Exposure of artificial turf granulate - how does microplastic affect the intestinal function of rainbow trout?

En utredning av tarmfunktionen hos regnbågslax efter exponering av konstgräsgranulat

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Preface

This Master thesis in Marine Ecology has been conducted at the Department of Biology and Environmental Science (NMIL) at the University of Gothenburg by Sara Ottosson. The study took place between March to August and includes 30 credits.

I would like to thank Bethanie Carney Almroth support, patience, wise thoughts and above all, all the time you gave me. I would also like to thank Henrik Sundh, Ida Hedén and Jari Parkkonen for all the help.

Sara Ottosson, Göteborg, augusti 2016

Abstract

The ongoing pollution of our lakes, oceans and rivers has been a hot topic for some time. For the last decade, there has been a discussion about microlitter, including microplastics and what consequences these small particles can have in our aquatic environment and organisms. Microlitter end up on our environment for many reasons, the decomposition of larger plastic objects that occur in the environment is one of them. But the plastic may also occur due to the emissions of hygiene- and household items, and anthropogenic waste from industrial processes.

A year ago, the City Council of Gothenburg prohibited purchase of new microplastic-products that can contaminate aquatic environments. Previously purchased products are likely to be retained but will be phased out over time and replaced with products without the content of microplastics. Unfortunately, plastic is generally a very persistent material and the degradation time can sometimes extend over 100 years. The degradation varies and depends on the chemical structure, weathering and UV exposure of the plastic.

The versatility of plastic material has led to its increased use in additional applications, including artificial turf fields where it is used as a filling material to provide structure and stability. IVL (Swedish Environmental Research Institute) estimates that artificial turf is the second largest emission source of micro plastics that is contributing to the environmental pollution. The abundance of plastic therefore gives us reason enough to examine what consequences this may cause.

This master's project of 30 credits has been implemented at the Department of Zoology at the University of Gothenburg. The aim of the thesis was to investigate whether the rubber granulate used in artificial turf (EPDM plastic) affect the intestine of rainbow trout *(Oncorhynchus mykiss)*. Previous studies show that microplastics can cause damage to the intestines of fish, but few studies have investigated the EPDM-rubber specifically. The thesis was test by feeding rainbow trout with rubber granulate from artificial turf. The fish were exposed for seven days. Intestinal tissues were analysed by proven methods. The results showed no significance between the groups, however, there is a tendency of differences. The exposed group shows a tendency that the intestinal epithelium is affected. Also the activity of the Na + / K + -ATPase decreased slightly in the exposed group. There was also a tendency to increased oxidative stress by increasing levels of glutathione in the exposed group. The non-significant can be a result of short exposure time. Previous studies show that fish exposed to PVC showed signs of changes in the intestine after 30 days. It is therefore proposed for future similar studies that the exposure time should be extended.

Sammanfattning (summary in Swedish)

Den pågående föroreningen av våra sjöar, hav och vattendrag har länge varit ett hett ämne. Det senaste decenniet har vi även öppnat diskussionen kring mikroplaster och vilka konsekvenser denna lilla partikel orsakar vattenlevande djur. Orsakerna till att mikroplast förekommer i miljön är många, dels kan det bero på en nedbrytningsprocess av större plastföremål som förkommer i miljön. Men det kan också bero på utsläpp av stora mängder avfall av antropogent ursprung från industriella processer, men även från hygien- och hushållsartiklar.

För ett år sedan förbjöd kommunfullmäktige i Göteborgs stad inköp av produkter som innehåller mikroplaster. Produkter som införskaffats före detta kommer sannolikt att behållas men sedan fasas ut och ersättas med produkter utan innehåll av mikroplast. Dessvärre är plast generellt ett mycket persistent material och nerbrytningstiden kan ibland sträcka sig över 100 år. Nedbrytningstiden varierar dock och beror bland annat på plastens kemiska struktur, vittring och UV-exponering.

Plastens många användningsområden och diversitet har under en tid tillämpats på konstgräsplaner där den används som ett fyllnadsmaterial för att ge struktur och stadga. IVL (Svenska Miljöinstitutet) uppskattar att konstgräsplaner står för den näst största uppsläppskällan av mikroplaster till miljön. Abundansen av plastpartiklar i miljön ger oss därför skäl nog att undersöka vilka konsekvenser detta kan orsaka (Magnusson, et al., 2016).

Detta mastersarbete om 30 hp har genomförts på Zoologiska institutionen på Göteborgs universitet. Syftet med uppsatsen var att utreda huruvida gummigranulatet från konstgräsplaner (EPDM-plast) påverkar tarmen hos regnbågslax. Tidigare studier visar att mikroplaster kan orsaka skador på tarmen hos fiskar, men få studier har utrett EPDM-plasten specifikt. För att bepröva detta matades regnbågslaxar med gummigranulat från en konstgräsplan i centrala Göteborg. Fiskarna exponerades under sju dagar. Tarmvävnaderna analyserades genom beprövade metoder, bland annat mättes barriärfunktionen i tarmepitelet i en Ussing kammare genom att följa sockerarten mannitol och aminosyran lysins transportväg genom epitelet. Den exponerade gruppen jämfördes sedan med en kontrollgrupp. Resultatet visar ingen signifikans mellan grupperna, dock finns det tendenser till skillnader. Den exponerade gruppen påvisar en tendens till påverkan av tarmepitelet genom ett både mer genomsläppligt och en trängre vävnad, beroende på molekylernas transportväg. Man såg också att aktiviteten av NA+/Ka+-ATPas sjönk något hos den exponerade gruppen. Det fanns

även en tendens till ökad oxidativ stress genom ökad glutationhalt hos den exponerade gruppen. Det icke-signifikanta resultatet kan bero på att exponeringstiden var för kort. Tidigare studier visar att fisk som utsatts för PVC-plast visade tecken på förändringar i tarmen först efter 30 dagar. Därför föreslås för framtida likande studier att exponeringstiden bör förlängas.

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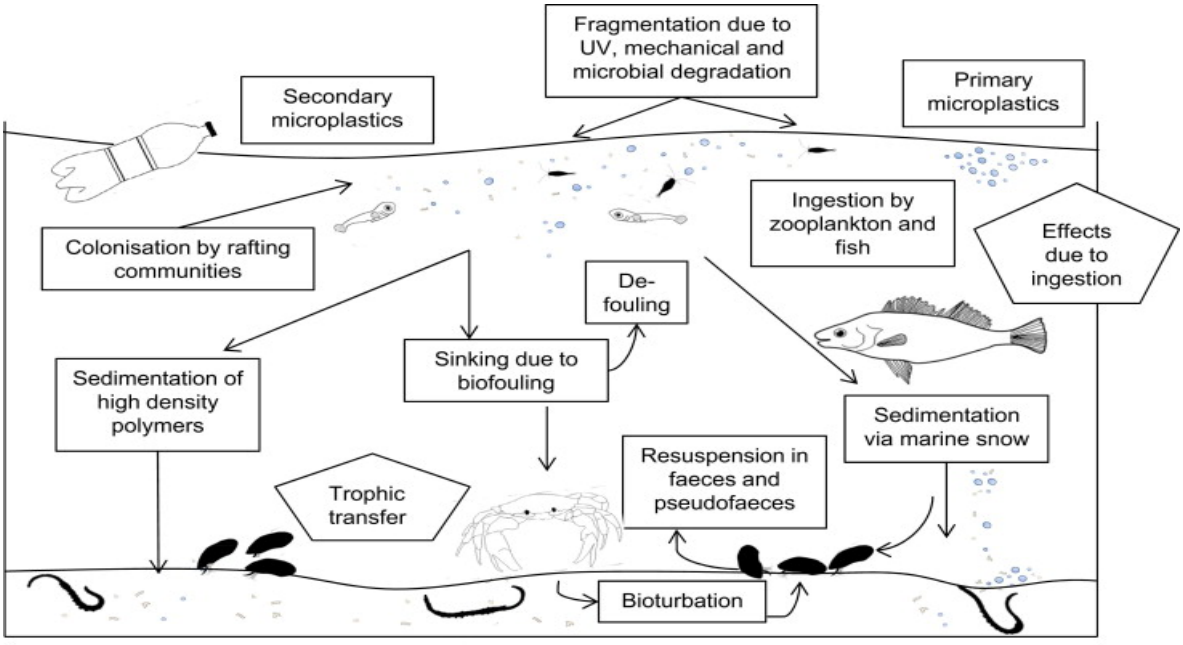
1 Introduction

1.1 Marine litter

The pollution of the marine environment comes in many forms, almost all of them from anthropogenic origin. Besides huge environmental problems like eutrophication, overexploitation, harvesting and global warming, the pollution of marine litter is nowadays a common problem, and one of the major ones (Derraik, 2002). According to European Union's Marine Strategy Framework Directive (2008/56/EC) is marine litter in our environment described as *"Marine litter is any persistent, manufactured or processed solid material discarded, disposed of or abandoned in the marine and coastal environment. Marine litter consists of items that have been made or used by people and deliberately discarded or unintentionally lost into the sea and on beaches including such materials transported into the marine environment from land by rivers, draining or sewage systems or winds"* (Gangani, et al., 2010). Marine littering has been a subject of concern for decades, but in recent time, attention has been directed towards microlitter and especially microplastics occurring in our aquatic environment. Microplastics are small fragments of any source of plastics, origin from either manufactured products (primary microplastics), or as a result of degradation of macroplastics (secondary microplastics). The degradation arise from UV-radiation, mechanical and microbial decomposition (Wright, et al., 2013), and oxidation (Lusher, et al., 2013).



Figur 1. Potential pathways of microplastics. Picture from http://www.sciencedirect.com.ezproxy.ub.gu.se/science/article/pii/S0269749113001140

The abundances of microplastics in our oceans affect all trophic levels. Due to their tiny particle size (5mm-1nm), microplastic can be ingested by a lower trophic fauna, thus affecting higher trophic levels if the particles have the potential for biomagnification (Lusher, et al., 2013), (Wright, et al., 2013). According to a study by Wright, et al. from 2013, even organisms at the base of the food web can absorb particles. Wright, et al., stated that nanoparticles (20 nm) could be absorbed by the fresh- and seawater algae Chlorella and Scenedesmus (Wright, et al., 2013). The ingestion of plastic particles has been found in both pelagic and demersal fish taxa. In a study from 2013, synthetic polymers in order of 0.13 mm-14.3 mm were found in 36,5% in a total of 504 fish examined (Lusher, et al., 2013). This indicates that the marine litter can be moved by great distance and cause damage even where humans are not normally present.

Microplastics can cause harmful effects in fish and other aquatic organisms. Beside the plastic toxicity per se, plastics have the ability to absorb chemical substances present in water due to its hydrophobic surface. This allows highly concentrated substances to be absorbed by aquatic organisms. A recent study have shown that chemical substances like PAHs, PCBs and DDT can be found in harbour sediment, which is a common pathway for microlitter in the ocean that originally comes from land (Peda, et al., 2016). In a study from 2013, fish of Japanese medaka (*Oryzias latipes*) were fed with both virgin polyethylene, respectively polluted polyethylene sorbed in marine environment for two months. The result showed hepatic stress respectively liver toxicity and pathology in the fish (Rochman, et al., 2013). Another study of Rochman et al., from 2014 showed that both virgin and polluted polyethylene could cause endocrine disruption in fish (Rochman et al., 2014).

This year, a group of scientists presented preliminary results showing that groups of fish (Sea bass, *Dicentrarchus labrax*) exposed to PVC (polyvinyl chloride) for 30, 60 and 90 days causing devastating damage in the intestinal tract that increased with time (Peda, C, et al., 2016). When Peda, et al., investigated the intestinal responses they saw 67% of the fish fed with native PVC already after 30 days showed moderate structural alterations. The fishes showed damage in the distal intestine like swelling and shortening of microvilli, widening of lamina propria (mucosal layer under the basal membrane and epithelial cells) and vacuolation of enterocytes. By a third group, exposed to polluted PVC, showed 83% of the fishes pronounced alterations within the same period of time. This group had severe damage in the distal intestine. Peda, C, et al., could observe that the epithelial layer and lamina propria appeared slightly detached from each other.

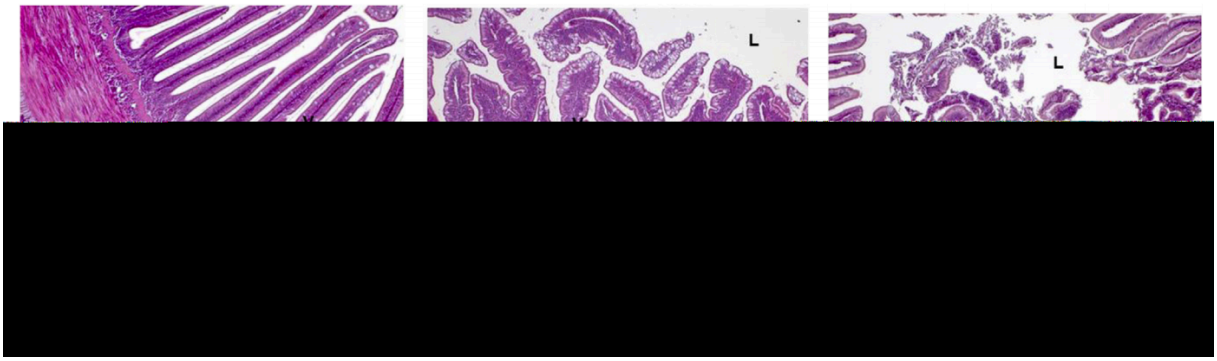


Figure 2. The figure shows a transverse section of the distal intestine of Dicentrarchus labrax after 30 days of exposure of PVC-microplastics (Polyvinyl chloride). From the left, the three groups of fish; Control group, MPV (Native microplastics) and MPI (Polluted microplastics), the damages are shown. Image taken from: http://ac.els-cdn.com.ezproxy.ub.gu.se/S0269749116300835/1-s2.0-S0269749116300835-main.pdf?_tid=a8dd0862-5c9d-11e6-a71c-00000aacb35f&acdnat=1470574469_684270e52a1b0738f7499f23d3f19c58 (Peda, C, et al., 2016).

After 60 of exposure, Peda, et al., could observe damages like beheading of villi and evident detachment of epithelial layer and lamina propria in the distal intestine. Further, after 90 days of exposure, 50% of the fishes in both groups showed severe alterations in the distal intestine like loss of structural serosa. Thus, the severity of the damage in the intestine tract increases gradually with time.

1.2 Artificial turf

Today's use of artificial turf can be counted into several areas. Aside from sport and play areas, we plan for turf areas within housing, gardens and indoor areas. In 2012, the number of sport fields in Sweden using artifical turf was counted to 861. There are several advantages of using artificial turf: maintenance costs are reduced, and the playing season is extended. Moreover, the risk of sports injury is reduced due to shock absorbing function of the rubber. Lately, studies has shown that the rubber granulate used in artificial turf can be hazardous for both humans and aquatic fauna. Although several studies show that the probability of causing health risk in humans is relatively small, it is known that aquatic organisms can be affected in toxic or sublethal ways. To clarify what and how the artificial turf is causing an environmental issue, one can also address the composition and the content of it. The principle of the composition is mostly the same, the plastic blades of grass consist of thermoplastics, usually polyethylene or polypropylene, whereas the granulate, used for provide stability to the plastic blades, consisting of recycled pieces of automobile tires or synthetic polymers. A progress report from Swedish Chemicals Agency (Kemikalieinspektionen) stated 2006 that Sweden holds 90% artificial turf containing recycled rubber from car tires, and the US has been using recycled tires in this matter since 1960's.

Several studies have shown the toxicity of car tires with its contents and leachate of PAH (polycyclic aromatic hydrocarbons), metals, phthalates, antioxidants and VOCs (volatile organic compounds). When measuring metal content in car tire rubber, one common result from a variety of studies is the considerably high concentration of zinc, whereas other metal compounds varying widely depending on granulate type (Menichini, et al., 2011; Bocca, et al., 2008). A survey from 2007 showed that high concentrations of zinc from tire rubber was the main reason for toxicity to *Ceriodaphnia dubia* (Wik, A., 2007). Another considerable by-product is the content of PAHs, which is a well-known carcinogenic and mutagenic compound, both harmful to the environment and humans. A study from 2004 investigating the potential uptake of PAHs from car tires in rainbow trout (*Oncorhynchus mykiss*) showed that there is a leachate of PAHs, followed by metabolism of the substance in the fish (Stephensen et al. 2003). PAHs are also known to cause heptatic preneoplams and neoplams in several fish species when metabolites forms (Aas, et al., 2000). Therefore, this should be a subject of concern.

As knowledge concerning the toxicity of car tires rubbers increase, we aim to develop environmentally friendly, or at least less toxic granulate for this cause. One widely used plastic is the synthetic polymer EPDM (Ethylene propylene diene monomer). In addition to using EPDM-granulate as an in-filler between plastic grass blades, it also can be utilized as seal, in vehicle applications, or in aquaculture as tank or pond liner, just to mention a few (Horowitz et al, 2000). The components of EPDM are carbon black, ethylene-ethylidenenorbomene-propylene terpolymer, ethylene-propylene copolymer, polyalkylbenzene, zinc oxide, 1,2-polybutadiene, modified clay, 1H-pyrrole-2,5-dione, 1,1'-(1,3-phenylene)bis- and peroxide.

Table 3. The table shows what EPDM plastic consists of. Substances that are marked with † are according to OSHA/WHMIS hazardous.

Chemical Name	**CAS-No.**	**Concentration***
†Carbon black	1333-86-4	< 31%
Ethylene-ethylidenenorbomene-propylene terpolymer	25038-36-2	< 26%
Ethylene-propylene copolymer	9010-79-1	< 26%
Polyalkylbenzene	None	< 6%
†Zinc oxide	1314-13-2	< 3%
1,2-Polybutadiene	9003-17-2	< 2%
Modified clay	66402-68-4	< 2%
†1H-Pyrrole-2,5-dione, 1,1'-(1,3-phenylene)bis-	3006-93-7	< 1.5%
†Peroxide, [1,3(or 1,4)-phenylenebis(1-methylethylidene)]bis[(1,1-dimethylethyl)	25155-25-3	< 1.5%

* All concentrations are percent by weight unless ingredient is a gas. Gas concentrations are in percent by volume. † This chemical is hazardous according to OSHA/WHMIS criteria.

One can speculate that its popularity on the market today is due to its high resistance against weather, oxygen, ozone and heat. The thermal properties of EPDM indicate a tolerance rate between -50°C to 150°C. Additives like antioxidants, fillers and plasticizers are used in the process to provide better technical characteristics of the rubber. As shown in the table above, chemicals are also often used in the process. Additives of chemicals have the purpose of giving stability to the final product. These chemicals, along with the EPDM-rubber, can unfortunately be diffused and cause damage to aquatic animals. Horowitz, et al., investigated the role of EPDM in a super intensive, recirculating production system where farmers cultivated shrimp *(Litopenaeus setiferus)*. EPDM was used as liner in the shrimp pond. A control pond without the EPDM liner was put up. Within two weeks, all of the shrimps in the EPDM pond had died, while the survival rate in the EPDM-free pond was 100%. One possible hypothesis of the result by Horowitz et al could be the toxicity of the chemicals used in production, which leaked into the pond and showed to be highly toxic to the shrimp (Horowitz., et al, 2000).

One of the potential advantages of using EPDM-rubber rather than recycled car tire rubber as granulate in artificial turf is the absence of PAH as described previously. When the Swedish Chemicals Agency (Kemikalieinspektionen) investigated the content from (i) recycled rubber (divided into fine-grained and coarse granulate) and (ii) newly manufactured EPDM-granulate, it showed that the leakage of PAH from recycled rubber measured to 62-76 mg PAH/kg. Leakage from new granulate measured to 1-1.3 mg PAH/kg, depending on the source.

A notable result from the study was the content of zinc and chromium. It turned out that the content of zinc in newly manufactured granulate exceeded the recycled fine-grained rubber. In the case of chromium, newly manufactured granulate exceeded both particle sizes of recycled rubber. Several studies show that high concentrations of zinc can be harmful to aquatic biota (Gualtieri et al. 2005). Swedish Chemicals Agency (Kemikalieinspektionen) also investigated the release of zinc and chromium from rubber, in the same progress report as previously mentioned. The test did include (i) recycled rubber (divided into fine-grained and coarse granulate) and (ii) newly manufactured EPDM-granulate. It was shown that zinc released from fine-grained rubber-granulate contained 7300 mg/kg, whereas EPDM-granulate contained 9500 mg/kg. The release of chromium was measured to less than 2 mg/kg in recycled rubber, whereas the leachate from EPDM-granulate showed to be as high as 5200 mg/kg.

1.3 The gastrointestinal tract in fish

Anatomically, the gastrointestinal tract of rainbow trout is built up of esophagus, pyloric sphincter, stomach, proximal intestine, distal intestine and rectum (Olsson, C., 2011). This study will keep its focus mainly to the proximal part of the intestine.

The functions of the fish intestine are several. Besides the most obvious functions as digestion of dietary intake and nutrition absorption, the intestine also helps maintain osmosis, ion regulation functions and buoyancy. Also, the intestine act as a barrier to the outward environment, e.g. as a defence against pathogens and is part of the immune system. (Di Giulio, R. T. & Hinton, D. E., 2008) (Olsson, C., 2011). The epithelial tissues are not homogeneous alike through the intestinal regions of fish, thus, the function as a defence barrier varies. The barrier function differs between the paracellular permeability and the endocytotic activity in intestine regions. The paracellular permeability is high, while the endocytotic activity is low in the proximal intestine, and vice versa in the distal intestine. The high paracellular permeability in the proximal intestine results in a reduction of tight junctions and thus, a higher nutrient uptake. Along the intestinal tract, the densities of tight junctions increase. Thus, the permeability and formation of tight junctions can determine the resistance of an epithelial tissue (He, L. et al. 2013). The need for tight junctions can be a reflection of bacteria and bacteria toxins present in the gastrointestinal tract (Olsson, C., 2011).

1.4 Osmoregulation

In order to maintain normal functions of fish requires a working osmoregulation. The flow of water and ions that control and maintain the osmolality differ between freshwater fish and seawater fish. Fish blood plasma has an osmolality about 300 to 325 mOsmol L^{-1}, regardless to the medium they live in. However, freshwater holds an osmolality less than 5 mOsmol L^{-1}, while seawater holds a high osmolality of 950 to 1050 L^{-1}. In practice, this means that the movement and diffusion of water and ions will look different due to water osmolality. In fresh water fish, there is a constant passive loss of ions and an uptake of water. In saltwater fish, however, there is a constant inflow of ions and a water loss to deal with. To help maintain these functions in order, fish use the gills, skin, kidneys and intestine to control the flows. Active ion exchange processes freshwater fish, located in the gills and intestine control the ion balance. Therefore, the gills and intestine is of utmost importance regarding osmoregulatory function (Di Giulio, R. T., & Hinton, D. E., 2008). A disruption in these functions would be hazardous for the fish. Dietary toxic agents like metals (T. Di Giulio, Richard & E. Hinton,

David, 2008) and synthetic polymers (EPDM) (Horowitz et al, 2000) will affect the gills and intestine of the fish with consequences like disrupted Na+/K+ATPase activity and by that, an interference of the osmoregulatory system (Di Giulio, R. T., & Hinton, D. E., 2008). As we know, the Na+/K+ATPase pump is of importance for cell physiology. In a study from 1988, an increase in Na+/K+ATPase activity was detected in rainbow trout (*Oncorhynchus mykiss*) when exposed to hexavalent chromium, hence, the activity was disrupted (Boge, et al., 1988). As mentioned before, when the Swedish Chemicals Agency (Kemikalieinspektionen) investigated the content of chromium in EPDM-granulate, it showed to be of a high value.

1.5 Oxidative stress

Fish, as other organisms, are very dependent on the prevailing factors that must comply with its homeostatic mechanisms and preferences. If these crucial factors are not present, fish will be affected and stressed. In addition to abiotic factors such as abnormal water temperature, oxygen and salinity, the fish may also be affected by pollution from human or natural origin in sufficient concentrations (Harper, C., & Wolf, J. C., 2009). Oxidative stress can be an expression of alterations in cell function of fish exposed to alien or hazardous substances present in our environment. Oxidative stress occurs when there is an imbalance between the detoxification mechanisms present in all living organisms, and the reactive oxygen species (ROS) entering the organism. The result of oxidative stress can be shown in complex networks in metabolic and/or physiological alterations. What can be seen in tissue or organ of fish is an increased alteration of e.g. lipid peroxidation and protein carbonyl content as a result of oxidative damage. Fortunately, all living aerobic organisms of today have developed multiple systems of antioxidant defence. When fighting reactive oxygen species, antioxidants like glutathione peroxidase, superoxide dismutase, NAD(P)H: quinone oxidoreductase and catalase can help preventing the onset of oxidative stress (Rodrigues et al., 2016). The general role of the defence system of antioxidants is to transform deleterious metabolites into water-soluble products, thus creating less harmful products. A defense system that does not work properly could have devastating consequences causing damaged DNA and at worst cell death (Rodrigues et al., 2016).

1.6 Biomarkers and antioxidant defense

A biomarker is defined as he biological activity of enzymes produced when xenobiotics are undergoing metabolism in organisms can be used as biomarkers, e.g when biomonitoring a polluted area. Elevated levels of a specific substance, i.e. protein, enzyme or metabolite, give

a measurement of exposure. One example is the activity of increasing aryl hydrocarbon (AhR) agonists (Ethoxyresorufin-o-deethylase (EROD), which indicates that PAHs could have contributed to elevated levels of EROD in the organism (Albertsson, E. 2011). A measure of oxidative stress, which occurs when an imbalance between prooxidants and antioxidants, can also be sued as a biomarker. Oxidative stress is a common mechanism of toxicity and can occur when a xenobiotic compound either increased ROS production, or impairs antioxidant metabolism. This results in some kinds of detrimental biochemical or physiological effect and likely damage to the organisms' cellular molecules (Carney Almroth, B et al. 2008). Another biomarker is the amount of glutathione present organism. The antioxidant, present in all living organism and found in all tissues, plays an important roll for cellular defence and detoxification in the liver. The celluar content of glutathione can either decrese or increase in levels, or remain unchanged after exposure. Rainbow trout that was exposed to zinc for 28 days showed elevated levels of hepatic glutathione (Lange, A., 2002),

2 Aim of the study

Today's concern for microlitter, especially microplastics, in our oceans is receiving increasing attention, and there are studies that state that marine fauna is affected negatively (Derraik, et al., 2002; Rochman et al., 2014; Peda et al., 2016). Swedish Environmental Research Institute (IVL) estimates that microplastic derived from artificial turf is the second largest source of pollution of the seas (Magnusson, et al., 2016). This type of "green" areas greatly increases worldwide, thus requires more toxicological research on dissemination, uptake and impact of marine fauna.

The primary purpose of this study was to investigate the potential physiological effects in the intestine of rainbow trout when the fish were exposed to granulate from artificial turf. During the study, measurements were taken of the fish nutrient uptake through the proximal part of the intestinal. The study also examined the activity of the Na+/K+-ATPase in the intestines and gills, potential levels of PAHs and electrolytes in blood plasma.

2.1 Questions

- Is there a risk that the nutrient uptake in fish is affected when they are exposed to plastic particles via the gastrointestinal tract?

3 Method

3.1 Sampling point

Artificial turf granulate was collected at Gamlestadsvallen in Gamlestan, a north-eastern district of Gothenburg. The samples were collected randomly in the corners of the football field where the granulate had been ploughed away. Our samples were most used by sportsmen and ploughed following snow-cleanup. Samples were put in a three-litre plastic bag. The granulate was transferred to the laboratory at the department of Zoology at the University of Gothenburg. According to Göteborgs stad, the granulate at Gamlestadsvallen consisted of recycled brown EPDM-rubber.

3.2 Rainbow trout (*Oncorhynchus mykiss)*

Rainbow trout is a common fish outside the coast of Sweden. Rainbow trout is euryhaline and can adapt to freshwater, brackish water and seawater. The fish used in this study were kept in freshwater during the experiment.

Rainbow trout (*Oncorhynchus mykiss*) were obtained from Vänneåns fish hatchery outside Laholm in southern Sweden. The fish were transferred into tanks to the laboratory at the department of Zoology at the University of Gothenburg. 63 fishes acclimatized for two to three weeks in one large 300L tank with recirculating, filtered and conditioned tap water. Eight randomly selected fish of mixed sex were then put in eight separate 50-litre aquariums, with aerated, filtrated and circulated water. Temperature in the aquarium was set to 10 ± 1°C. The lightning in the room was set to 12:12 (dark/light). Sampling took place twice with a total number of sixteen fishes.

During the experiment, the fish were monitored once a day to observe and control its health. The course "Laboratory Animal Science, 3 credits" was taken before the experiment started. The course was conducted to obtain knowledge about animal welfare, humane endpoints for animal experiments and ethical principles indicated by the European Union Directive (2010/63/UE).

3.3 Laboratory work

The sampling took place twice in Tånglaken at the department of Zoology at the University of Gothenburg. Most of the laboratory work took place in "Ål-labbet" and "Fel-labbet" at the department of Zoology.

3.3.1 Time of exposure

During the last week of acclimatization, the fish were fasted for one week. The food was prepared with a mixture of 95 g pellets, 5 g EPDM-rubber and 100 ml tap water. (Control mixture; 100 g pellets, 100 ml tap water). Sixteen anesthetized (6 mg metomidate/litre water) fish (eight exposed fishes, eight control fishes) were orally fed with the pellet mixtures through a feeding tube. The feeding was performed twice within a week.

3.3.2 Preparation

At the time of sampling, eight fish of each group of treatment were euthanized with a sharp blow to the head. The fish were weighed and measured before the sampling and had an average weight of 217,9 ± 0,27 g, and an average length of 26,8 ± 7,31 cm.

Fish	Treatment	Length	Weight	Sex	Comment
1	Exposed	27,4	223,6	Male	
2	Exposed	26,9	214,8	Male	
3	Exposed	27,5	258,1	Male	Hard to catch, possibly stressed
4	Exposed	27,5	241,1	Male	
5	Control	27,2	223	Female	
6	Control	27,5	255,5	Male	
7	Control	24	157	Male	
8	Control	26,3	198,7	Female	
9	Control	28,1	236,9	Female	
10	Control	25,1	166	Male	
11	Control	28	240,9	Male	
12	Control	26	197,5	Male	
13	Exposed	28	245,2	Male	
14	Exposed	27,1	215,1	Female	Fed two days late
15	Exposed	27	215,5	Male	Fed two days late
16	Exposed	26,5	198,5	Male	
Mean		**26,88125**	**217,9625**		

After blood sampling, the body cavity was opened laterally and samples were taken from the bile with a needle, the fluid was inserted into eppendorf tubes. The samples were frozen on dry ice and stored at –80°C.

Samples were also taken from the proximal “small ”intestine and distal “large” intestine. Samples from the distal intestine was taken and then put into 500 μL formalin. After 24 h, the formalin was removed and replaced with 70% ethanol until further processing. Samples from the proximal intestine of each fish were fixed in sampling buffer intended for intestinal tissue. The samples were put in liquid nitrogen. Samples were also taken from the proximal intestine and were put into Ringer's solution on ice.

One small fragment of the gills of each fish was fixed in sampling buffer intended for gills. The sample was then transferred to a –80°C freezer.

The liver of the fishes was divided into two parts for later evaluation of GSH and enzymes and placed in liquid nitrogen.

3.3.4 Ussing Chamber

The Ussing Chamber technique is a way of measure active ion transport and the electrical resistance (R) through an epithelial barrier. This gives us information about the permeability and vitality of the membrane. Accordingly, once we have information about the permeability of a membrane, it can be classified as leaky or tight. Transepithelial transport of e.g. water and electrolytes occurs in two different pathways. Molecules can be moved transcellularly; transport is made through the apical- and basolateral surfaces of the cell. Molecules can also be moved paracellularly, where solutes are moved between cells, via tight junction barriers (Vidyasagar, S. & MacGregor, G., 2016). In this analysis, the hydrophilic marker molecules ^{14}C mannitol and lysine have been used due to their separate means of transport through the membrane. Mannitol moves through the paracellular space via passive diffusion, while lysine is transported actively. This gives an overview of the whole cellular transport functions and thus, reveals how nutrients flow through the membrane.

Sixteen fish were killed with a sharp blow to the head with a ‘priest’. After removal of the proximal intestine, tissues were placed in salmon Ringer's solution and put on ice. The measurements were run in two rounds, eight samples per session. Each sample was scraped for removal of the mucosal layer in the intestine. The apical and basolateral sides of the tissue were tensioned in a ‘plug-in’ or ‘slider’. The slider was placed in between the two chambers. The chamber system was filled with a mixture of 0.5 mM cold lysine and 30μl warm lysine (^{3}H), 150μl mannitol (^{14}C) and 32,5μl salmon Ringer's solution. The chambers were equipped with one pair of calcium chloride electrodes for current passage, and one pair of Ag/AgCl-electrodes for measurement of the trans epithelial potential (TEP), trans epithelial resistance

(TER) and short circuit current (SCC). Measurements began when exchanging the Ringer's solution in all chambers and adding 100μl fresh Ringer's solution, and the mixture of mannitol and lysine to the mucosa side at the following intervals; t0, 20, 25, 30, 60, 80, 85 and 90 minutes and t0, 20, 28, 36, 41, 71, 85, 90 minutes. (Differences in time intervals because of delay. Does not influence on the result).

3.3.5 Na+, K+-ATPase Assay of Intestinal and Gills

Before the actual testing of the intestinal and gills were done, an ADP-standard was tested in the spectrophotometer to ensure the accurate values.

Salt solution, imidazole solution, homogenization buffer, and sampling buffers (specific for intestine and gill tissues) were prepared before testing. ATPase assay mixture was prepared by centrifuging lactic dehydrogenase and pyruvate kinase. The pellet of the enzymes was put in a mixture of ATP, NADH, phosphoenolpyruvate and imidazole solution. The mixture was divided into two, where ouabain (AM+) was added to one. After samples were thawed, intestine mucosa, gills and buffer where homogenised with a glass-glass homogeniser on ice. After centrifuging the homogenate, the supernatant of each sample where put into a Nunc plate (four replicates each). With a repeater pipette, the samples were filled with 50μl salt solution. Two wells of each sample were filled with 200μl AM+ (ouabain) and remaining wells with 200μl AM- (without ouabain). The microplates were read in a wavelength of 340 nm for 11 minutes with 15 seconds interval, in a temperature-controlled spectrophotometer (25°C).

Protein determination was done by first preparing BSA reaction medium. The BSA reaction medium was mixed with distilled water into four different standard concentrations. The standard concentrations were pipetted into a Nunc plate (three of each replicate). Homogenate containing mucosa or gills were pipetted into three wells of each sample. With a repeater pipette, all of the wells were filled with 200μl BSA reaction medium. The plate incubated at 37°C for 30 minutes. After cooling for two minutes, the plate was read at a wavelength of 562 nm in a spectrophotometer.

3.3.6 Glutathione analysis

Glutathione exists in two forms: reduced and oxidized. Its antioxidant characteristics defend organisms from reactive oxygen species, and can also be used as a marker of oxidative stress.

In this analysis, concentrations of glutathione were measured in total content and oxidized form.

After thawing from liquid nitrogen, 16 liver samples were put on ice. 5% sulfosalicylic acid (SSA) was added to each sample, four times the liver weight. Samples were cut into small pieces before sonication. After 15 minutes on ice, samples were run in a centrifuge at 4°C, 10000 g (rcf) in 20 minutes. The supernatant from 16 samples was placed into 1.5 ml eppendorf tubes. The supernatant was then divided into two fractions; 16 tubes of supernatant diluted 80 times in SSA and HCI for measurement of total GSH (total glutathione). And 16 tubes of 100μl supernatant for measurement of GSSG (concentration of glutathione content including both oxidized and reduced forms). After 24 h in a –80°C freezer, samples were slowly thawed on ice. Each GSSG sample were mixed with 5μl 2-vinylpyridine and was shaken for one hour.

20 μl of each standard where pipetted in triplicates into a Nunc plate. Two plates with standards was pipetted with 20μl of respective sample (GSx and GSSG). Each well in the plate was filled with 200μl reagent solution. After five minutes, 20μl of GR-solution was added to the wells. The plates were read in a spectrophotometer at 415 nm for seven minutes. Samples were quantified against a standard curve consisting of a blank and four standards consisting of GSH (glutathione) s mixed and diluted with SSA and HCI to a final concentration of 10, 2, 5 respective 2 μM.

3.3.7 Polycyclic aromatic hydrocarbons ‘PAH’ in bile

After samples were thawed, the bile from sixteen fish was diluted 800 times each with 48% ethanol in elongated tubes. Samples were analysed trough a luminescence spectrofluorometer. The samples were measured one by one. The spectrofluorometer measures possible levels of polycyclic aromatic hydrocarbons through three different types of wavelength pair, 290/335 nm, 341/838 nm and 380/430 nm. PAHs are strongly fluorescent, and what seems to distinguish PAH compounds from each other is the size and structure of the molecule. Thus, dissimilar wavelength pairs seeks to detect specific compounds, e.g. detection of benzo*[a]*pyrine can be detected at 380/430 nm, while the naphthalene type can be detected at 290/335 nm (Aas et al. 2000). The result from this assay will show a merged value from all wavelength pairs, with a comparison between controls and exposed fishes.

3.3.8 Plasma analysis

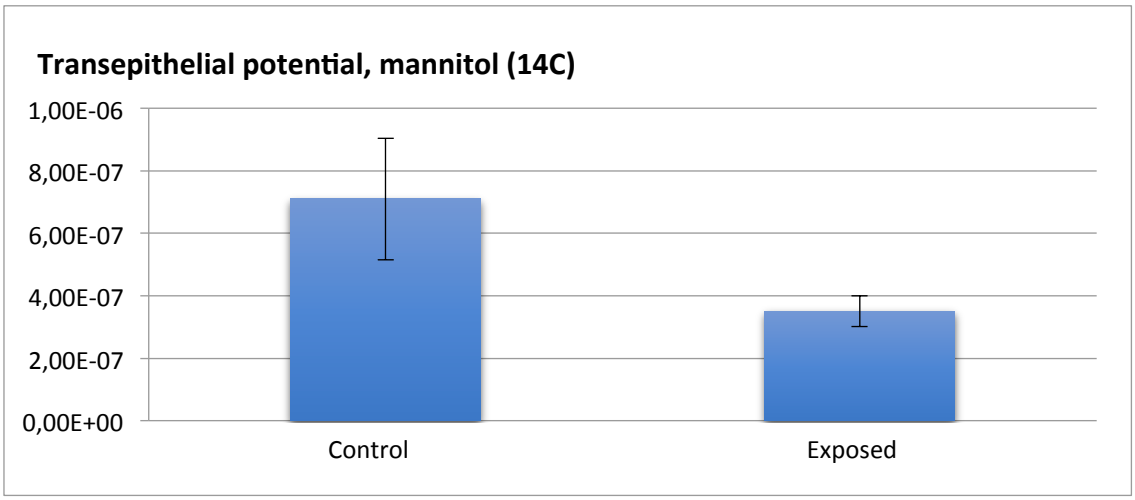
After killing of 16 fishes, samples of approximately two ml blood were taken using heparinized syringes, and then centrifuged within 30 minutes to separate blood clot and plasma. 120μl plasma from each fish were pipetted into eppendorf tubes. Samples froze down at -80°C for approximately two weeks. After thawing of samples, the electrolytes in the plasma (potassium, sodium, chloride and calcium) were read in an incubator.

4 Results

4.1 Ussing Chamber

4.1.1 Mannitol

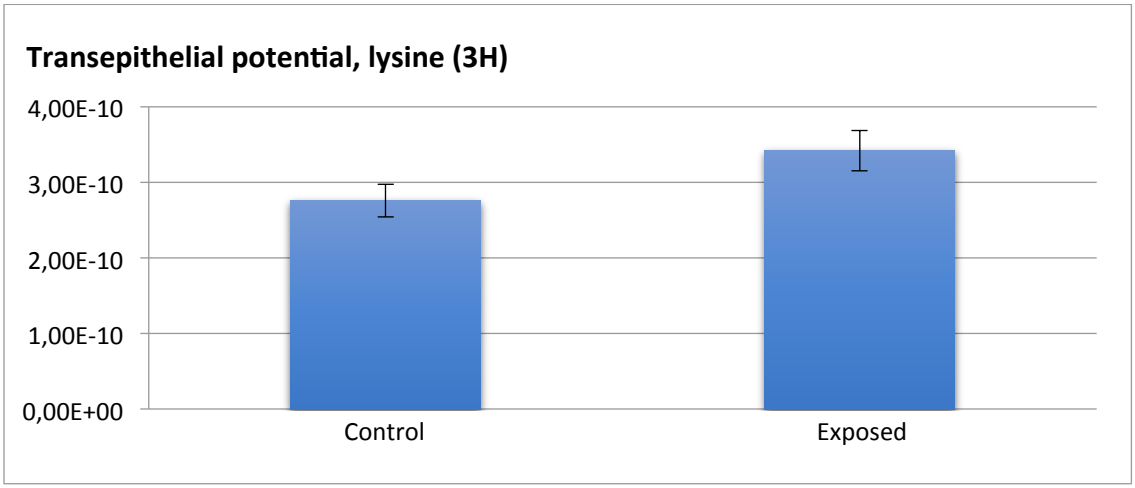
The result from the Ussing chamber analysis shows that there was a tendency of difference between the two groups. Fish exposed to EPDM-plastic granulate for seven days, compared to the non-exposed group within the same period of time, showed a decrease in TER (transepithelial potential). Although, the exposed group was not significantly affected (P = 0.095), and α was set to < 0.05.



*Figur 4. The diagram shows that there is no significant difference between the control group and the fish exposed to EPDM-plastic granulate for seven days. The y-axis are expressed in mol*min^{-1}.cm^2. All data are expressed as means* ± SEM

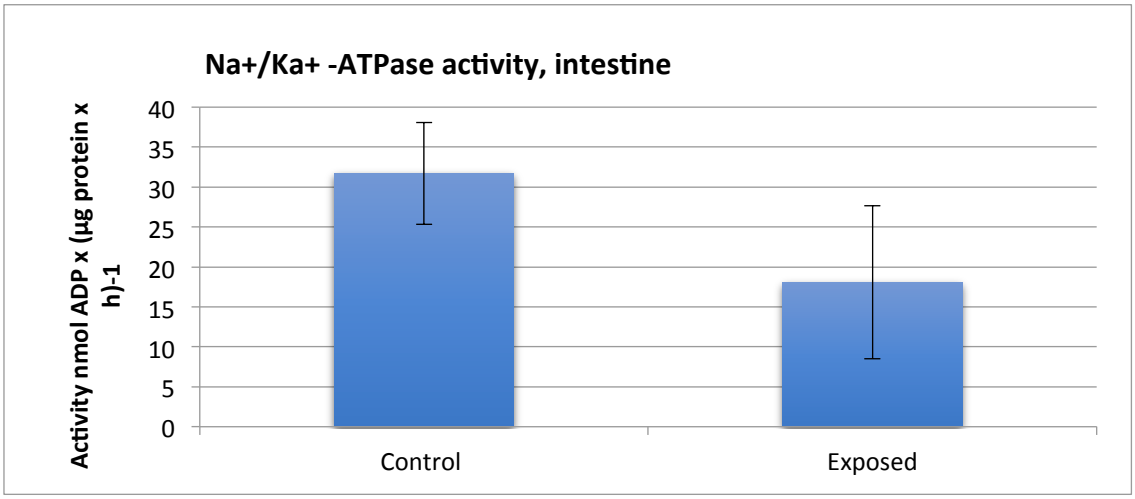
4.1.2 Lysine

The result from the Ussing chamber analysis showed that there was an increase in TER (transepithelial potential) in the group of fish exposed to EPDM-plastic granulates compared to the control group. The exposure lasted seven days. There was a tendency towards a difference between the two groups, although the difference is not significant ($P = 0.076$), and α was set to $< 0{,}05$.



*Figur 5. The diagram shows that there is no significant difference between the control group and the fish exposed to EPDM-plastic granulate for seven days. The y-axis are expressed in mol*min^{-1}.cm^{2}. All data are expressed as means* ± SEM

4.2 Na+, K+-ATPase Assay of Intestinal and Gills



Figur 6. The diagram shows that there is no significant difference between the control group and the fish exposed to EPDM-plastic granulate for seven days. The y-axis are expressed as activity nmol ADP x (µg protein x h)-1. All data are expressed as means ± SEM

Unfortunately, the result from the Na+, K+-ATPase Assay of the gills were not publishable. Most likely, there have been problems at the laboratory, e.g. pipetting errors.

4.3 Glutathione analysis

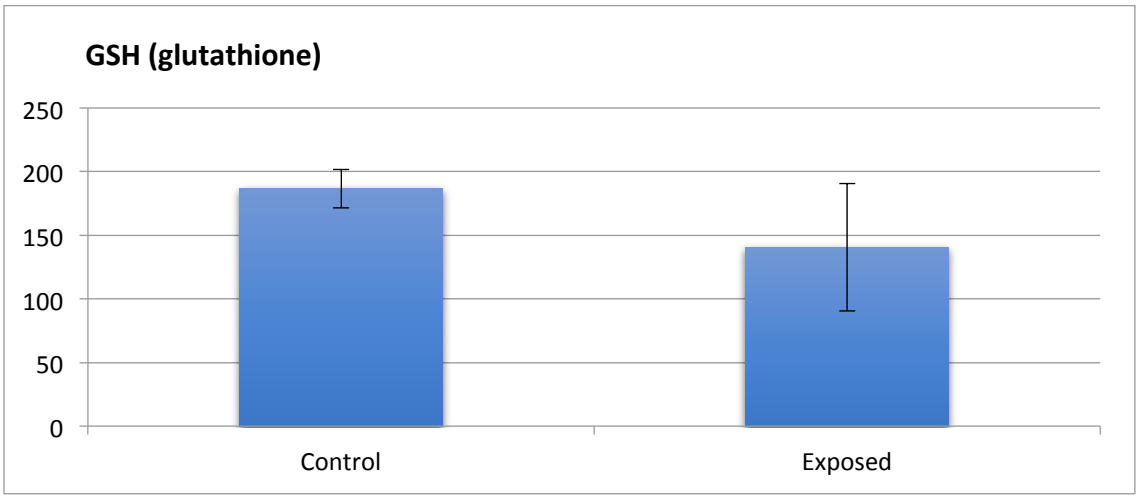


Figure 7. The amount of total glutathione in the liver of rainbow trout. The diagram shows that there is no significant difference between the control group and the fish exposed to EPDM- plastic granulate for seven days. All data are expressed as means ± SEM

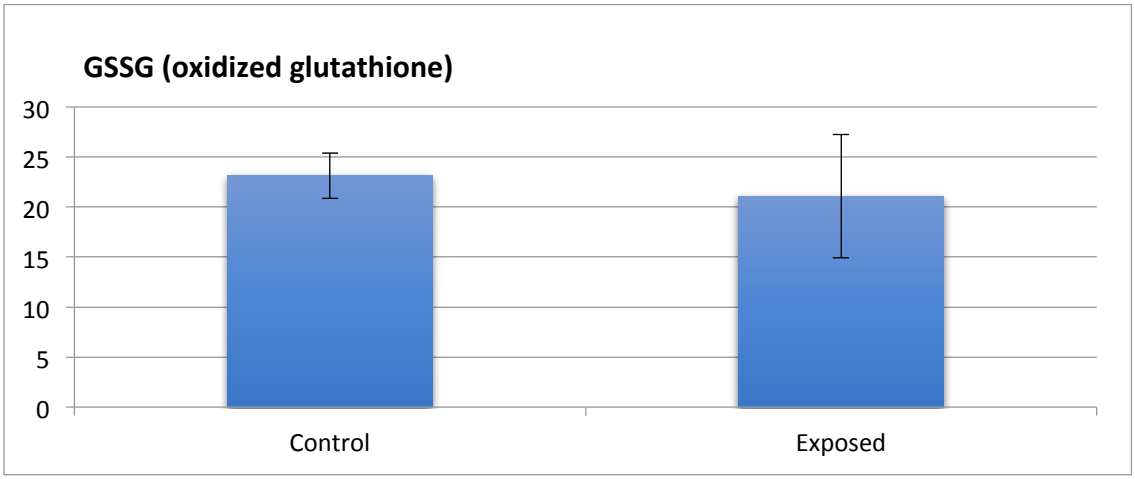


Figure 8. The amount of oxidized glutathione in the liver of rainbow trout. The diagram shows that there is no significant difference between the control group and the fish exposed to EPDM- plastic granulate for seven days. All data are expressed as means ± SEM

4.4 Polycyclic Aromatic Hydrocarbons 'PAH' in bile

The following diagrams show the screening of PAH contamination in samples, expressed in three different wavelength pairs, 290/225 nm, 341/383 nm and 380/430 nm. The difference between the control group (n=8) and fish exposed to EPDM-plastic granulate (n=8) was not

significant (p was set to < 0,05). Tests of significance (t-test) showed values of $p = 0{,}66$, $p = 0{,}63$ respective $p = 0{,}13$, values are expressed in the order of the diagrams.

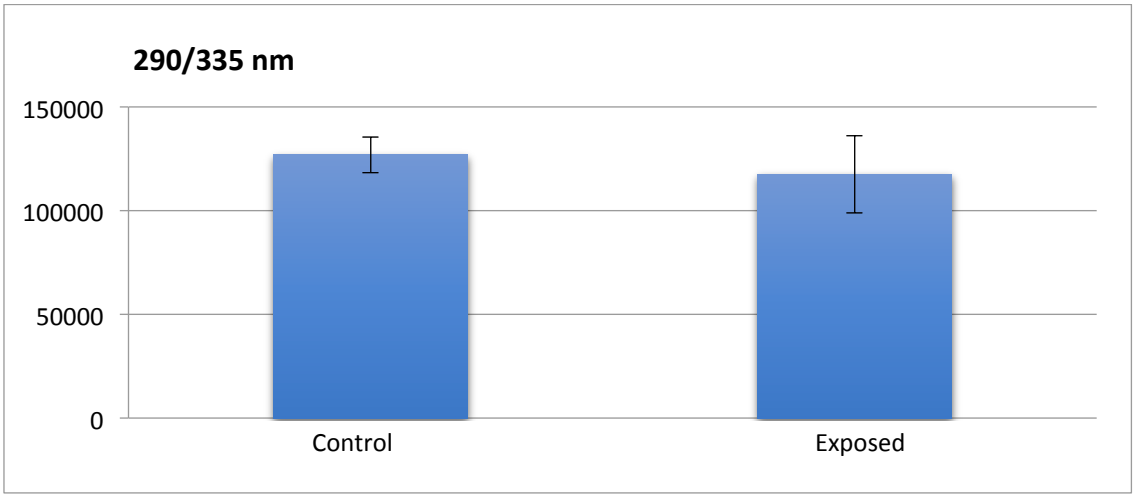


Figure 9. Content of PAH, samples were read in 290/335 nm. The diagram shows that there is no significant difference between the control group and the fish exposed to EPDM- plastic granulate for seven days. All data are expressed as means ± SEM.

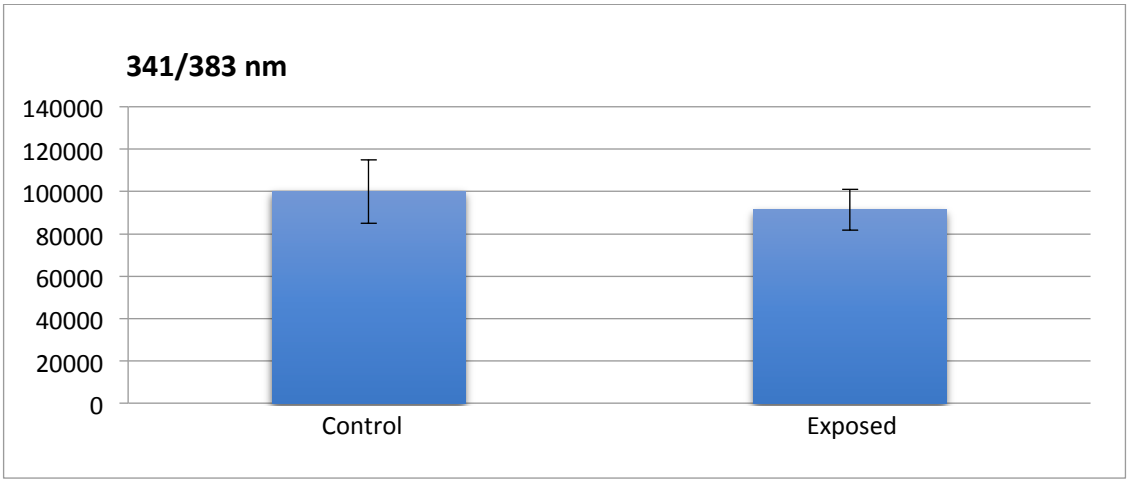


Figure 10. Content of PAH, samples were read in 341/383 nm. The diagram shows that there is no significant difference between the control group and the fish exposed to EPDM- plastic granulate for seven days. All data are expressed as means ± SEM.

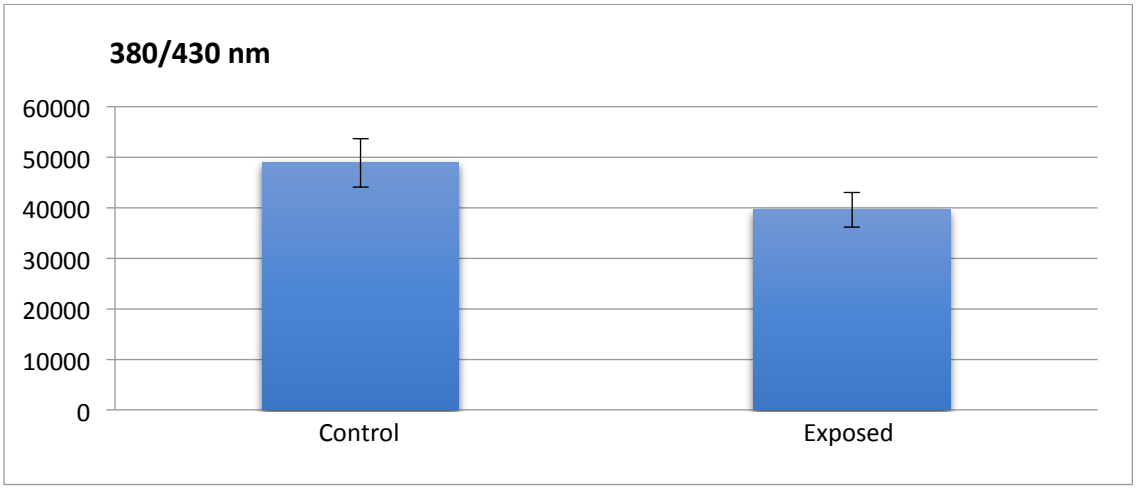


Figure 11. Content of PAH, samples were read in 380/430 nm. The diagram shows that there is no significant difference between the control group and the fish exposed to EPDM- plastic granulate for seven days. All data are expressed as means ± SEM.

4.5 Plasma analysis

The diagram shows the concentrations of potassium, sodium, chloride, calcium and pH-value in fish blood plasma. There was no significant difference between the control group (n=8) and the group of fish exposed to EPDM-plastic granulate (n=8). α was set to < 0.05.

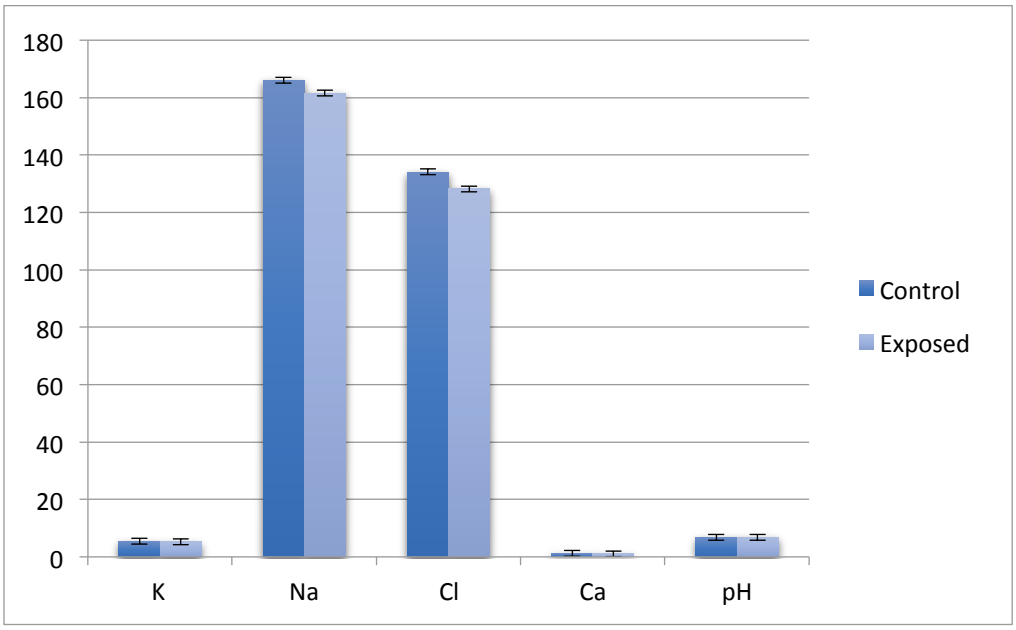


Figure 10. Content of electrolytes and pH-value in fish blood plasma. The diagram shows that there is no significant difference between the control group and the fish exposed to EPDM- plastic granulate for seven days. All data are expressed as means ± SEM.

5 Discussion

5.1 Assays

This study intended to investigate whether EPDM rubber could affect the nutrient uptake in rainbow trout (*Oncorhynchus mykiss)*. A very cautious interpretation can be made of the results, which suggest that the nutrient uptake in the exposed fish is disturbed. Also, the exposed group probably indicates tendencies to oxidative stress. The increase in GSSG levels (oxidized glutathione) can be an indication of stress.

Although the results of this study did not yield any significant results, there are many points to discuss. In this study, the experimental group of fish were fed with a mixture of fish food and EPDM-rubber. They were fed twice under a period of one week. Without knowing the quantities absorbed and accumulated by marine fauna, IVL (Swedish Environmental Research Institute) estimated the annual loss of rubber from artificial turf to 2300-3900 tonnes per year, whereas 5% consist of EPDM rubber (115-195 tons). There is no data for how much rubber that reaches the seas, and thus, affecting the fauna (Magnusson, et al., 2016). Also, the uptake in fish likely to be dependent on the density of the plastic, the density determines which zone of the ocean the plastic accumulates. The uptake in benthic and pelagic organisms are therefore probably different. Thus, we know very little about the amount of granulate to which a fish might be exposed in their naturally habitats, they are more likely to be exposed for longer periods of time in their natural environment. Without knowledge of exposure period or concentration in the gastrointestinal tract of the fish, it can be difficult to understand the effect from it that reflects reality. However, our study aimed to understand the underlying mechanisms that might be affected by exposure to EPDM rubber via diet.

A recent study by Browne, M.A., et al., showed that the blue mussel (*Mytilus edulis*) retained microplastics in their circulatory system for the entire 48 days (Browne, et al., 2008). Thus, is it hard to state whether seven days of exposure, as in this experiment, was too short or, if the EPDM rubber simply did not affect the physiology of the fish compared with the control group of fish. The experimental group of fish from this study showed a tendency ($P = 0.09$) to being affected by the microplastic in the Ussing analysis. One could see that flux of mannitol (3^H) that flowed through the paracellular space in the epithelial tissue of the intestine somehow decreased compared to the control group. This could indicate that the tight junctions that control the in- and outflow by substances in the paracellular space have become tighter

than before. As mentioned in the introduction part of this study, the paracellular permeability is naturally high in the proximal part in rainbow trout, which mean a reduction of tight junctions and thus, a higher nutrient uptake (Olsson, 2011). Also Olsson, states that the endocytotic region of the cell, that controls the active transport of in- and outflows by substances, has a lower uptake of nutrients compared to the passive transport in the paracellular space. The (non-significant) result from this study is inversely compared to what Olsson (2011) states, where we have a lower nutrient uptake in the paracellular space, and a higher nutrient uptake in the endocytotic region of the cell. Another interesting part of what Olsson (2011) writes is the fact that the need for tight junctions can be a result of intrusive bacteria toxins. Even though EPDM-plastic is not a bacteria toxin – one can hypothesize that fish intestine in this case responds to alien substances in a similar matter.

When Peda, et al., (2016), investigated the effects of PVC on the intestinal tract of Sea bass (*Dicentrarchus labrax*) they saw that the distal part of the intestine proved to be the most affected. Effects of the proximal intestine and the mid intestine were also studied, but these did not yield that significant differences as the distal intestine developed compared with the control group. The research group from that study used both native- and polluted microplastics, where the polluted microplastic was collected at Milazzo harbour in Sicily, previously known as an area with polluted sediment with contaminants like DDT, PBC and PAHs. Microplastics, or plastic in general, have unfortunately the tendency to absorb substances from the water column, substances that can be harmful to aquatic fauna. It is possible that the severe damage in the distal intestine was affected by those contaminants. Therefore, to reflect reality we need to study microplastics deployed in harbours or pelagic zones. A recent survey conducted by the Swedish Environmental Research Institute (IVL) revealed that large quantities of microplastics have been discovered during inventories of the Swedish west coast. The same study believes that microplastics from artificial turf are the second largest source of emissions to the aquatic environment (Magnusson, et al., 2016). From these previous studies, the following conclusions can be drawn: (I) Even though it could be difficult frame, we have a need to reflect reality, and therefore requires that future studies will examine the toxicity of polluted plastic or plastic that already occur in the aquatic environment. (II) The result from Peda, C., et al., showed that the distal intestine was the most affected. Only the proximal intestine was studied in this investigation. However, the focus differed from both studies. Peda, C., et al., investigated the pathological effects, while the aim

of this study was to study the nutrient uptake by the intestine, which mostly occurs through the proximal intestine. Anyhow, just consider how a non-functional intestinal tract affects the nutrient flow? For further research, we need to investigate the effect microplastics can cause the whole intestinal tract.

EPDM-rubber comes in many types and sizes. Finding a full index of what EPDM-rubber is not an easy thing due to the diversity of it. By the name, you understand that the rubber contains of ethylene, propylene, and diene. But what the name does not tell is the hidden substances and chemicals used in the manufacturing process. It is known from before that zinc is used in the manufacturing process, but not whether the zinc is bonded into the rubber or not. While this master thesis was written, a similar bachelor thesis was done at the same institution. That study performed a so-called XRF-analysis where you do a chemical analysis of a particular object, in this case the granulate. The result showed that the rubber granulate peaked specifically in zinc (Svalin, J., 2016) Through research, we know that zinc is toxic and threatens the marine fauna (Wik, A. 2007; Gualtieri et al. 2005). Thus, it is difficult to conclude whether it is rubber granulate itself or zinc from the manufacturing process that could pose a danger to the marine fauna.

5.2 Mismeasurements and influencing factors

As mentioned above, the results of the Ussing measurements showed differences results compared to previous research, at least the result of how lysine takes its way through the paracellular space in the epithelial tissue of the intestine. When using the Ussing chamber technique, one can question the vitality of the epithelial tissue and its possible impacts of the result. The vitality of the tissue decreases significantly with time (or during treatment with certain reagents – unclear which specific regents) according to a study by Clarke, L. L. (2009). The epithelial tissue was directly put into Ringer's solution when it was removed from the fish. The most critical time, however, is the time before the tissue runs in the chambers (Clarke, L. L, 2009). In this study, there was a time of approximately one hour before the measurements begun. How this could have affected the result in this study is unfortunately beyond my knowledge. Although, that fact should be kept in mind.

As for the measurement of the Na+, K+-ATPase - these data must be interpreted with caution, as the BCA (Bicinchoninic acid assay) measurements are slightly miscalculated. The miscalculation became known only after the lab and the calculations were completed and all samples were destroyed. A compromise solution was done to settle the miscalculation. The

value from the only working standard solution was used to calculate the concentration in all fish intestine. The three absorbance values from each fish was divided with the standard solution (value = 0.054). An average of these values was then calculated for each fish.

The measurement of the Na+, where K+ -ATPase activity in the intestine were read in a spectrophotometer for 11 minutes. The raw data show the whole activity for 11 minutes. After a dialogue with a supervisor at the department, we decided to only process the data where activity was highest. This occurred on average over 2 minutes and 45 seconds in each fish intestine. The calculations are thus only calculated based on that specific time period.

Unfortunately, because of the limited time of this study could not the measurement of EROD be performed as planned. That would have given us information about PAHs flourished in the fish's liver. PAHs are encountered mainly in the liver where it is metabolized or bioaccumulated. The Ah-receptor, which is present in fish tissues, can be used as biomarker. An increased activity of that receptor suggests that contaminants like PAHs could be present in the fish's circulation. However, measurements of potential existence of PAHs were performed on fish bile. The difference between the groups of fishes was minimal. It is therefore believable that the granulate did not contain any PAHs. This result does not exclude that fluctuations of PAHs can exist in the fish liver.

5.3 Improvement points and further research

The laboratory work carried out on the intestine, liver, gills and blood plasma all showed a tendency to affected organs, even if the result was not significant. As mentioned before, one can only speculate that the time of exposure (seven days) was too short to give a significant effect. A suggestion for future research is therefore longer time of exposure of the EPDM-plastic granulate. Other factors with potential to influence the outcome are the presence of stressful conditions. Before the phase of plastic exposure, the fishes were held in two large tubs with approximately 25 fishes in each. Fish in big captivity can cause social stress among each other with hierarchical structures as a result. What were noticeable in some of the fish were small wounds above the jaw. The wound can be caused by the fish itself, or by a fish of a higher order. Thus, fish suffering from social stress or physiological injuries can create a general stress in fish. Also, the fact that the fish were captured and then anaesthetized twice, in the sake of force-feeding, is most likely a stressful factor for the fish. Besides increasing

cortisol levels in fish, it is not fully clear how stress in fish affects the outcome in this particular study – after all it is notable.

What has been discovered during the study is the lack of studies focused on toxicity of the intestine. Most studies are focused on studying the gills. More attention should be paid to the problem of intestine toxicity as a large part of hazardous metals binds to particles (e.g. microplastics) that fish and other organisms confuse with food, and thus, affects the gastrointestinal tract.

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