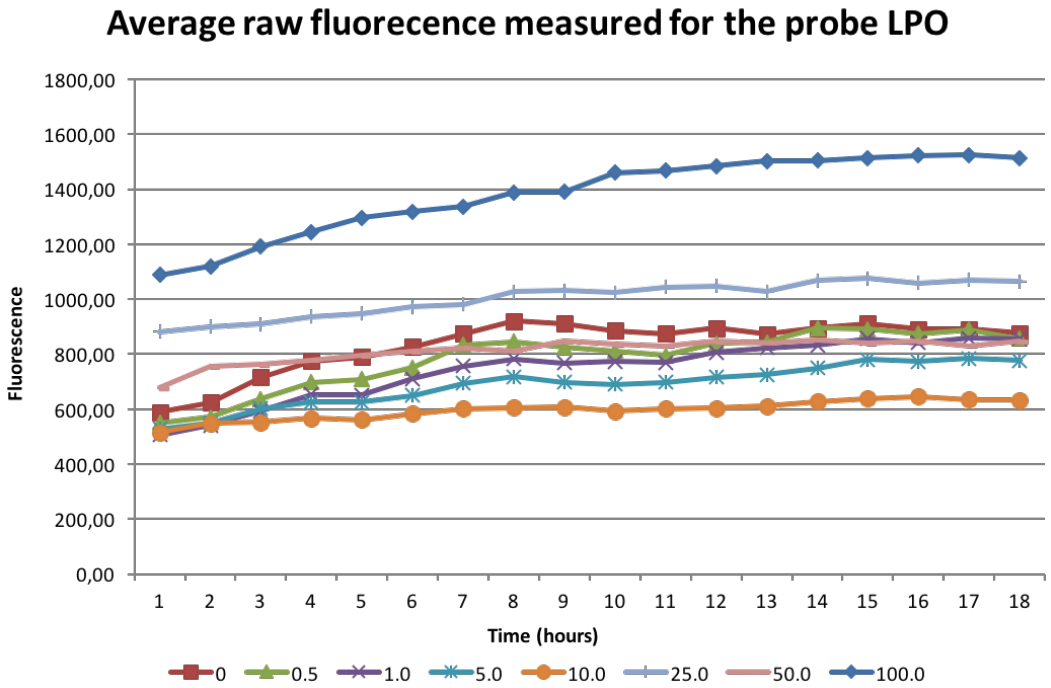

Figure B13: Average raw fluorescence measured for the probe C11-BODIPY without normalisation. X-axis indicates time in hours, Y-axis indicates average raw fluorescence measured in a.u. The seven tested concentrations together with the control are displayed with its own colour which is described in the explanation under the graph.

Normalisation with blank controls

In the first step of the normalisation of the data, the average of the blank controls for each concentration was subtracted from the raw fluorescence. The blank control was evaluated to see how much of the signal that can originate from the copepods and not the surrounding media. All the values that are negative indicates that the blank control showed more fluorescence than the wells containing copepods. To get enough replicas of the exposed copepods there were only two replicas for each concentration of the blank control, as showed in Figure 8 under 2.2.4 in the material and methods section. Such a small number of replicas does not give robust results, and more replicas should be used to get fully valid data.

For the probe DHR123 (Figure B11), none of the wells containing copepods show more fluorescence than the blank control for each concentration. The only concentration that showed a positive fluorescence difference was the concentration 0.5 µg/mL up to 5 hours reading.

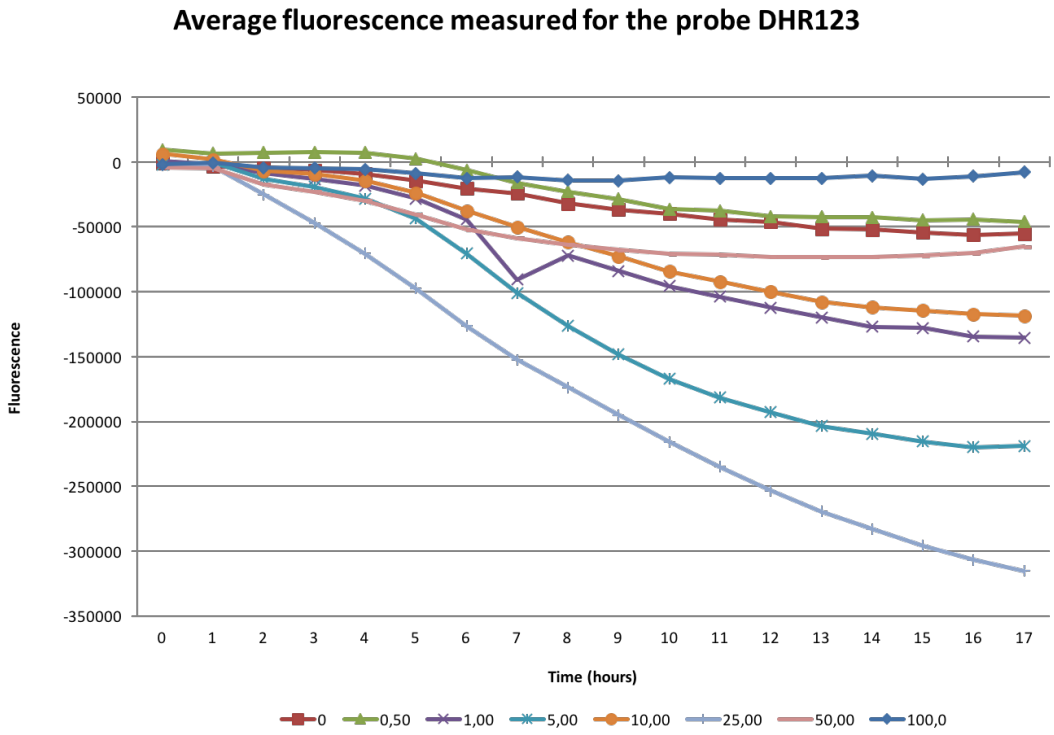

Figure B14: Average fluorescence measured for 18 hours when the blank control is subtracted for the probe DHR123. X-axis indicates time in hours, Y-axis indicates average raw fluorescence measured in a.u. The seven tested concentrations together with the control are displayed with its own colour which is described in the explanation under the graph.

For the probe H_2DCFDA it is clear that the highest concentration shows more fluorescence than the blank control, as displayed in Figure B15. The fluorescence measured is difficult to read

from this Figure for the lower concentrations, so a new Figure showing a shorter time of exposure will also be presented.

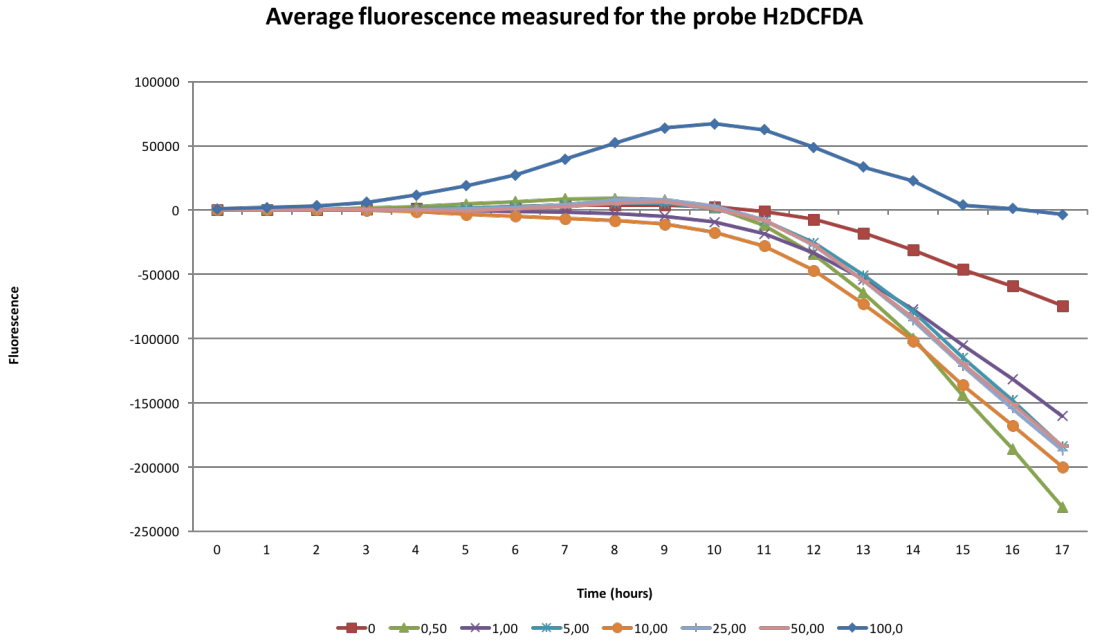

Figure B15: Average fluorescence measured for 18 hours when the blank control is subtracted for the probe H_2DCFDA. X-axis indicates time in hours, Y-axis indicates average raw fluorescence measured in a.u. The seven tested concentrations together with the control are displayed with its own colour which is described in the explanation under the graph.

Figure B16 shows that all the concentrations except 1 µg/mL and 10 µg/mL of NPs show a positive difference in fluorescence between 4 and 8 hours, which means that the average fluorescence from the wells containing animals was bigger than the fluorescence from the blank control. This fluorescence is especially high for the highest concentration (Figure 12 and 13).

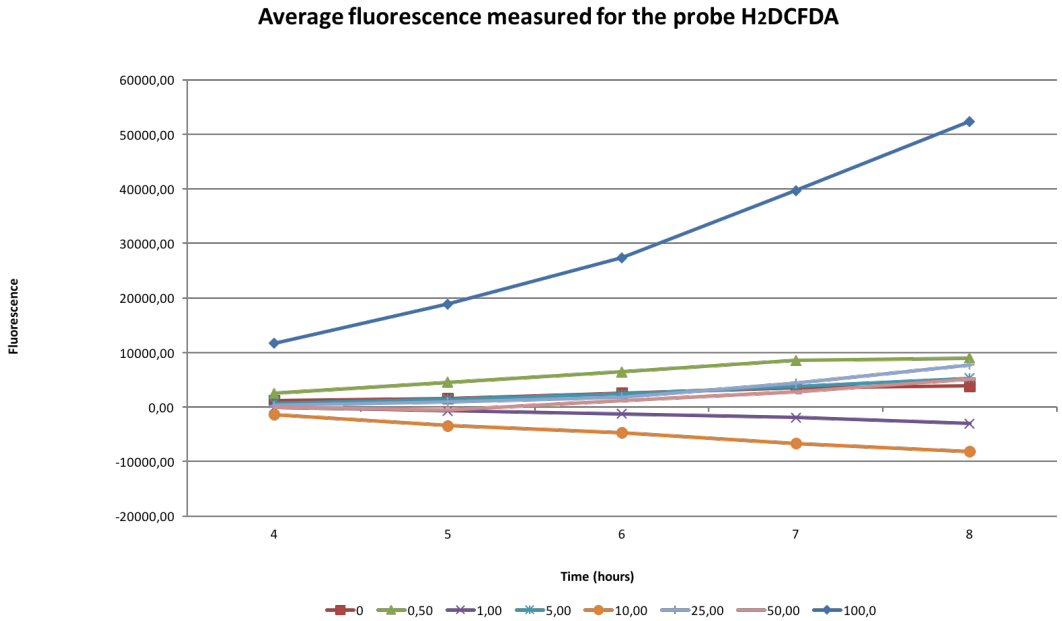

Figure B16: Average fluorescence measured between 4 and 8 hours of reading for the probe H_2DCFDA. X-axis indicates time in hours, Y-axis indicates average raw fluorescence measured in a.u. The seven tested concentrations together with the control are displayed with its own colour which is described in the explanation under the graph.

The average fluorescence for C11-BODIPY where the blank controls are taken into consideration do not show a similar pattern as for the two other probes. The concentration 25 µg/mL showed higher fluorescence, but these high fluorescence values here may occur because of the big standard deviation observed in Figure 17 in the results part of the thesis. The high standard deviation occurred because of two wells that showed a really elevated fluorescence compared to the other wells, up to a fourfold increase. This elevation could be the result of a human error, as it is possible that there were added double amount of probe in these wells (although this is not sure). Even though many of the concentrations showed positive fluorescence values after the normalisation, it was the control concentration that holds the highest fluorescence values and further normalisation with copepod lengths were considered as not needed.

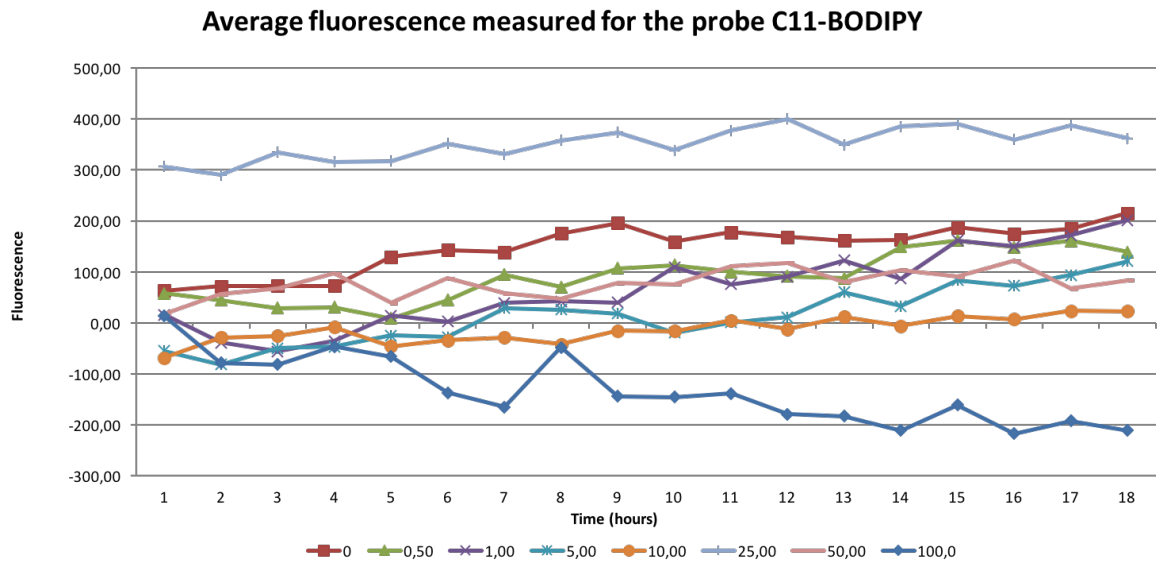

Figure B17: Average fluorescence measured for 18 hours when the blank control is subtracted for the probe C11-BODIPY. X-axis indicates time in hours, Y-axis indicates average raw fluorescence measured in a.u. The seven tested concentrations together with the control are displayed with its own colour which is described in the explanation under the graph.

Normalisation with blank controls and length of the animals

The copepods varied in size, and a size range of 300-650 µm was measured in the animals used in these experiments. Therefore, it is important to divide the fluorescence measured by the size of each copepod, as a larger copepod potentially may produce more ROS than a smaller copepod. Measuring of the copepods was done for the probes DHR123 and H_2DCFDA, but not for C11-BODIPY, as explained above.

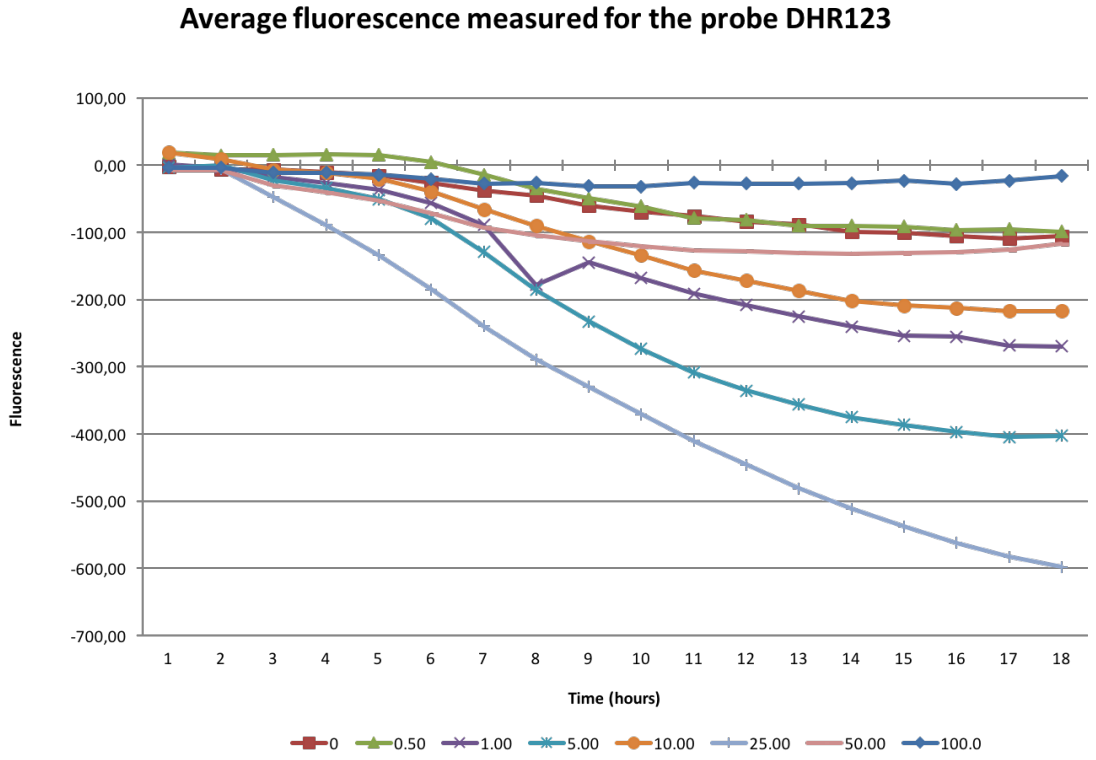

Figure B18: Average fluorescence measured for 18 hours when the blank control is subtracted and divided on the length of the copepods, for the probe DHR123. X-axis indicates time in hours, Y-axis indicates average raw fluorescence measured in a.u. The seven tested concentrations together with the control are displayed with its own colour which is described in the explanation under the graph.

Table B1: Lengths of all the copepods measured for the probe DHR123

	1	2	3	4	5	6	7	8	9	10
CONTROL	324.38	483.08	463.45	NA	579.98	455.78	459.62	428.26	566.91	623.31
T1	468.64	NA	551.19	577.89	653.34	NA	401.44	344.74	484.64	571.12
T2	425.48	487.75	525.93	444.62	453.46	NA	451.45	631.44	560.75	497.46
T3	390.64	512.77	570.49	632.68	553.97	516.24	572.18	662.44	452.75	549.9
T4	348.65	443.82	563.17	387.35	469.71	NA	471.78	NA	592.1	493.77
T5	516.25	494.75	655.31	501.37	629.85	308.33	592.11	526.13	551.24	844.1
T6	468.2	548.12	659.7	519.47	573.85	496.46	454.55	672.26	549.08	635.47
T7	NA	409.1	551.5	614.24	529.89	571.3	310.75	599.85	501.88	515.5

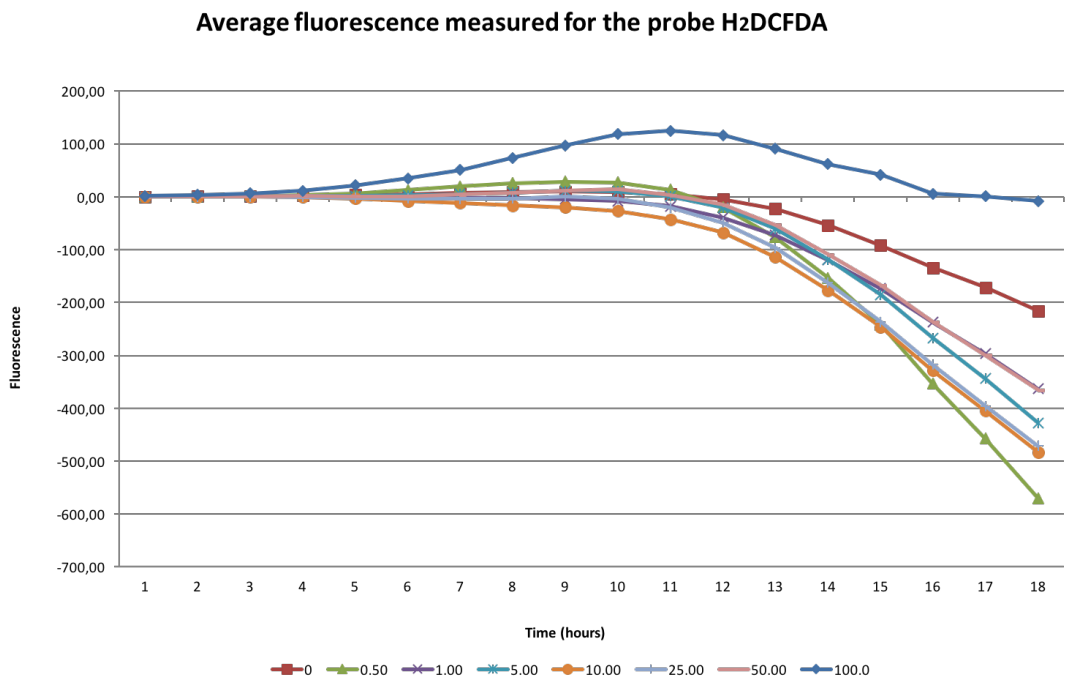

Figure B19: Average fluorescence measured for 18 hours when the blank control is subtracted and divided on the length of the copepods, for the probe H_2DCFDA. X-axis indicates time in hours, Y-axis indicates average raw fluorescence measured in a.u. The seven tested concentrations together with the control are displayed with its own colour which is described in the explanation under the graph.

Table B2: Lengths of all the copepods measured for the probe H_2DCFDA

	1	2	3	4	5	6	7	8	9	10
C	471.89	374.29	371.18	298.56	335.59	NA	314.83	NA	381.39	314.7
T1	NA	429.38	427.8	415.03	465.14	355.89	484.28	404.57	344.59	NA
T2	380.89	449.16	375.26	411.63	391.17	354.11	299.5	513.54	364.39	479.51
T3	440.53	435.19	399.88	388.71	490.52	453.15	378.76	536.5	414.88	423.67
T4	308.36	424.28	491.32	457.52	319.19	450.63	442.46	480.19	387.92	390.7
T5	NA	562.7	623.91	408.57	457.98	616.58	459.24	517.21	306.5	NA
T6	551.27	459.05	520.88	445.22	459.38	412.31	652.07	NA	480.63	563.61
T7	591.97	543.7	513.66	556.85	474.39	521.88	503.34	575.63	506.1	523.12

After comparing the results for each concentration to their corresponding blank controls, as well as size of the copepods, the only concentration that gave a decent result was the highest concentration (100 µg/mL). This concentration gave a positive signal up to 10 hours after exposure, when the signal started to weaken. This could indicate death of the animals at about 10 hours after exposure. The results from the normalisation of the test to prove oxidative stress using fluorescent probes showed that these methods need to be developed further to give solid

data. Only the positive fluorescent signal from the probe H_2DCFDA (Figure 15) seems to indicate that the method was working.

Pictures were taken for all concentrations to look at the fluorescence in the copepods (Figures 15, 18 and B20). Here it is clear that the fluorescence seen is concentrated in the organisms, so there is not a lot of the fluorescence measured that can originate from the media surrounding the copepods.

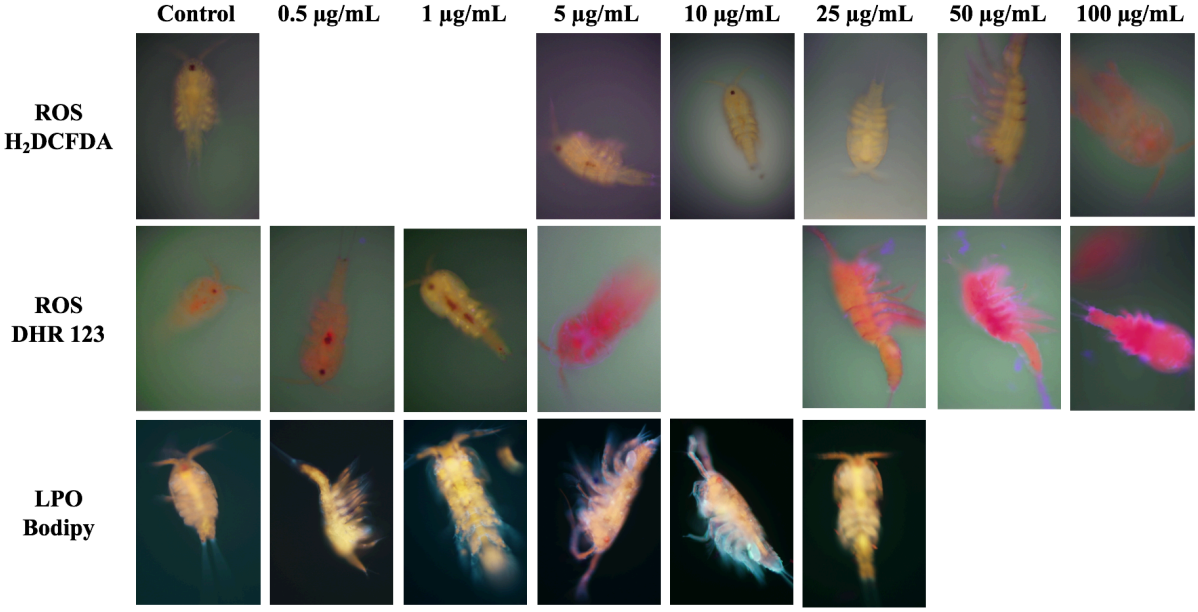

Figure 20: Fluorescent pictures taken from the different concentrations for the three probes with the fluorescent microscope Olympus DP72.

Overall, all the findings obtained in this study for the test using fluorescent probes lead to several potential hypotheses regarding why the methods need further development. One hypothesis is that the differences in ROS production between the control and the lowest concentrations are so small it is difficult to detect it the animals exposed to NP particles. That could be the reason for why there seems to be no concentration-response pattern in the lower concentrations for all of the probes tested. Another hypothesis is that the animals are so small that the ROS detected in all of the concentrations does not come from only within the copepods, but also the media solution in the wells. There may be a possibility that the NSW used to make all the concentrations are reacting with something in the NPs and from the ROS detected, so the fluorescent signal that is detected is not coming from the copepods themselves. However, when looking at the fluorescent pictures of the copepods in Figures 15, 18 and B20, all the fluorescence seem to come from the animals and there is no or little detected fluorescence on the outside of the animals. This would indicate that the measured raw fluorescence should originate from the copepods. A third assumption is that because the part of the pipetting takes

a bit of time, this procedure could stress the animals enough to create ROS inside the animals. The control group of copepods were pipetted first for all three probes as by looking at the microplate setup in Figure 8 was a logic way to set up the experiment, but it is uncertain if the longer time being in the wells could stress the animals more than when being kept in a larger beaker. If this assumption had been the case, it could have explained the constant decrease in fluorescence detected for the probe C11-BODIPY up to 10 μg/mL as the animals have a shorter time in the microplate wells, before the concentrations of NPs are high enough to affect the copepods at 25 μg/mL (possible analysis of the curve in Figure 17).

Further optimisation steps should include: a) addition of more copepods than just one in each well, to see if the signal gets bigger with more biomass; b) probe concentration can also be adjusted, to see if more and less probe in each well alters their fluorescent signal ; c) test replication to get a more robust data set; d) optimisation of better techniques to add animals to the wells and remove excess NSW added; e) test the use of artificial seawater (ASW) instead of NSW, as NSW can contain complexes that may affect the NPs or the probes. In addition, the method used by Jeong et al. (2018) discussed in 4.3 using H_2DCFDA gave better results than the *in vivo* method used in this thesis, and may therefore be a more suitable approach for smaller animals as rotifers and copepods. So, this approach should be run with copepods *T. battagliai* to see if it can be applicable to other organism and give similar results. The only disadvantage on using this approach, is that it requires several replications in terms of copepods in comparison with that used in the *in vivo* method. Finally, if this method ought to be used for the PS-NH_2 used in this study, the concentrations would need to be altered to a lower concentration range, as many organisms were dead after 24 hours at the highest concentrations used in this thesis.

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