Effects of Microplastics from Feed in Atlantic Cod (*Gadus morhua*)

Syver Hauge

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Department of Biosciences
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Abstract

Microscopic plastic particles, microplastics (MPs), have been found in all the world's oceans, and is ingested by marine organisms. Even though microplastic pollution is of increasing scientific interest, little is known about the effects of ingested MPs. In this thesis, the aim was to analyze the sub-lethal effects of ingested MPs in the Atlantic cod (*Gadus morhua*). The fish was exposed to five different plastic treatments, each with different sizes: low density polyethylene (LDPE) of 125-500 μm, 20-25 μm, and 4-6μm, polystyrene (PS) of 0.2μm, and polyvinyl chloride (PVC). The fish went through a 21 days exposure where they were fed fish skretting pellets coated in coconut oil containing MPs. Following the plastic feed treatment, several assays were performed to examine effects in enzyme activity of glutathione reductase (GR), glutathione s-transferase (GST), and glutathione peroxidase (GPx), as well as concentration of total cellular glutathione (GSH). The analyses showed that there were no effect from any of the microplastic treatments on any of the biomarkers. These results may indicate that MPs have no effect on the examined biomarkers. However, more research is needed to get a better understanding of the risks related to microplastic pollution.

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Abbreviations

ANOVA	Analysis of variance.
CAT	Catalase.
EFSA	European Food Safety Authority.
GPx	Glutathione peroxidase.
GR	Glutathione reductase.
GSH	Glutathione.
GSSG	Oxidized glutathione.
GST	Glutathione s-transferase.
LDPE	Low density polyethylene.
MPs	Microplastics.
NIVA	Norsk institutt for vannforskning.
NP	Nanoparticle.
PAH	Polycyclic aromatic hydrocarbon.
PCB	Polychlorinated biphenyl.
POP	Persistent organic pollutant.
PS	Polystyrene.
PVC	Polyvinyl chloride.
ROS	Reactive oxygen species.
SOD	Superoxide dismutase.

Introduction

1.1 Plastics

In the modern world, there is plastic everywhere. Due to their many beneficial properties e.g. long durability, low cost and easy to produce, plastics are used in a wide variation: packaging, construction, automotive, and electronics are the most plastic demanding sectors (Plastics Europe, 2018). With increasing population and an economic system which benefits consumption, there will also be an increasing production of plastics. Plastics Europe (2018) has presented figures that are showing an increase in the world's plastic production from 335 million tonnes in 2016 to 348 million tonnes in 2017, and the numbers are expected to grow (Jambeck et al., 2015).

With the high consumption of plastic products, some of the plastics find their way into nature. It is estimated that 4.8-12.7 metric tonnes of plastic annually find its way to the marine environment from land-based sources (Haward, 2018). In the oceans, macroplastics, plastic items having sizes $>$20 mm (Barnes et al., 2009), have the potential to inflict damage to marine organisms after being ingested (GESAMP, 2016; Laist, 1997) or by entanglement (GESAMP, 2016; Laist, 1987, 1997).

Macroplastic pollution is a visible problem, and there has therefore been a lot of studies regarding this issue. However, in recent years scientists have discovered that smaller plastic fragments, microplatics (MPs), plastic items $<$5 (Andrady et al., 1995), are present in the oceans. There are a handful of sources of MPs, and they are divided into two categories: primary MPs, and secondary MPs. Primary MPs are designed and produced as MPs such as microbeads used in facial scrubs, body wash products, and toothpastes (Fendall and Sewell, 2009; Gregory, 1996). Secondary MPs are MPs that originates from macroplastics which have broken down and undergone fragmentation. This fragmentation process is caused by a variety of factors where the most important are UV-radiation and physical stress.

Studies have shown that MPs are found in the oceans worldwide, e.g. the Atlantic ocean (Lusher et al., 2014), and the Mediterranean (Collignon et al., 2012). Some studies even report that MPs are present in the arctic (Kanhai et al., 2018; Mu et al., 2019), and in Antarctica (Absher et al.,

2019; Cincinelli et al., 2017; Lusher et al., 2015; Morgana et al., 2018; Munari et al., 2017). They are also found in several different parts of the ocean such as surface-waters (Lusher et al., 2015), sub-surface waters (Desforges et al., 2014; Kanhai et al., 2018), deep sea and sediments (Van Cauwenberghe et al., 2013).

1.2 Ingestion and translocation

With MPs present in all marine ecosystems, contact with marine life cannot be avoided. A wide range of marine organisms from different phyla, habitats, and trophic levels are shown to ingest MPs. Among these are plankton, polychaetes, echinoderms, bivalves, and fish (Boerger et al., 2010; Possatto et al., 2011). MPs also have the ability of translocation (Browne et al., 2008; Lu et al., 2016). One of the most common ways of uptake of MPs in fish is via oral uptake. After ingestion the microplastic particles are subsequently transported through the stomach and released into the intestine. In the intestine there is a potential for uptake of the particles. There are different uptake routes and mechanics depending on the particle size, and there are three general routes of uptake. These are through microfold cells (m-cells) in the Peyer's patches, through normal epithelial cells (enterocytes), and lastly paracellular transport past gap junctions (Florence, 1997). The smallest particles (diameter <220 nm) are able to be taken up through paracellular transport (Aprahamian et al., 1987) or via endocytosis, both pinocytosis and phagocytosis (Pratten and Lloyd, 1986), in the enterocytes. The intermediate microparticles (<10μm) can be taken up via phagocytosis in the m-cells in the Peyer's patches (Eldridge et al., 1990; Jani et al., 1990; LeFevre et al., 1978). The rates of uptake in the Peyer's patches are dependent on particle size. Jani et al. (1990) found that smaller particles (diameter 50 nm) had a higher rate of uptake than larger particles (diameter 1100 nm). In addition to in vivo studies mainly on mice, there has also been done in vitro studies on cell cultures on cellular uptake of microparticles. Desai et al. (1997) studied a culture of the cell line Caco-2 and found that particle size, time, particle concentration, and temperature were factors affecting the uptake rates of particles: larger particles have lower uptake rate than smaller particles, and the uptake increases with increased incubation time, increased particle concentration, and increased temperature.

There is a smaller number of studies on uptake rates and mechanics of larger particles (>20μm). However, a European Food Safety Authority (EFSA) report concludes that the upper size limit for absorption of microparticles is 150μm (Alexander et al., 2016). On the other hand, more recent studies show that MPs with size up to 0.6 mm are taken up which implies that the size

limit for particulate uptake is much larger than first assumed.

Following microparticle uptake, the particles have the ability to remain in the cells responsible for uptake for several weeks (LeFevre et al., 1978). They may however, be translocated via blood or lymph to other tissue or organs. Reineke et al. (2013) have shown that polystyrene MPs with sizes between 500 nm and 5 μm were absorbed in the intestine and translocated to organs such as heart, kidney and liver. Studies using larger polystyrene particles (5 μm and 20 μm) show that they accumulate in liver, kidney and gut in mice (Deng et al., 2017) and zebrafish (Lu et al., 2016).

It is not only the microplastic particles that may impose a hazard to marine organisms. When ingested, MPs may serve as vectors for chemical pollutants (Engler, 2012). Chemicals may be added to the plastic to alter its properties, and they can also sorb to the plastic particles from seawater. Some chemical pollutants include different persistent organic pollutants (POPs), polychlorinated biphenyls (PCBs), and polycyclic aromatic hydrocarbons (PAHs) (Hirai et al., 2011).

Even though there is limited research on the field, it has been shown that trophic transfer of MPs from one trophic level to the next is possible (Farrell and Nelson, 2013; Nelms et al., 2018; Setälä et al., 2014). It is therefore reasonable to believe that bioaccumulation is possible.

1.3 Oxidative stress induced by microparticles

Oxidative stress happens when the production of reactive oxygen species (ROS) in the cell is higher than the elimination, thus damaging the cell (Sies and Cadenas, 1985). Oxidative stress can occur when excessive ROS are produced and react with biological structures such as membranes, DNA or proteins (Valko et al., 2006). Effects of oxidative stress are numerous e.g. apoptosis, inflammation and formation of cancer (Manke et al., 2013). ROS are shown to be produced by metal ions (Stohs and Bagchi, 1995) and metal nanoparticles (NP) e.g. silver, ZnO and TiO_2 NPs (AshaRani et al., 2009; Park et al., 2008; Sharma et al., 2012). The metal ions and NPs react with molecular oxygen (O_2) and produces peroxides. Wether MPs behave in the same way is, however, less understood.

MPs are shown to induce oxidative stress in a variety of organisms such as lugworms (*Arenicola marina*) and the nematode *Caenorhabditis elegans* (Lei et al., 2018). Lu et al. (2016) Found

oxidative stress in zebrafish (*Danio rerio*) induced by 5 μm MPs analyzing superoxide dismutase (SOD) and catalase (CAT). Another study on *Mullus surmuletur* showed that MPs have no effect on SOD or CAT, but show elevated levels of GST (Alomar et al., 2017).

In addition to oxidative stress in liver, MPs are shown to induce damage in several other tissue. Examples of damages caused by MPsnare damaging on the villi and enterocytes in *D. rerio* leading to mortality (Lei et al., 2018), behavioural disorder in Crucian carp (*Carassius carassius*) caused by nanoplastics in brain tissue (Mattsson et al., 2017), and neurotoxic responses (Deng et al., 2017).

1.4 Biomarkers

In this study, the focus is to look for effects on antioxidant concentrations and activity in Atlantic cod (*Gadus morhua*) caused by exposure to MPs. The biomarkers chosen were total cellular glutathione (GSH) concentration, glutathione reductase (GR) activity, glutathione s-transferase (GST) activity, and glutathione peroxidase (GPx) activity.

ROS are constantly produced in the cells due to electron transfer processes in the mitochondria (Halliwell, 1998). However, ROS levels can increase when exposed to toxicants. When there is an imbalance between antioxidants and ROS , which may potentially cause cellular damage, it is referred to as oxidative stress (Sies and Cadenas, 1985).

In fish liver, there is a large number of antioxidants to prevent damage from oxidative stress (Tkachenko et al., 2014). Among the many antioxidants, we find GSH, GR, GST, and GPx (Storey, 1996). The concentrations of GSH, and activity of GSH-linked enzymes are suggested biomarker for oxidative stress (Almar et al., 1998; Storey, 1996).

GSH is a tripeptide that plays a major role in detoxification of xenobiotics and elimination of hydroperoxides (Akerboom and Sies, 1981). To keep ROS levels low, GSH is used as a cofactor in a reaction where H_2O_2 is reduced to H_2O. This process is catalyzed by the enzyme GPx. When GSH is used in this reaction, it is converted to GSSG. This is the oxidized form of GSH. In a healthy cell, it is assumed that the ratio of GSH/GSSG is 100:1 (Zitka et al., 2012). In a cell exposed to toxicants however, GSSG levels may rise, and the ratio may be shifted. This makes GSH/GSSG ratio suitable as a biomarker for toxic exposure to cells.

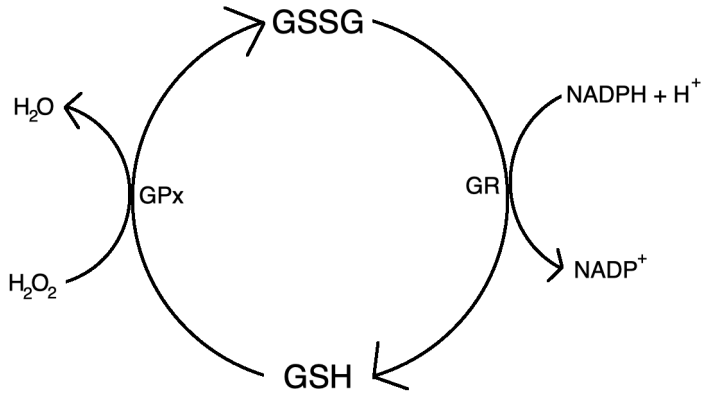

Figure 1.1. GSH-GSSG cycle. GSH is converted to GSSG in the reduction of H_2O_2 catalyzed by glutathione peroxidase (GPx). GSSG is reduced to GSH catalyzed by the enzyme glutathione reductase (GR). This reaction uses NADPH. Figure adapted from fig 1 in "Glutathione" by Meister and Anderson (1983), Annual Review of Biochemistry, 52, p. 714. Copyright 1983 by Annual Reviews Inc.

To prevent accumulation of GSSG in the cell, NADPH + H^+ is used in a reaction where GSSG is reduced to GSH. This reaction is catalyzed by GR. Together, these components (figure 1.1), GSH, GSSG, GPx and GR, make good biomarkers for the health status of the cell.

GST is another enzyme which is important for detoxification. It is a group of phase II enzymes which catalyzes the conjugation of GSH and reactive electrophiles to prevent high levels of ROS (Sies, 1997).

1.5 Model organism

In Norway, the Atlantic cod is an important economic and commercial species (Ageeva et al., 2017). At the same time, it is reported that the populations have decreased both along the Norwegian coast and in other regions (Myers et al., 1996). This has resulted in Atlantic cod is included in the OSPAR list of threatened and/or declining species (OSPAR, 2008). Myers et al. (1996) is reporting that the biggest treat against Atlantic cod populations is overfishing. Atlantic cod is susceptible to environmental contaminants, which makes it a species well suited for toxicological studies (Berg et al., 2010).

1.6 Aim

The main aim of this thesis was originally to examine GI uptake and translocation of MPs of different sizes to other tissue in fish. However, problems arose which made analyses for uptake impossible. Additionally, the fish were supposed to be examined for DNA-damage. Unfortunately, there was a problem with the procedures, which lead to the exclusion of DNA-damage assay in this thesis. Therefore, another aim was presented. The new aim was to examine if there are sub-lethal effects caused by acute exposure to MPs of different sizes. To address this question, an exposure study was set up using Atlantic cod and six different treatments: Control, 125-500 μm LDPE, 20-25 μm LDPE, 4-6 μm LDPE, 0.2 μm PS, and PVC. A hypothesis along with an alternative hypothesis were proposed:

- H_0: There is no difference in GSH concentration and/or GR, GST and/or GPx activity between control group and plastic feed groups
- H_A:There is a difference in GSH concentration and/or GR, GST and/or GPx activity between control and at least one of the plastic feed groups.

Methods

2.1 Set-up and exposure

2.1.1 Set-up

The experiment was done at Norsk institutt for vannforskning (NIVA)'s experimental facility at Solbergstrand in the outer Oslofjord, Norway. 90 Atlantic cod were kept in 30 separate glass aquaria. Prior to the experiments, the fish were not fed for seven days.The aquaria were supplied with sea water from 60 m depth with temperature and salinity reported in table 2.1.

Treatment

The aquaria were randomly assigned one of six treatments (n=5 per treatment):

- Control (C): Skretting pellet covered in coconut oil only
- 125-500 LDPE: Skretting pellet covered in coconut oil containing 125-500μm LDPE particles
- 20-25 LDPE: Skretting pellet covered in coconut oil containing 20-25μm LDPE particles
- 4-6 LDPE: Skretting pellet covered in coconut oil containing 4-6μm LDPE particles
- 0.2 PS: Skretting pellet covered in coconut oil containing 0.2μm PS particles
- PVC: Skretting pellet covered in coconut oil containing PVC particles (unknown sizes).

The experiment lasted three weeks: The fish were transferred to their aquaria June 1st. The feeding began seven days later, on the 8th of June. 14 days later, on the 22nd of June the experiment ended.

Table 2.1

Temperature and salinity in aquaria.

Factor	Min	Max	Mean
Temperature	7.2°C	7.6°C	7.4°C
Salinity	33.3ppm	34.7ppm	34.3ppm

2.1.2 Treatment preparation: Skretting pellets with plastic particles

Control feed

10 mL of coconut oil was poured into a beaker containing 60 skretting pellets. The content was mixed well so that every skretting pellet was covered in coconut oil. One at a time the skretting pellets were transferred to a weighing dish placed on ice for the coconut oil to thicken.

Plastic feed

200 mg (50 droplets in the case of the 0.2μm PS particle treatment) plastic particles were dissolved into 10 mL of coconut oil in a beaker containing 60 skretting pellets. The content was mixed well so that every skretting pellet was covered in coconut oil. One at a time the skretting pellets were transferred to a weighing dish placed on ice for the coconut oil to thicken. This procedure was repeated for every plastic treatment one day before every feeding. The prepared pellets were kept in one falcon tube for every treatment, which were stored at 4°C.

2.1.3 Exposure

Each aquarium received 12 pellets per feeding. The fish were fed three times: seven days after they were transferred to the aquaria, 8th of June, four days later, 12th of June, and three days later, 15th of June. The experiment ended seven days after the last feeding.

2.2 Sampling

At the end of the experiment, all the fish from one aquarium at a time, chosen randomly, were euthanized by a blow to the head with a wooden stick and subsequently weighed. Because all three fish from the same aquarium were pseudo-replicates only one fish from each aquarium was sampled. The intestines from all three fish from the same aquarium were evaluated, and the fish with highest content of skretting pellets were chosen for biological samples.

Table 2.2

Weight (g) before and after exposure, and length (cm) of fish

Fish no	Treatment	WBE	WAE	Difference	Length
1	PVC	203	184	-19	29
2	0,2 PS	285	256	-29	32
3	0,2 PS	273	256	-17	32
4	20-25 LDPE	270	244	-26	31
5	4-6 LDPE	215	190	-25	30
6	PVC	215	208	-7	30
7	20-25 LDPE	251	242	-9	30
8	Control	240	190	-50	29
9	Control	317	286	-31	31
10	4-6 LDPE	292	274	-18	31
11	PVC	197	172	-25	29
12	0,2 PS	273	240	-33	31
13	0,2 PS	224	192	-32	29
14	125-500 LDPE	265	238	-27	32
15	Control	222	214	-8	31
16	4-6 LDPE	280	238	-42	32
17	0,2 PS	236	208	-28	28
18	20-25 LDPE	206	190	-16	30
19	125-500 LDPE	265	246	-19	31
20	125-500 LDPE	334	308	-26	33
21	PVC	245	212	-33	30
22	20-25 LDPE	291	266	-25	31
23	20-25 LDPE	263	236	-27	31
24	4-6 LDPE	398	306	-92	34
25	PVC	252	228	-24	31
26	125-500 LDPE	266	248	-18	29
27	4-6 LDPE	251	234	-17	32
28	Control	136	126	-10	26
29	Control	200	194	-6	30
30	125-500 LDPE	270	256	-14	33

Note. WBE = weight before exposure, WAE = weight after exposure

Following fish termination, a 1 mL blood sample was taken using a 1 mL syringe. 100 μL was put into a 1.5 mL eppendorf tube containing 400μL PBS which was kept on ice for comet assay and handled for assay procedure consecutively (see section 2.3.1). The rest of the blood was transferred to a 1.5 mL eppendorf tube which was kept on ice. The eppendorf tubes not intended for comet assay were centrifuged eight at a time using a mini centrifuge resulting in

the red blood cells separating from the plasma. The plasma was transferred to glass containers using disposable transfer pipettes. The plasma samples were stored in a freezer at -20°C. The

stomach of the fish was cut open from head to anus with a scalpel. The liver was removed from the fish using a scalpel. A small piece equivalent to 1-1.5 mL was cut off and put into a cryotube. The cryotube was put in liquid nitrogen, and later stored at the University of Oslo in a freezer at -80°C for protein analyses.

The rest of the liver was put into a pre-labelled glass container which was kept on ice, and later stored in a freezer at -20°C.

Using a knife, the head of the fish was cut horizontally in the frontal bone so that the brain was exposed. The spinal cord and the olfactory bulb were cut close to the brain with a pair of scissors so that the brain was loose. The brain was removed and put into a glass container. The brain samples were stored in a freezer at -20°C.

A 1 cm$\times$1 cm piece of muscle tissue was cut out from above the pectoral fin using a scalpel. The sample was put in a glass container which was stored in a freezer at -20 °C.

The scalpel was cleaned in methanol after every tissue extraction and before a new fish was processed.

Due to unexpected problems, the samples could not be analyzed for MPs content. Therefore only the liver was used in analyses for biomarkers of oxidative stress, and blood was used for DNA-damage analyzes.

2.3 Laboratory

The first step of comet assay laboratory work was done at Solbergstrand. The rest of the laboratory work was performed at the University of Oslo

2.3.1 Comet assay

Solutions

- Phosphate buffered saline (PBS)
  - Stock solution: dH_2O, NaCl, Na_2HPO_4, KH_2PO_4

  - Working solution: PBS stock solution, dH_2O, $NA_2EDTA{\cdot}2H_2O$, NaOH

- Lysis buffer
  - Stock solution: dH2O, NaCl (58.44 g/mol), NaOH (40.0 g/mol), $NA_2EDTA{\cdot}2H_2O$ (372.2 g/mol), Trizma-base (121.2 g/mol)
  - Working solution: Lysis stock solution, DMSO, Triton X-100
- Electrophoresis buffer
  - Stock solution: dH_2O, NaOH (40.0 g/mol), NA_2EDTA (372.24 g/mol)
  - Working solution: Stock solution, dH_2O, 37% HCl
- Neutralization buffer
  - dH_2O, Trizma-base (121.14 g/mol), Trizma-HCl (157.56 g/mol)
- Tris-EDTA buffer
  - 0.5 M Tris buffer (pH 8), 0.5 M NA_2EDTA (pH 8), dH_2O
- Staining solution
  - Trizma EDTA buffer, SYBR gold
- 96% ethanol

Procedure

The eppendorf tubes containing 100 μL blood and 400 μL PBS were further diluted 1000$\times$ in PBS. 10 μL was transferred to an eppendorf tube containing 90 μL agarose. 25 μL of sample mixed with agarose was pipetted onto pre-cooled Gelbond films. When finished, there were two Gelbond films each containing 15 samples. The Gelbond films were placed in lysis buffer and stored in the ark at 4°C overnight at the University of Oslo.

The next day the films were rinsed in ice-cold electrophoresis buffer for 5 minutes. The Gelbond films were immediately transferred to the electrophoresis chamber containing 1.4 L new ice-cold electrophoresis buffer. Electrophoresis was then run for 20 minutes. When finished, the Gelbonds films were rinsed in neutralizing buffer in separate containers for five minutes in room temperature. After five minutes in neutralizing buffer, the Gelbond films were moved to new containers with fresh neutralizing buffer and rinsed for another ten minutes. Following

rinsing in neutralizing buffer, the Gelbond films were transferred to new containers containing dH_2O and rinsed briefly before transferring them to new containers with 96% ethanol. The Gelbond films were then transferred to another set of containers with fresh 96% ethanol where they were fixed for 24 hours.

Unfortunately something happened during the procedure which led to the samples vanishing from the Gelbond films. Therefore it was impossible to score the samples and there is therefore no results from the comet assay

2.3.2 Tissue preparations for protein, glutathione reductase, glutathione s-transferase, and glutathione peroxidase assays

Solutions

- Sodium phosphate buffer: 0.1 M phosphate buffer (pH 7.8) containing 0.15 M KCL
- Homogenizing buffer: sodium phosphate buffer with DTT (1 mM) and glycerol (5% v/v)

Procedure

An approximately 0.25 mg piece of liver sample was put into a 1.5 mL precellys tube containing 15 ceramic precellys beads.

Homogenizing buffer was added to a total volume of 1.5 mL.

The sample was homogenized using a Precellys 24 homogenizer programmed to 6000 rpm, 3×10 sec. The samples were kept cool, at 3-4°C using a Cryolys and liquid nitrogen.

The tubes were transferred quickly to centrifugation at $10{,}000\times$g for 30 minutes at 4°C.

Avoiding the pellet at the bottom and the fatty layer at the top of the tube, the supernatant was transferred to a new eppendorf tube.

The samples were centrifuged at $100{,}000\times$g at 4°C for 60 minutes.

The supernatant was transferred to four different pre-cooled cryotubes: 50 μL were transferred to a cryotube for protein assay. The rest of the supernatant was evenly distributed into three different cryotubes for GR, GST, and GPx assays.

All the cryotubes were put into liquid nitrogen, and later stored in a freezer at -80°C for further analyses.

2.3.3 Protein

The procedure is based on a modified Lowry assay (Lowry et al., 1951). Results were used to standardize the results from GR, GST, and GPx assays.

Solutions

- Tris buffer pH 8
- Reagent A
  - Alkaline copper tartrate solution
- Reagent B
  - Diluted Folin-Ciocalteu reagent
- Bovine γ-globulin protein standard
- Standard solutions:
  - A dilution series was prepared by adding 39.7 μL tris buffer and 0.3μL 200mg/mL Bovine γ-globulin protein standard to an eppendorf tube. The eppendorf tube was vortexed.
  - 20μL tris buffer was added to three other eppendorf tubes
  - 20μL tris buffer + Bovine γ-globulin protein standard mix was transferred from the first eppendorf tube to the second eppendorf tube. The eppendorf tube was vortexed.
  - 20μL tris buffer + Bovine γ-globulin protein standard mix was transferred from the second eppendorf tube to the third eppendorf tube. The eppendorf tube was vortexed.
  - 20μL tris buffer + Bovine γ-globulin protein standard mix was transferred from the third eppendorf tube to the fourth eppendorf tube. The eppendorf tube was vortexed.
  - The concentrations in eppendorf tubes were now 1.5 mg/mL, 0.75 mg/mL, 0.375 mg/mL and 0.1875 mg/mL respectively.

Procedure

The 96-well microplate was loaded follows: 10μL tris-buffer was pipetted into three wells as blanks, 10μL standards of concentrations 1.5, 0.75, 0.375 and 0.1875. were pipetted into wells

marked 1.5, 0.75, 0.375 and 0.1875. The samples were added as three replicates into wells. 25μL Reagent A was added to each well using a multichannel pipette. 200 μL Reagent B was added to each well using a multichannel pipette. The plate incubated 15 minutes and then the absorbance was read at 750 nm once every minute for 15 minutes using BioTek's Synergy Mx Microplate Reader.

2.3.4 Glutathione

The procedure is based on the technical bulletin of Sigma-Aldrich® glutathione assay kit, catalog number CS0260.

Solutions

- 2vp
  - 8.6 μL 2-vinylpyridine in 31.4 μL ethanol
- 5% SSA
  - 2.5 g 5-Sulfosalicylic acid in 50 mL dH_2O
- Assay buffer
  - 100 mM potassium phosphate buffer mixed with 1 mM EDTA
- DTNB stock solution (1.5 mg/mL)
  - 8 mg DTNB in 5.33 mL DMSO
- Enzyme solution
  - 3.8 μL glutathione reductase in 250 μL assay buffer
- Glutathione standard solution
  - Glutathione standard stock solution and SSA, diluted to 50 μM
- Glutathione (GSH) standard stock solution (10 mM)
  - Glutathione reduced in 0.1 mL distilled water
- NADPH solution

  - 10 μL NADPH stock solution in 2.5 mL assay buffer

- NADPH stock solution (40 mg/mL)
  - 25 mg NADPH in 0.625 mL dH_2O

- Working mixture
  - 8 mL assay buffer, 228 μL enzyme solution, and 228 μL DTNB stock solution

Procedure

A 0.1-0.13 g piece of liver tissue from each sample was cut with a scalpel, and then weighed precisely using disposable plastic weighing vessels and an analytical balance. The liver was put into a precellys tube containing 15 ceramic precellys beads. 10 volumes (1-1.3 mL) SSA was added to the tube. The samples were homogenized using a Precellys 24 homogenizer programmed to 6000 rpm, 3×10 sec. The samples were kept cool, at 3-4°C using a Cryolys and liquid nitrogen.

After homogenization, the tubes were transferred quickly to a centrifuge, and ran at 10,000×g for 10 minutes at 4°C. The supernatant from each sample, avoiding the pellet at the bottom and the fatty layer at the top of the tube, was transferred and divided to two new eppendorf tubes. The weight of the eppendorf tubes were measured using an analytical balance.

solution was added to one eppendorf tube for each sample under a fume hood. This was to be able to estimate oxidized glutathione, glutathione disulphide (GSSG). All eppendorf tubes incubated in room temperature for 60 minutes.

After incubation, a 96-well microplate was loaded as follows:

Three replicates of 10 μL SSA was pipetted as blanks.

Two replicates of 10 μL standard 1× was pipetted into wells. Two replicates of 10 μL standard 0.5× was pipetted into wells. Two replicates of 10 μL standard 0.25× was pipetted into wells. Two replicates of 10 μL standard 0.125× was pipetted into wells. Two replicates of 10 μL standard 0.0625× was pipetted into wells.

Three replicates of 10 μL of sample without 2vp was pipetted into wells. Three replicates of 10 μL of sample 1 containing 2VP was pipetted into wells.

Using a multichannel pipette, 150 μL working mixture was added to each well. The samples were mixed well by pipetting up and down. The plate incubated in room temperature for five

minutes before 50 μL NADPH solution was added to each well with a multichannel pipette. The samples were mixed well by pipetting up and down.

The 96-well microplate was read once every minute at 412 nm for five minutes with kinetic read using a BioTek's Synergy Mx plate reader.

The glutathione concentrations were calculated using equation 1

$$\text{nmoles GSH/mL sample} = \frac{\Delta A_{412}/min(sample) \times dil}{\Delta A_{412}/min(1nmole) \times vol} \quad (1)$$

where ΔA_{412}/min(sample) is the slope generated by the sample.

ΔA_{412}/min(1 nmole) is the slope generated by the standard curve for 1 nmole GSH.

dil is the dilution factor of sample

vol is volume of sample in mL

2.3.5 Glutathione Reductase

Glutathione reductase activity is quantified using the assay described in Mapson and Goddard (1951)

Solution preparations

- Phosphate buffer:
  - 0.1 M sodium phosphate, pH 7.6
- GSSG-solution:
  - 310 mg GSSG in 50 mL phosphate buffer.
- NAPH-solution:
  - 40 mg NADPH in 10 mL phosphate buffer.
- EDTA-solution:
  - 95 mg EDTA in 100 mL phosphate buffer.
- Assay buffer:
  - 1 mL EDTA solution in 9 mL phosphate buffer.

Procedure

The liver samples were prepared for GR assay earlier (see section 2.3.2)
A 96-well microplate was loaded using the following procedure:

160 μL assay buffer was added to all wells.

20 μL phosphate buffer was added to wells 1 A-H as blanks.

20 μL Eight replicates of sample was added to wells.

20 μL NADPH-solution was added to all wells.

25 μL GSSG-solution was added to half of the blank wells and half of wells containing sample.

25 μL Phosphate buffer was added to wells not containing GSSG.

The plate was read once every 40 seconds for 15 minutes at an absorbance of 340 nm with kinetic read using a BioTek's Synergy Mx plate reader.

The GR activity was calculated using equation 2

$$\text{GR activity}(\text{mmol min}^{-1}\text{mg protein}^{-1}) = \frac{(V_{max}(\text{sample + GSSG}) - V_{max}(\text{sample}) \times 60}{6300 \times 0.35} \quad (2)$$

2.3.6 Glutathione S-Transferase

Glutathione S-Transferase activity was quantified using GST assay (Habig et al., 1974)

Solutions

- Assay buffer: 0.1M Na-phosphate, pH 7.5.
- Glutathione solution: 1 mM GSH
  - 16 mg GSH in 50 mL assay buffer. The Glutathione solution was made the same day as the assay was executed, and stored on ice.
- CDNB-solution: 100 mM CDNB
  - 25.4 mg CDNB in 1.25 mL DMSO.
- Reaction mixture
  - 400 μL CDNB solution in 20 mL glutathione solution. The reaction mixture was made shortly before loading samples to the 96-well microplate, and mixed well using a magnetic stirrer in room temperature.

Procedure

The liver samples were prepared for GST assay earlier (see section 2.3.2).
The samples were diluted 50× by mixing 10μL sample with 490 μL assay buffer. 50 μL sample was pipetted into wells on a 96-well microplate. Four replicates were done for each sample. 50 μL assay buffer was pipetted into four wells as blanks. 200μL reaction mixture was added to all wells using a multipipette. The 96-well microplate was read once every 38 seconds for 15 minutes at an absorbance of 340 nm with kinetic read using a BioTek's Synergy Mx plate reader.

The GST activity was calculated using equation 3

$$\text{GST activity}(\mu\text{mol min}^{-1}\text{mL}^{-1}) = \frac{\Delta A_{340}(\text{min}^{-1}) \times V_{\text{mix}}(\text{mL}) \times \text{DF}}{\varepsilon_{\text{mM}}(\mu\text{M}^{-1}\text{cm}^{-1}) \times \ell(\text{cm}) \times V_{\text{sample}}(\text{mL})} \tag{3}$$

where ΔA_{340} is the change in absorbance per minute

V_{mix} is the volume of mixture in each well in mL

DF is the dilution factor of each sample

ε_{mM} is the extinction coefficient for CDNB at 340 nm per μmol per cm

ℓ is the path lenght of 96-well microplate in cm

V_{sample} is the volume of sample in mL ℓ is 0.552 cm ε_{mM} is 0.0096

The results were standardized using the protein concentrations calculated in section 2.3.3

$$\frac{\text{GST activity}(\text{nmol min}^{-1}\text{mL}^{-1})}{\text{protein concentration}(\text{mg mL}^{-1})} \tag{4}$$

2.3.7 Glutathione Peroxidase

The Glutathione peroxidase activity was quantified using the GPx assay (Mills, 1959).

Solutions

- Assay buffer: 0.1 M K-phosphate, pH 7.0.
- GSH solution: 215.1 mg GSH to 10 mL dH_2O.
- NADPH solution: 16 mg NADPH to 1 ml assay buffer.
- NaN_3 solution: 13 mg NaN_3 to 10 mL assay buffer.

- GR solution76.8 μL GR to 1 mL assay buffer.
- H_2O_2: 90.4 μL H_2O_2 to 10 ml dH_2O.

Samples and solutions were kept on ice.

Procedure

The liver samples were prepared for GPx assay earlier (see section 2.3.2 on page 12).
50 μL H_2O_2 + 250 μL assay buffer + 25 μL NaN_3 + 25 μL GSH + 25 μL GR + 90 μL dH_2O + 10 μL supernatant sample was mixed together in an eppendorf tube. This was repeated for every sample.

Four replicates were done for every sample. 100μL of mixture was pipetted into each well, and one blank, where 100 μL mixture containing assay buffer instead of supernatant sample, was added for every tenth sample.10 μL NADPH was added to all wells using a multipipette. The 96-well microplate was read once every 38 seconds for 15 minutes at an absorbance of 340 nm kinetic read using a BioTek's Synergy Mx plate reader.

The GPx activity is calculated using equation 5

$$\text{GPx activity}(\text{nmol min}^{-1}\text{mL}^{-1}) = \frac{\Delta A_{340}(\text{min}^{-1}) \times V_{\text{mix}}(\text{mL}) \times \text{DF}}{\varepsilon_{\text{mM}}(\mu\text{M}^{-1}\text{cm}^{-1}) \times \ell(\text{cm}) \times V_{\text{sample}}(\text{mL})} \tag{5}$$

where ΔA_{340} is the change in absorbance per minute

V_{mix} is the volume of mixture in each well in mL

DF is the dilution factor of each sample

ε_{mM} is the extinction coefficient for NADPH at 340 nm per μmol per cm

ℓ is the path lenght of 96-well microplate in cm

V_{sample} is the volume of sample in mL. ℓ is 0.552 cm

ε_{mM} is 0.00622

The results were standardized using the protein concentrations calculated in section 2.3.3.

$$\frac{\text{GPx activity}(\text{nmol min}^{-1}\text{mL}^{-1})}{\text{protein concentration}(\text{mg mL}^{-1})} \tag{6}$$

2.4 Statistics

To look for equal variance between treatment groups, a Barlett's test was used. For this test, the null hypothesis, H_0: All groups have equal variance. The alternative hypothesis, H_A: At least two groups have different variance. A significance level of rejecting H_0 was set to .05 ($\alpha = .05$)

For biomarkers with equal variance between all groups, a one-way analysis of variance (ANOVA) was conducted to compare the means between treatment groups. The null hypothesis, H_0: There is no difference in the means between treatment groups. The alternative hypothesis, H_A: At least two groups have different means. A significance level of rejecting H_0 was set to .05 ($\alpha = .05$)

For biomarkers with unequal variance (GR only), a Kruskal Wallis test was conducted instead of ANOVA.

To conduct statistical analyses, the statistical software R (version 1.1.383 – ©2009-2017 RStudio, Inc.) was used. The only R-package used was ggplot2, a graphics package used to make boxplots. Microsoft Excel (version 16.28) was used to process raw data.

Results

3.1 Glutathione

One-ways ANOVAS between each treatment groups revealed no significant differences in total cellular glutathione concentration (figure 3.1), $F(5, 24)=0.49$, $p=.778$ (Appendix 5, table A2); GSH concentration (figure 3.2), $F(5, 24)=2.16$, $p=.0921$ (Appendix 5, table A3); GSSG concentration (figure 3.3), $F(5, 24)=0.07$, $p=.996$ (Appendix 5, table A4) or GSH/GSSG ratio (figure 3.4), $F(5, 24)=1.02$, $p=.428$ (Appendix 5, table A5).

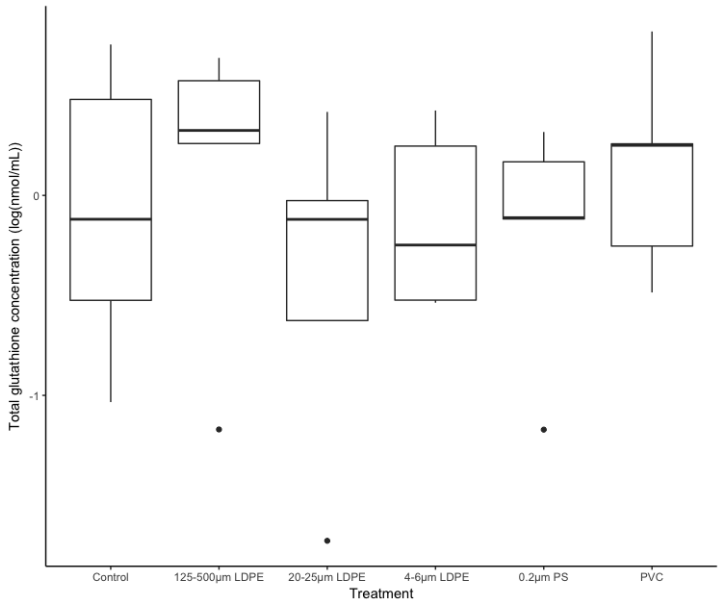

Figure 3.1. Boxplot showing each treatment group's total cellular glutathione concentrations in liver expressed as nmol/mL. n=5 for each group. Each box represents a treatment group. Top and bottom line of each box represent third and first quartile respectively. The horizontal line inside the box represents the group median. Whiskers represent data points outside first and third quartile, but within 1.5 $\times$ interquartile range. Data points outside 1.5 $\times$ interquartile range are shown as single dots. There were no significant difference between treatment groups ($p=.778$).

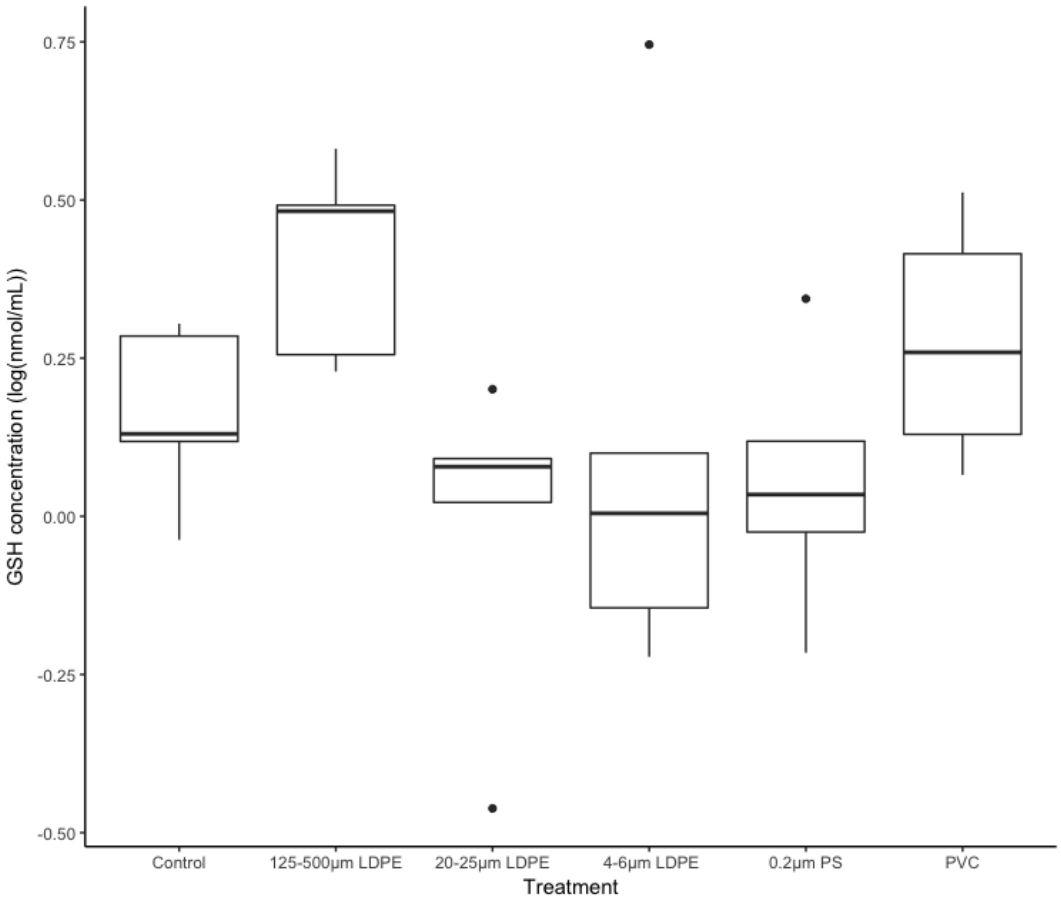

Figure 3.2. Boxplot showing each treatment group's GSH concentrations in liver expressed as nmol/mL. n=5 for each group. Each box represents a treatment group. Top and bottom line of each box represent third and first quartile respectively. The horizontal line inside the box represents the group median. Whiskers represent data points outside first and third quartile, but within 1.5 × interquartile range. Data points outside 1.5 × interquartile range are shown as single dots. There were no significant difference between treatment groups (p=.0921).

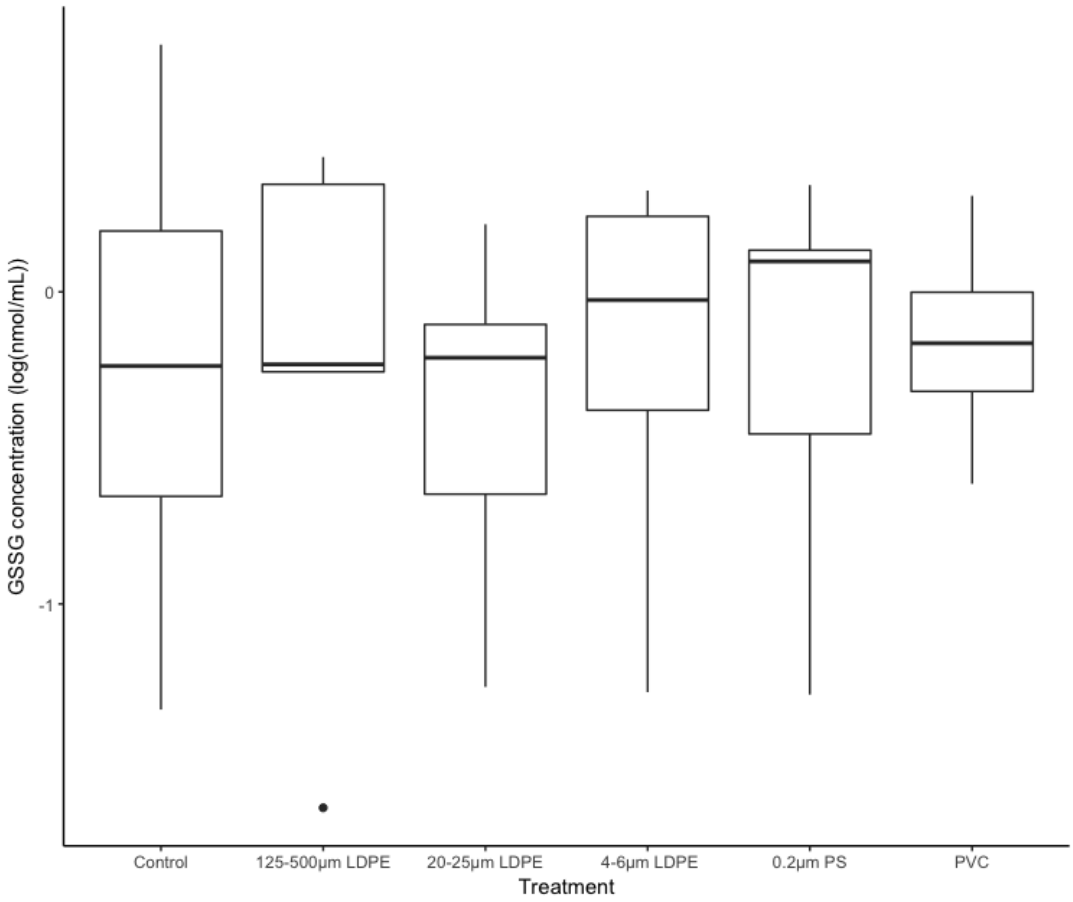

Figure 3.3. Boxplot showing each treatment group's GSSG concentrations in liver expressed as nmol/mL. n=5 for each group. Each box represents a treatment group. Top and bottom line of each box represent third and first quartile respectively. The horizontal line inside the box represents the group median. Whiskers represent data points outside first and third quartile, but within 1.5 × interquartile range. Data points outside 1.5 × interquartile range are shown as single dots. There were no significant difference between treatment groups (p=.996).

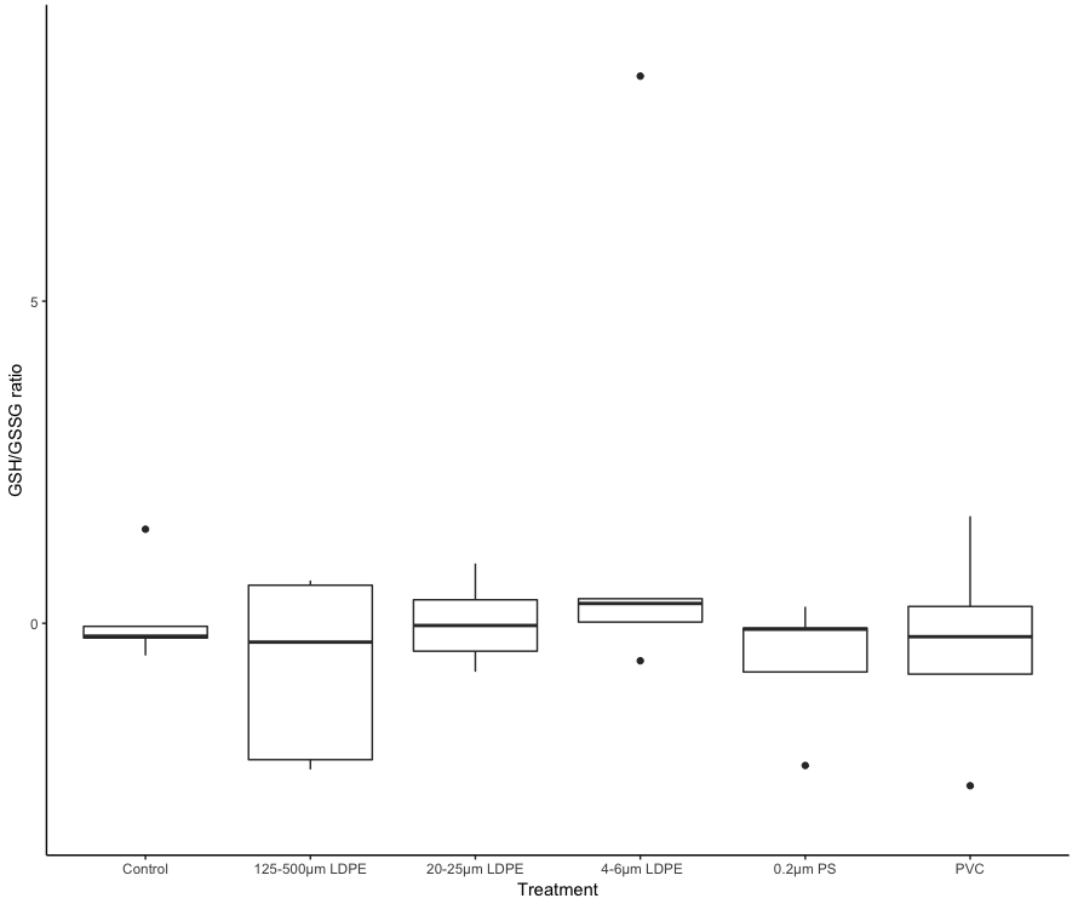

Figure 3.4. Boxplot showing each treatment group's GSH/GSSG ratio in liver. n=5 for each group. Each box represents a treatment group. Top and bottom line of each box represent third and first quartile respectively. The horizontal line inside the box represents the group median. Whiskers represent data points outside first and third quartile, but within 1.5 $\times$ interquartile range. Data points outside 1.5 $\times$ interquartile range are shown as single dots. There were no significant difference between treatment groups (p=.428).

3.2 Glutathione Reductase

A Kruskal-Wallis test was conducted to examine the difference in GR activity according to plastic feed treatment. There were no significant differences between the six treatment groups ($\chi^2 = 0.86$, $df = 5$, $p = .522$) (Appendix 5, table A6). Visualized in figure 3.5

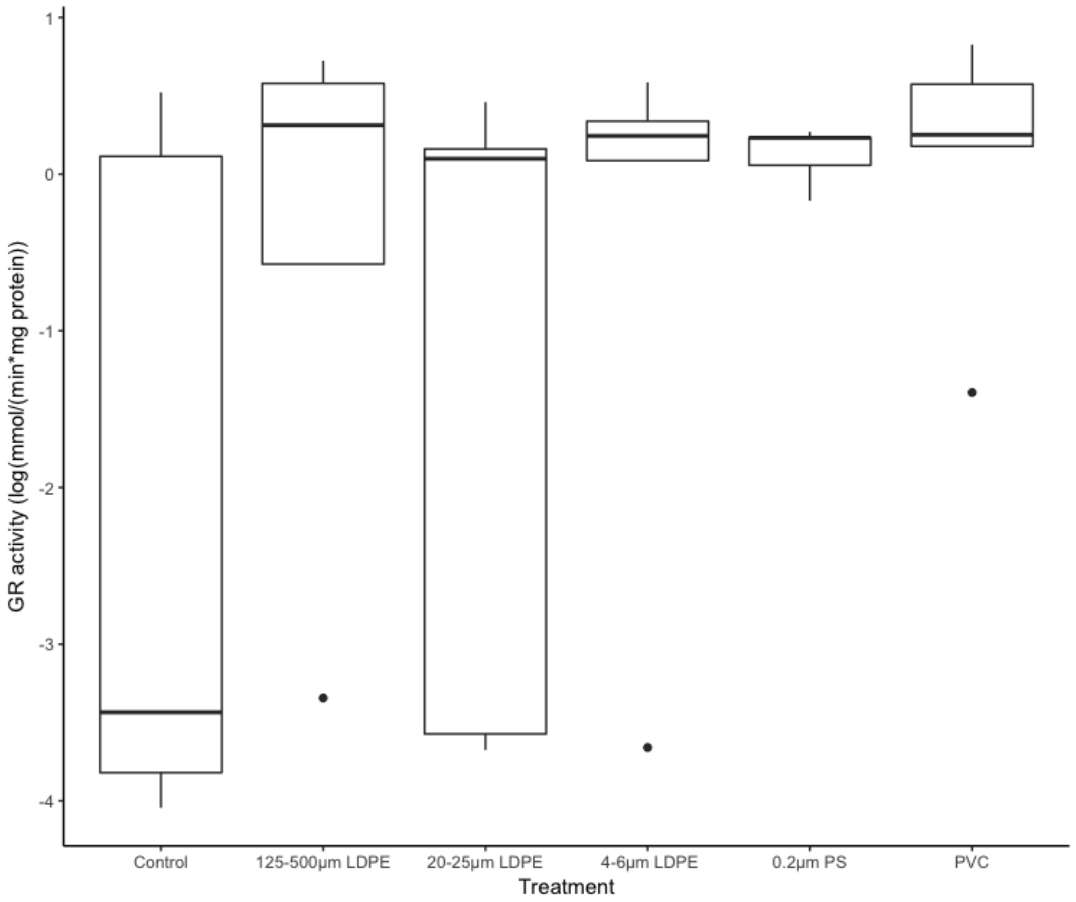

Figure 3.5. Boxplot showing each treatment group's GR activity in liver expressed as mmol/(min*mg protein). n=5 for each group. Each box represents a treatment group. Top and bottom line of each box represent third and first quartile respectively. The horizontal line inside the box represents the group median. Whiskers represent data points outside first and third quartile, but within 1.5 $\times$ interquartile range. Data points outside 1.5 $\times$ interquartile range are shown as single dots. There were no significant difference between treatment groups (p=.522).

3.3 Glutathione S-Transferase

A one-way ANOVA was conducted to examine the difference in GST activity according to plastic feed treatment. There were no significant differences between the six treatment groups

(*F(5, 24)*=1.21, *p*=.334) (Appendix 5, table A7)

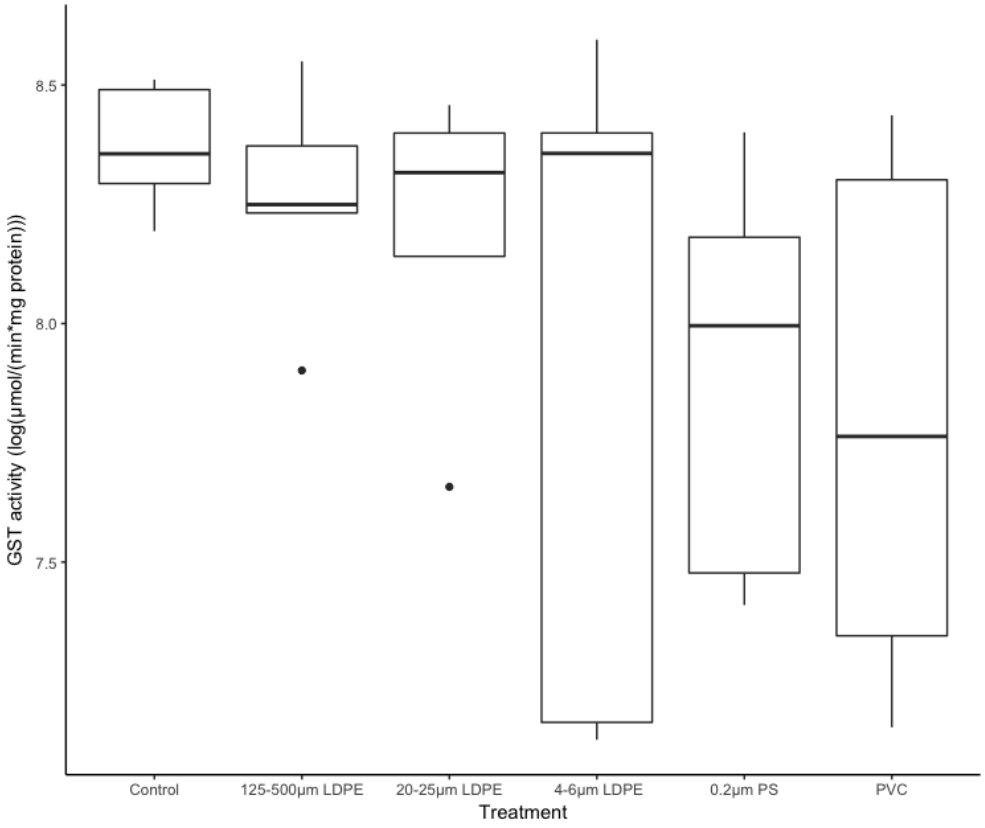

Figure 3.6. Boxplot showing each treatment group's GST activity in liver expressed as μmol/(min*mg protein). n=5 for each group. Each box represents a treatment group. Top and bottom line of each box represent third and first quartile respectively. The horizontal line inside the box represents the group median. Whiskers represent data points outside first and third quartile, but within 1.5 $\times$ interquartile range. Data points outside 1.5 $\times$ interquartile range are shown as single dots. There were no significant difference between treatment groups (*p*=.334).

3.4 Glutathione Peroxidase

A one-way ANOVA was conducted to examine the difference in GPx activity according to plastic feed treatment. There were no significant differences between the six treatment groups (

GPx: $F(5, 24)=0.35$, $p=.878$) (Appendix 5, table A8).

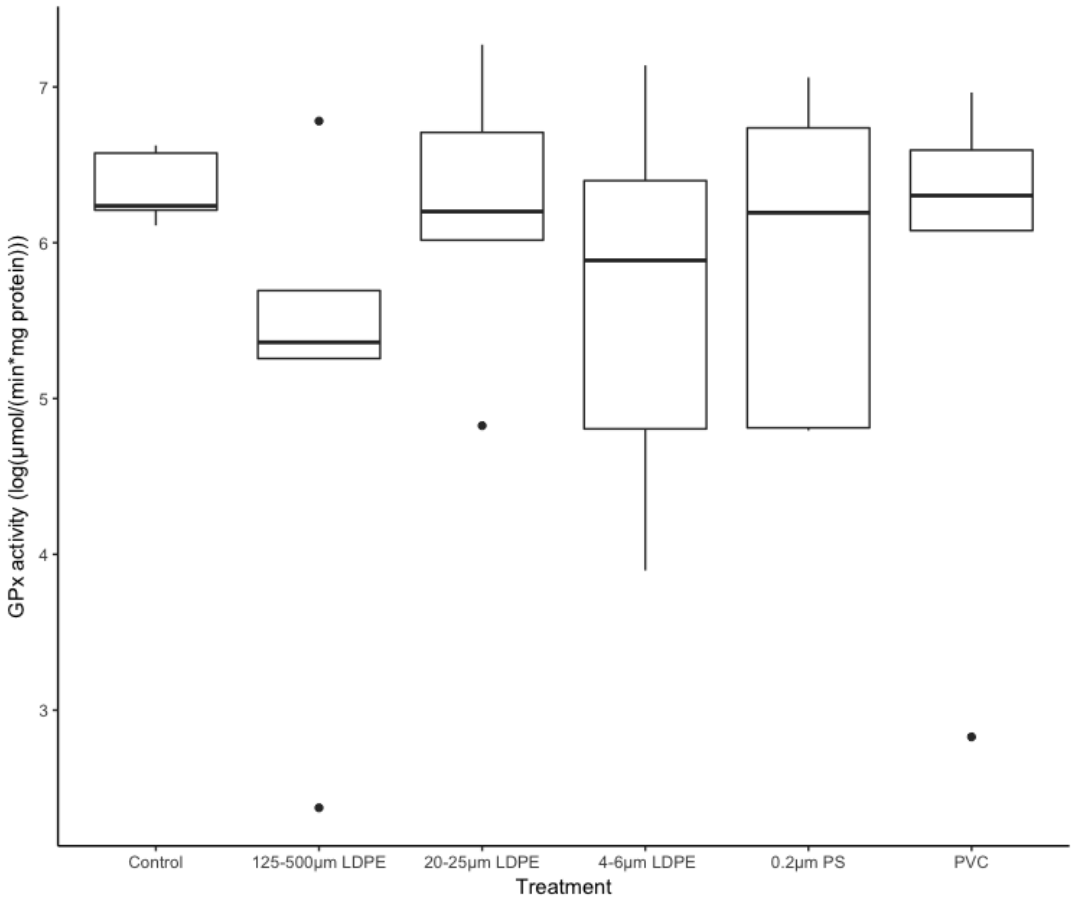

Figure 3.7. Boxplot showing each treatment group's GPx activity in liver expressed as μmol/(min*mg protein). n=5 for each group. Each box represents a treatment group. Top and bottom line of each box represent third and first quartile respectively. The horizontal line inside the box represents the group median. Whiskers represent data points outside first and third quartile, but within 1.5 $\times$ interquartile range. Data points outside 1.5 $\times$ interquartile range are shown as single dots. There were no significant difference between treatment groups ($p=.878$).

Discussion

In this thesis where Atlantic cod were treated with microplastics and analyzed for oxidative stress. The results show that there are no effects from microplastic exposure on any of the biomarkers examined. This implies one or more of the following:

- MPs were not taken up in the intestine
- MPs were taken up in the intestine but were not translocated to the liver
- MPs were translocated to the liver but did not induce oxidative stress on the liver
- Oxidative stress was induced in the liver by the MPs but not detected in the analyses

It seems unlikely that the MPs were not taken up in the instestines for some of the treatment groups, but more likely for others. The fish were treated with 0.2, 4-6, 20-25 and 150-500 um. The smaller particles – 0.2 and 4-6 um – are shown to be taken up at relatively high rates (Desai et al., 1997; Jani et al., 1990). Deng 2017 and Lu 2016 also show that particles with size 20 μm are taken up. Therefore, it seems likely that the 20-25 μm group also will be taken up, but at a slower rate than the smaller particles (Desai et al., 1997; Jani et al., 1990). As for the larger treatment group, the 150-500 um, there is more uncertainty. Volkheimer et al. (1968) argues that 150 um is the upper size limit for particle uptake, which an EFSA report also concludes with (Alexander et al., 2016). However more recent studies show uptake of larger MPs (Avio et al., 2015; Jovanovic et al., 2018; Lu et al., 2016). Avio et al. (2015) reports that microplastic particles with sizes up to 0.6mm are located in the liver. This finding indicates that the upper size limit for particle uptake in the intestine is much larger than first assumed. For this reason it is likely that even the biggest MPs in this thesis (150-500 μM) also may have been taken up in the intestine.

Even though MPs are taken up by the intestinal epithelial cells, it is not given that they are translocated to organs. Particles may instead be transported along the blood system and removed via the bile (Alexander et al., 2016). There is no evidence that this is the explanation for the lack of effects in this thesis. However, the process of removing MPs from the organism cannot be disregarded.

MPs have the potential to be translocated to different tissues in the organism when they are not removed. It is reported that MPs have been found to translocate to brain, liver and kidney (Deng et al., 2017; Lu et al., 2016). Jovanovic et al. (2018) shows, however, that only 5% of the fish had MPs in the liver after ingestion which indicated that although possible, translocation to liver is an uncommon process. This may be one of the most important reasons why there were no effects in neither this thesis nor many other's studies. If this slow translocation rate is accurate, it is not unreasonable that this explains the results presented in this thesis. In addition Collard et al. (2017) and Jovanovic et al. (2018) both discovered that the livers containing MPs contained on average ~1 particle per liver.

Extremely high concentrations of MPs will induce inflammation and oxidative stress in fish, but not because of the MPs per se. The high concentration of MPs overstimulates the immune system and phagocytes which leads to these effects (Jovanović and Palić, 2012). When exposed to smaller and more ecological relevant concentrations, studies show no effects on oxidative stress from MPs (Avio et al., 2015) . As a consequence, studies should use ecological relevant concentrations to avoid finding effects from overstimulation rather than the MPs.

In my thesis I used GSH concentration and GST, GR and GPx activity as indicators for oxidative stress in liver. these bioindicators do not show oxidative stress or damage on cellular structures but are indicators that oxidative stress has occurred due to their role as antioxidant proteins. There are other bioindicators used to indicate oxidative stress as well e.g. catalase, lipid peroxidation levels and superoxide dismutase. My results show no indication of oxidative stress in any of the biomarkers. Some get the same result as the results in this thesis (Avio et al., 2015). Other research on the same field have opposing results however. Others do in fact find indicators that MPs induce oxidative stress in fish (Barboza et al., 2018; Qiao et al., 2019; Wen et al., 2018). This shows that microplastic-induced oxidadive stress is possible. As a consequence, I cannot disregard that MPs may have induced oxidative stress in fish in my experiment if they were taken up and translocated to the liver.

Regarding the last point, that the MPs in fact did induce oxidative stress that was not detected is possible, but improbable. Given the small sample size (n=5) one or more group may not represent the true mean. Having larger sample sizes will reduce the uncertainty.

If the fish tissue had been analyzed for content of MPs, it would be more easy to understand the results. If MPs were shown to have translocated to other the liver, it would seem that there

was uptake and translocation yet no effects on oxidative stress . On the other hand, if the liver turned out to not contain MPs, it would seem to be either no uptake from the intestine or no translocation to organs. Without the tissue analyzes one can only speculate in the results.

When doing toxicological experiment on fish it is common to use *D. rerio* as model organism (Spitsbergen and Kent, 2003). Although not as well suited because of the bigger size, I used Atlantic cod (*Gadus morhua* as model organism in my experiment because it is an economically and ecologically important fish in Norway and the rest of the north-east Atlantic (Ageeva et al., 2017). Research has found that the populations of Atlantic cod are decreasing (Myers et al., 1996) and it is therefore important to understand what is affecting the populations. With Atlantic cod being one of the most common fish consumed in Norway it is also important to have a better understanding of the uptake of MPs in Atlantic cod for food safety reasons.

Looking back, there are a few things that could have been done different when doing the exposure. One thing is to reduce the amount of treatments from six to five. Since there was a limitation of space and number of aquaria, there was also a limit to the number of replicates. It seems reasonable to exclude PVC group because I was unable to find out the size of the particles which makes the group less useful. This reduction in treatment groups would allow me to increase the sample size to get a more robust experiment.

Most studies uses microplastic concentrations higher than found in the oceans. Cole et al. (2011) has reviewed over 100 articles addressing MPs pollution in the sea, and the concentrations ranged from >1 item m^{-3} 800m off the coast of California to over 2,500 items m^{-3} in the North Pacific central gyre. When waters not immediate close to plastic gyres, estuaries or bays have such a low concentration of MPs, one may question the studies' ecological relevance. However, the organisms living in the oceans may be exposed to MPs for several years, which short term studies are unable to replicate.

Despite being a field of interest, where several studies are done on MPs, translocation from the intestine to other tissue in fish is a poorly studied area. Everaert et al. (2018) estimates that the amount of MPs on the oceans will increase 50-folds between 2010 and 2100. To get a better understanding of the risks associated to the microplastic pollution and exposure in marine organisms that is reported, more studies are needed. However, there is more attention to this subject now than ever before. Most likely our knowledge in this field will grow drastically in future years.

Conclusion

Research show that nano- and microparticles have the ability to translocate and cause oxidative stress in marine organisms. To get at better understanding of the risk of microplastic ingestion on fish, an exposure study on Atlantic cod was done. This study was performed to examine the effects on GSH, GPx, GST, and GR by acute exposure to microplastics of different sizes. Results show that there are no effects on any of the biomarkers from any plastic treatments compared to the control group. This may be because the plastic particles are too big to be absorbed from the intestines and translocated to other tissue. Another possibility is that MPs have no effects from acute MPs exposure on the biomarkers tested. Other studies have shown both translocation and effects on different biomarkers several biomarkers, including biomarkers suited for oxidative stress. Because there was no content analyzes on the liver, it is impossible to say wether the results shown in this thesis is due to no translocation or that the MPs did not have any effects on the biomarkers.

Bibliography

Absher, T. M., Ferreira, S. L., Kern, Y., Ferreira, Jr., A. L., Christo, S. W., and Ando, R. A. (2019). Incidence and identification of microfibers in ocean waters in Admiralty Bay, Antarctica. *ENVIRONMENTAL SCIENCE AND POLLUTION RESEARCH*, 26(1):292–298.

Ageeva, T. N., Jobling, M., Olsen, R. L., and Esaiassen, M. (2017). Gender-specific responses of mature Atlantic cod (Gadus morhua L.) to feed deprivation. *FISHERIES RESEARCH*, 188:95–99.

Akerboom, T. P. and Sies, H. (1981). Assay of glutathione, glutathione disulfide, and glutathione mixed disulfides in biological samples. In *Detoxication and Drug Metabolism: Conjugation and Related Systems*, volume 77 of *Methods in Enzymology*, pages 373 – 382. Academic Press.

Alexander, J., Barregard, L., Bignami, M., Ceccatelli, S., Cottrill, B., Dinovi, M., Edler, L., Grasl-Kraupp, B., Hogstrand, C., Hoogenboom, L. R., Knutsen, H. K., Nebbia, C. S., Oswald, I., Petersen, A., Rogiers, V. M., Rose, M., Roudot, A.-C., Schwerdtle, T., Vleminckx, C., Vollmer, G., Wallace, H., and Chain, E. P. C. F. (2016). Presence of microplastics and nanoplastics in food, with particular focus on seafood. *EFSA JOURNAL*, 14(6).

Almar, M., Otero, L., Santos, C., and Gonzalez-Gallego, J. (1998). Liver glutathione content and glutathione-dependent enzymes of two species of freshwater fish as bioindicators of chemical pollution. *JOURNAL OF ENVIRONMENTAL SCIENCE AND HEALTH PART B-PESTICIDES FOOD CONTAMINANTS AND AGRICULTURAL WASTES*, 33(6):769–783.

Alomar, C., Sureda, A., Capo, X., Guijarro, B., Tejada, S., and Deudero, S. (2017). Microplastic ingestion by Mullus surmuletus Linnaeus, 1758 fish and its potential for causing oxidative stress. *ENVIRONMENTAL RESEARCH*, 159:135–142.

Andrady, A., Amin, M., Hamid, S., Hu, X., and Torikai, A. (1995). Effects of increased solar ultraviolet radiation on materials. *AMBIO*, 24(3):191–196.

Aprahamian, M., Michel, C., Humbert, W., Devissaguet, J. P., and Damge, C. (1987). Transmucosal passage of polyalkylcyanoacrylate nanocapsules as a new drug carrier in the small intestine. *Biology of the Cell*, 61(1-2):69–76.

AshaRani, P. V., Mun, G. L. K., Hande, M. P., and Valiyaveettil, S. (2009). Cytotoxicity and Genotoxicity of Silver Nanoparticles in Human Cells. *ACS NANO*, 3(2):279–290.

Avio, C. G., Gorbi, S., and Regoli, F. (2015). Experimental development of a new protocol for extraction and characterization of microplastics in fish tissues: First observations in commercial species from adriatic sea. *Marine Environmental Research*, 111:18 – 26. Particles in the Oceans: Implication for a safe marine environment.

Barboza, L. G. A., Vieira, L. R., Branco, V., Figueiredo, N., Carvalho, F., Carvalho, C., and Guilhermino, L. (2018). Microplastics cause neurotoxicity, oxidative damage and energy-related changes and interact with the bioaccumulation of mercury in the european seabass, dicentrarchus labrax (linnaeus, 1758). *Aquatic Toxicology*, 195:49 – 57.

Barnes, D. K. A., Galgani, F., Thompson, R. C., and Barlaz, M. (2009). Accumulation and fragmentation of plastic debris in global environments. *PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B-BIOLOGICAL SCIENCES*, 364(1526):1985–1998.

Berg, K., Puntervoll, P., Valdersnes, S., and Goksoyr, A. (2010). Responses in the brain proteome of Atlantic cod (Gadus morhua) exposed to methylmercury. *AQUATIC TOXICOLOGY*, 100(1):51–65.

Boerger, C. M., Lattin, G. L., Moore, S. L., and Moore, C. J. (2010). Plastic ingestion by planktivorous fishes in the North Pacific Central Gyre. *MARINE POLLUTION BULLETIN*, 60(12):2275–2278.

Browne, M. A., Dissanayake, A., Galloway, T. S., Lowe, D. M., and Thompson, R. C. (2008). Ingested microscopic plastic translocates to the circulatory system of the mussel, Mytilus edulis (L.). *ENVIRONMENTAL SCIENCE & TECHNOLOGY*, 42(13):5026–5031.

Cincinelli, A., Scopetani, C., Chelazzi, D., Lombardini, E., Martellini, T., Katsoyiannis, A., Fossi, M. C., and Corsolini, S. (2017). Microplastic in the surface waters of the Ross Sea (Antarctica): Occurrence, distribution and characterization by FTIR. *CHEMOSPHERE*, 175:391–400.

Cole, M., Lindeque, P., Halsband, C., and Galloway, T. S. (2011). Microplastics as contaminants in the marine environment: A review. *MARINE POLLUTION BULLETIN*, 62(12):2588–2597.

Collard, F., Gilbert, B., Compere, P., Eppe, G., Das, K., Jauniaux, T., and Parmentier, E. (2017). Microplastics in livers of European anchovies (Engraulis encrasicolus, L.). *ENVIRONMENTAL POLLUTION*, 229:1000–1005.

Collignon, A., Hecq, J.-H., Galgani, F., Voisin, P., Collard, F., and Goffart, A. (2012). Neustonic microplastic and zooplankton in the North Western Mediterranean Sea. *MARINE POLLUTION BULLETIN*, 64(4):861–864.

Deng, Y., Zhang, Y., Lemos, B., and Ren, H. (2017). Tissue accumulation of microplastics in mice and biomarker responses suggest widespread health risks of exposure. *SCIENTIFIC REPORTS*, 7.

Desai, M. P., Labhasetwar, V., Walter, E., Levy, R. J., and Amidon, G. L. (1997). The mechanism of uptake of biodegradable microparticles in caco-2 cells is size dependent. *Pharmaceutical Research*, 14:1568–1573.

Desforges, J.-P. W., Galbraith, M., Dangerfield, N., and Ross, P. S. (2014). Widespread distribution of microplastics in subsurface seawater in the NE Pacific Ocean. *MARINE POLLUTION BULLETIN*, 79(1-2):94–99.

Eldridge, J. H., Hammond, C. J., Meulbroek, J. A., Staas, J. K., Gilley, R. M., and Tice, T. R. (1990). Controlled vaccine release in the gut-associated lymphoid tissues. i. orally administered biodegradable microspheres target the peyer's patches. *Journal of Controlled Release*, 11(1):205 – 214.

Engler, R. E. (2012). The Complex Interaction between Marine Debris and Toxic Chemicals in the Ocean. *ENVIRONMENTAL SCIENCE & TECHNOLOGY*, 46(22):12302–12315.

Everaert, G., Van Cauwenberghe, L., De Rijcke, M., Koelmans, A. A., Mees, J., Vandegehuchte, M., and Janssen, C. R. (2018). Risk assessment of microplastics in the ocean: Modelling approach and first conclusions. *ENVIRONMENTAL POLLUTION*, 242(B):1930–1938.

Farrell, P. and Nelson, K. (2013). Trophic level transfer of microplastic: Mytilus edulis (L.) to Carcinus maenas (L.). *ENVIRONMENTAL POLLUTION*, 177:1–3.

Fendall, L. S. and Sewell, M. A. (2009). Contributing to marine pollution by washing your face: Microplastics in facial cleansers. *MARINE POLLUTION BULLETIN*, 58(8):1225–1228.

Florence, A. (1997). The oral absorption of micro- and nanoparticulates: Neither exceptional nor unusual. *PHARMACEUTICAL RESEARCH*, 14(3):259–266.

GESAMP (2016). Sources, fate and effects of microplastics in the marine environment: part two of a global assessment. In Kershaw, P. and Rochman, C., editors, *Reports Studies*,

number 93, page 220, IMO/FAO/UNESCO-IOC/UNIDO/WMO/IAEA/UN/ UNEP/UNDP Joint Group of Experts on the Scientific Aspects of Marine Environmental Protection.

Gregory, M. (1996). Plastic 'scrubbers' in hand cleansers: A further (and minor) source for marine pollution identified. *MARINE POLLUTION BULLETIN*, 32(12):867–871.

Habig, W., Pabst, M., and Jakoby, W. (1974). Glutathione s-transferases. the first enzymatic step in mercapturic acid formation. *The Journal of biological chemistry*, 249(22):7130—7139.

Halliwell, B. (1998). Can oxidative dna damage be used as a biomarker of cancer risk in humans? problems, resolutions and preliminary results from nutritional supplementation studies. *FREE RADICAL RESEARCH*, 29(6):469–486.

Haward, M. (2018). Plastic pollution of the world's seas and oceans as a contemporary challenge in ocean governance. *NATURE COMMUNICATIONS*, 9(1):667.

Hirai, H., Takada, H., Ogata, Y., Yamashita, R., Mizukawa, K., Saha, M., Kwan, C., Moore, C., Gray, H., Laursen, D., Zettler, E. R., Farrington, J. W., Reddy, C. M., Peacock, E. E., and Ward, M. W. (2011). Organic micropollutants in marine plastics debris from the open ocean and remote and urban beaches. *MARINE POLLUTION BULLETIN*, 62(8):1683–1692.

Jambeck, J. R., Geyer, R., Wilcox, C., Siegler, T. R., Perryman, M., Andrady, A., Narayan, R., and Law, K. L. (2015). Plastic waste inputs from land into the ocean. *SCIENCE*, 347(6223):768–771.

Jani, P., Halbert, G., Langridge, J., and Florence, A. (1990). Nanoparticle uptake by the rat gastrointestinal mucosa - quantitation and particle-size dependency. *JOURNAL OF PHARMACY AND PHARMACOLOGY*, 42(12):821–826.

Jovanovic, B., Gokdag, K., Guven, O., Emre, Y., Whitley, E. M., and Kideys, A. E. (2018). Virgin microplastics are not causing imminent harm to fish after dietary exposure. *MARINE POLLUTION BULLETIN*, 130:123–131.

Jovanović, B. and Palić, D. (2012). Immunotoxicology of non-functionalized engineered nanoparticles in aquatic organisms with special emphasis on fish—review of current knowledge, gap identification, and call for further research. *Aquatic Toxicology*, 118-119:141 – 151.

Kanhai, L. D. K., Gårdfeldt, K., Lyashevska, O., Hassellöv, M., Thompson, R. C., and O'Connor, I. (2018). Microplastics in sub-surface waters of the arctic central basin. *MARINE POLLUTION BULLETIN*, 130:8 – 18.

Laist, D. W. (1987). Overview of the biological effects of lost and discarded plastic debris in the marine environment. *MARINE POLLUTION BULLETIN*, 18(6B):319–326.

Laist, D. W. (1997). *Impacts of marine debris: entanglement of marine life in marine debris including a comprehensive list of species with entanglement and ingestion records*, pages 99–139. Springer New York, New York, NY.

LeFevre, M. E., Vanderhoff, J. W., Laissue, J. A., and Joel, D. D. (1978). Accumulation of 2-m latex particles in mouse peyer's patches during chronic latex feeding. *Experientia*, 34(1):120–122.

Lei, L., Wu, S., Lu, S., Liu, M., Song, Y., Fu, Z., Shi, H., Raley-Susman, K. M., and He, D. (2018). Microplastic particles cause intestinal damage and other adverse effects in zebrafish Danio rerio and nematode Caenorhabditis elegans. *SCIENCE OF THE TOTAL ENVIRONMENT*, 619:1–8.

Lowry, O., Rosebrough, N., Farr, A., and Randall, R. (1951). Protein measurement with the folin phenol reagent. *JOURNAL OF BIOLOGICAL CHEMISTRY*, 193(1):265–275.

Lu, Y., Zhang, Y., Deng, Y., Jiang, W., Zhao, Y., Geng, J., Ding, L., and Ren, H. (2016). Uptake and accumulation of polystyrene microplastics in zebrafish (danio rerio) and toxic effects in liver. *Environmental Science & Technology*, 50(7):4054–4060.

Lusher, A. L., Burke, A., O'Connor, I., and Officer, R. (2014). Microplastic pollution in the Northeast Atlantic Ocean: Validated and opportunistic sampling. *MARINE POLLUTION BULLETIN*, 88(1-2):325–333.

Lusher, A. L., Tirelli, V., O'Connor, I., and Officer, R. (2015). Microplastics in Arctic polar waters: the first reported values of particles in surface and sub-surface samples. *SCIENTIFIC REPORTS*, 5.

Manke, A., Wang, L., and Rojanasakul, Y. (2013). Mechanisms of Nanoparticle-Induced Oxidative Stress and Toxicity. *BIOMED RESEARCH INTERNATIONAL*.

Mapson, L. W. and Goddard, D. R. (1951). The reduction of glutathione by plant tissues. *BIOCHEMICAL JOURNAL*, 49(5):592–601.

Mattsson, K., Johnson, E. V., Malmendal, A., Linse, S., Hansson, L.-A., and Cedervall, T. (2017). Brain damage and behavioural disorders in fish induced by plastic nanoparticles delivered through the food chain. *Scientific Reports*, 7(1):11452.

Meister, A. and Anderson, M. (1983). Glutathione. *ANNUAL REVIEW OF BIOCHEMISTRY*, 52:711–760.

Mills, G. C. (1959). The purification and properties of glutathione peroxidase of erythrocytes. *THE JOURNAL OF BIOLOGICAL CHEMISTRY*, 234 3:502–6.

Morgana, S., Ghigliotti, L., Estévez-Calvar, N., Stifanese, R., Wieckzorek, A., Doyle, T., Christiansen, J. S., Faimali, M., and Garaventa, F. (2018). Microplastics in the arctic: A case study with sub-surface water and fish samples off northeast greenland. *ENVIRONMENTAL POLLUTION*, 242:1078 – 1086.

Mu, J., Zhang, S., Qu, L., Jin, F., Fang, C., Ma, X., Zhang, W., and Wang, J. (2019). Microplastics abundance and characteristics in surface waters from the northwest pacific, the bering sea, and the chukchi sea. *MARINE POLLUTION BULLETIN*, 143:58 – 65.

Munari, C., Infantini, V., Scoponi, M., Rastelli, E., Corinaldesi, C., and Mistri, M. (2017). Microplastics in the sediments of Terra Nova Bay (Ross Sea, Antarctica). *MARINE POLLUTION BULLETIN*, 122(1-2):161–165.

Myers, R., Hutchings, J., and Barrowman, N. (1996). Hypotheses for the decline of cod in the North Atlantic. *MARINE ECOLOGY PROGRESS SERIES*, 138(1-3):293–308.

Nelms, S. E., Galloway, T. S., Godley, B. J., Jarvis, D. S., and Lindeque, P. K. (2018). Investigating microplastic trophic transfer in marine top predators. *ENVIRONMENTAL POLLUTION*, 238:999–1007.

OSPAR (2008). Ospar list of threatened and/or declining species and habitats.

Park, E.-J., Yi, J., Chung, Y.-H., Ryu, D.-Y., Choi, J., and Park, K. (2008). Oxidative stress and apoptosis induced by titanium dioxide nanoparticles in cultured BEAS-2B cells. *TOXICOLOGY LETTERS*, 180(3):222–229.

Plastics Europe (2018). Plastics - the facts 2018. , Plastics Europe.

Possatto, F. E., Barletta, M., Costa, M. F., Ivar do Sul, J. A., and Dantas, D. V. (2011). Plastic debris ingestion by marine catfish: An unexpected fisheries impact. *MARINE POLLUTION BULLETIN*, 62(5):1098–1102.

Pratten, M. K. and Lloyd, J. B. (1986). Pinocytosis and phagocytosis: the effect of size of a particulate substrate on its mode of capture by rat peritoneal macrophages cultured in vitro. *Biochimica et Biophysica Acta (BBA) - General Subjects*, 881(3):307 – 313.

Qiao, R., Sheng, C., Lu, Y., Zhang, Y., Ren, H., and Lemos, B. (2019). Microplastics induce intestinal inflammation, oxidative stress, and disorders of metabolome and microbiome in zebrafish. *Science of The Total Environment*, 662:246 – 253.

Reineke, J. J., Cho, D. Y., Dingle, Y.-T., Morello, III, A. P., Jacob, J., Thanos, C. G., and Mathiowitz, E. (2013). Unique insights into the intestinal absorption, transit, and subsequent biodistribution of polymer-derived microspheres. *PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF THE UNITED STATES OF AMERICA*, 110(34):13803–13808.

Setälä, O., Fleming-Lehtinen, V., and Lehtiniemi, M. (2014). Ingestion and transfer of microplastics in the planktonic food web. *ENVIRONMENTAL POLLUTION*, 185:77–83.

Sharma, V., Anderson, D., and Dhawan, A. (2012). Zinc oxide nanoparticles induce oxidative DNA damage and ROS-triggered mitochondria mediated apoptosis in human liver cells (HepG2). *APOPTOSIS*, 17(8):852–870.

Sies, H. (1997). Oxidative stress: Oxidants and antioxidants. *EXPERIMENTAL PHYSIOLOGY*, 82(2):291–295.

Sies, H. and Cadenas, E. (1985). Oxidative stress - damage to intact-cells and organs. *PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY OF LONDON SERIES B-BIOLOGICAL SCIENCES*, 311(1152):617–631.

Spitsbergen, J. M. and Kent, M. L. (2003). The state of the art of the zebrafish model for toxicology and toxicologic pathology research—advantages and current limitations. *Toxicologic Pathology*, 31(1_suppl):62–87. PMID: 12597434.

Stohs, S. and Bagchi, D. (1995). Oxidative mechanisms in the toxicity of metal ions. *Free Radical Biology and Medicine*, 18(2):321 – 336.

Storey, K. (1996). Oxidative stress: Animal adaptations in nature. *BRAZILIAN JOURNAL OF MEDICAL AND BIOLOGICAL RESEARCH*, 29:1715–33.

Tkachenko, H., Kurhaluk, N., Grudniewska, J., and Andriichuk, A. (2014). Tissue-specific responses of oxidative stress biomarkers and antioxidant defenses in rainbow trout Oncorhynchus mykiss during a vaccination against furunculosis. *FISH PHYSIOLOGY AND BIOCHEMISTRY*, 40(4):1289–1300.

Valko, M., Rhodes, C., Moncol, J., Izakovic, M., and Mazur, M. (2006). Free radicals, metals and antioxidants in oxidative stress-induced cancer. *Chemico-Biological Interactions*, 160(1):1 – 40.

Van Cauwenberghe, L., Vanreusel, A., Mees, J., and Janssen, C. R. (2013). Microplastic pollution in deep-sea sediments. *ENVIRONMENTAL POLLUTION*, 182:495–499.

Volkheimer, G., Schulz, F., Aurich, I., Strauch, S., Beuthin, K., and Wendlandt, H. (1968). Persorption of particles. *DIGESTION*, 1(2):78+.

Wen, B., Jin, S.-R., Chen, Z.-Z., Gao, J.-Z., Liu, Y.-N., Liu, J.-H., and Feng, X.-S. (2018). Single and combined effects of microplastics and cadmium on the cadmium accumulation, antioxidant defence and innate immunity of the discus fish (symphysodon aequifasciatus). *Environmental Pollution*, 243:462 – 471.

Zitka, O., Skalickova, S., Gumulec, J., Masarik, M., Adam, V., Hubalek, J., Trnkova, L., Kruseova, J., Eckschlager, T., and Kizek, R. (2012). Redox status expressed as GSH:GSSG ratio as a marker for oxidative stress in paediatric tumour patients. *ONCOLOGY LETTERS*, 4(6):1247–1253.

Appendix A

Statistical analyses

Table A1

One-way analysis of variance of protein concentration by treatment groups.

	df	*SS*	*MS*	*F*	*p*
Between groups	5	0.2848	0.05696	0.899	.498
Within groups	24	1.525	0.06336		
Total	29	1.8053			

Note. *df* = degrees of freedom, SS = sum of squares, MS = mean square, F = F value, *p* = *p* value.

Table A2

One-way analysis of variance of total cellular glutathione concentration by treatment groups.

	df	*SS*	*MS*	*F*	*p*
Between groups	5	1.052	0.2103	0.493	.778
Within groups	24	10.245	0.4269		
Total	29	11.297			

Note. *df* = degrees of freedom, SS = sum of squares, MS = mean square, F = F value, *p* = *p* value.

Table A3

One-way analysis of variance of GSH concentration by treatment groups.

	df	*SS*	*MS*	*F*	*p*
Between groups	5	0.6046	0.12092	2.164	.0921
Within groups	24	1.3412	0.05588		
Total	29	1.9458			

Note. *df* = degrees of freedom, SS = sum of squares, MS = mean square, F = F value, *p* = *p* value.

Table A4

One-way analysis of variance of GSSG concentration by treatment groups.

	df	*SS*	*MS*	*F*	*p*
Between groups	5	0.164	0.0328	0.074	.996
Within groups	24	10.649	0.4437		
Total	29	10.813			

Note. *df* = degrees of freedom, SS = sum of squares, MS = mean square, F = F value, *p* = *p* value.

Table A5

One-way analysis of variance of GSH/GSSG ratio by treatment groups.

	df	*SS*	*MS*	*F*	*p*
Between groups	5	8774	01755	1.019	.428
Within groups	24	41315	1722		
Total	29	50089			

Note. *df* = degrees of freedom, SS = sum of squares, MS = mean square, F = F value, *p* = *p* value.

Table A6

Kruskal-Wallis rank sum test of GR concentration by treatment group

χ^2 = 5.2323, *df* = 5, p = .3882

Note. χ^2 = Kruskal-Wallis chi squared, *df* = degrees of freedom, *p* = *p* value.

Table A7

One-way analysis of variance of GST activity by treatment groups.

	df	*SS*	*MS*	*F*	*p*
Between groups	5	9672374	1934475	1.21	.334
Within groups	24	38300671	1595861		
Total	29	47973045			

Note. *df* = degrees of freedom, SS = sum of squares, MS = mean square, F = F value, *p* = *p* value.

Table A8

One-way analysis of variance of GPx activity by treatment groups.

	df	*SS*	*MS*	*F*	*p*
Between groups	5	331537	66307	0.35	.878
Within groups	24	4568027	190334		
Total	29	4899564			

Note. *df* = degrees of freedom, SS = sum of squares, MS = mean square, F = F value, *p* = *p* value.

Appendix B

Fish biometrics

Table B1

Weights (g) before and after exposure, and lengths of fish (cm).

Aq no	Treatment	WBE 1	WBE 2	WBE 3	WAE 1	WAE 2	WAE 3	WD 1	WD 2	WD 3	L 1	L 2	L 3	FC
1	PVC	231	203	156	200	184	136	-31	-19	-20	30	29	27	2
2	0,2 PS	285	401	265	256	372	244	-29	-29	-21	32	35	32	1
3	0,2 PS	273	222	407	256	184	346	-17	-38	-61	32	29	34	1
4	20-25 LDPE	270	181	264	244	164	238	-26	-17	-26	31	29	31	1
5	4-6 LDPE	251	272	215	226	234	190	-25	-38	-25	30	31	30	3
6	PVC	334	215	244	298	208	218	-36	-7	-26	33	30	31	2
7	20-25 LDPE	251	292	250	242	256	204	-9	-36	-46	30	30	30	1
8	Control	215	160	240	NA	122	190	NA	-38	-50	NA	26	29	3
9	Control	317	154	196	286	124	NA	-31	-30	NA	31	25	NA	1
10	4-6 LDPE	292	231	386	274	210	348	-18	-21	-38	31	24	36	1
11	PVC	239	197	380	218	172	332	-21	-25	-48	30	29	32	2
12	0,2 PS	262	200	273	228	180	240	-34	-20	-33	30	30	31	3
13	0,2 PS	224	299	202	192	264	166	-32	-35	-36	29	32	25	1
14	125-500 LDPE	265	305	264	238	282	230	-27	-23	-34	32	29	31	1
15	Control	357	190	222	328	170	214	-29	-20	-8	34	28	31	3
16	4-6 LDPE	280	197	266	238	178	232	-42	-19	-34	32	29	31	1
17	0,2 PS	178	234	236	146	206	208	-32	-28	-28	27	31	28	3
18	20-25 LDPE	206	140	286	190	130	246	-16	-10	-40	30	26	30	1
19	125-500 LDPE	123	211	265	92	180	246	-31	-31	-19	25	27	31	3
20	125-500 LDPE	334	303	280	308	268	238	-26	-35	-42	33	32	31	1
21	PVC	245	169	234	212	148	194	-33	-21	-40	30	27	29	1
22	20-25 LDPE	291	262	290	266	248	258	-25	-14	-32	31	31	31	1
23	20-25 LDPE	263	324	NA	236	288	NA	-27	-36	NA	31	30	NA	1
24	4-6 LDPE	290	211	398	NA	NA	306	NA	NA	-92	NA	NA	34	3
25	PVC	261	138	252	236	NA	228	-25	NA	-24	34	NA	31	3
26	125-500 LDPE	308	266	287	274	248	NA	-34	-18	NA	34	29	NA	2
27	4-6 LDPE	171	296	251	154	272	234	-17	-24	-17	27	33	32	3
28	Control	357	271	136	324	242	126	-33	-29	-10	33	31	26	3
29	Control	255	222	200	NA	198	194	NA	-24	-6	NA	30	30	3
30	125-500 LDPE	270	220	170	256	208	160	-14	-12	-10	33	29	29	1

Note. Aq no = aquarium number, WBE = weight before exposure, WAE = weight after exposure, WD = weight difference, L = length, FC = fish chosen for sampling. NA = Not available; fish were removed during the exposure because of bad health/death.