Master's thesis

NTNU
Norwegian University of Science and Technology
Faculty of Natural Sciences
Department of Biology

Inger Larsen Lyngstad

Chemical Effects of Common Plastic Products

Baseline Toxicity Screening and Chronic Toxicity to Polychaetes

Master's thesis in Environmental Chemistry and Toxicology
Supervisor: Martin Wagner
Co-supervisor: Andy Booth and Stefania Piarulli
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Abstract

Plastics are made from polymers that are combined with a range of additives to achieve desired functions and properties. These additives, along with non-intentionally added substances and other sorbed contaminants, can act as vectors of chemical exposure to marine organisms due to the vast plastic pollution in marine environments. To evaluate the chemical toxicity from commonly used plastic items, methanol extracts from 50 plastic products were screened for baseline toxicity using the Bacterial Luminescence Toxicity (BLT) screen. Out of these 50 items, three products with high, yet varying, baseline toxicity were selected for a chronic toxicity experiment in which larvae of the marine polychaete *Capitella* spp. were exposed to seawater leachates. Additionally, data from non-target chemical screening conducted by SINTEF Ocean personnel were used for comparison with baseline toxicity data.

Out of the 50 tested plastic samples, 46 extracts induced baseline toxicity (i.e., >20% luminescence inhibition compared to control), with a high variation in toxicity between samples. The polymer type was a significant predictor of baseline toxicity, with elastomers being the most toxic materials. The number of chemical features detected did not explain the toxicity. Balloons, car tire, and lab gloves were selected for chronic toxicity testing as they induced a potent baseline toxicity with EC_{20} values of 4.6, 0.44, and 0.04 g plastic L^{-1}, respectively. The leachates from car tire and lab gloves induced significant acute (5 d) and chronic (21 d) mortality to *Capitella* spp. at the highest concentrations tested (10 g L^{-1}, $p < 0.05$). Additionally, the lab glove leachate induced a non-significant reduction in *Capitella* spp. length at day 21 in animals exposed to the medium concentration (1 g L^{-1}, $p = 0.08$). No effect on mortality or length was observed from balloon leachate at any concentration tested.

These results indicate that common plastic products contain chemicals that can leach and cause toxicity to marine organisms if exposed at high concentrations. In addition, they indicate a high variation in toxicity between plastic products of different polymers but also between products from the same polymer type. Future research is needed to better understand whether these chemicals are bioavailable for marine organisms and which compartments are most relevant for exposure (i.e., water, sediment, microplastics). Efforts should be made to limit production of plastic products with highly toxic chemical content, especially where safer alternatives can be used to achieve the desired properties.

Sammendrag

Plast lages av polymerer som kombineres med en rekke tilsetningsstoffer for å oppnå ønskede funksjoner og egenskaper. Disse tilsetningsstoffene, samt utilsiktet tilsatte stoffer og andre absorberte stoffer, kan fungere som vektorer for kjemisk eksponering av marine organismer grunnet svært stor plastforurensning i marine miljøer. For å evaluere den kjemiske toksisiteten fra alminnelige plastartikler, ble metanolekstrakter fra 50 plastprodukter screenet for grunntoksisitet ved bruk av Bacterial Luminescence Toxicity (BLT) screen. Av disse 50 produktene ble tre produkter med høy, men likevel varierende grunntoksisitet valgt for et kronisk toksisitetseksperiment der larver av den marine børstemarken *Capitella* spp. ble eksponert for sjøvann med migrerte plastkjemikalier. I tillegg ble data fra kjemisk non-target screening utført av SINTEF Ocean-personell brukt til sammenligning med grunntoksisitet resultatene.

Av de 50 testede plastprøvene induserte 46 ekstrakter grunntoksisitet (dvs. $>$ 20% luminescensinhibering sammenlignet med kontroll), med høy variasjon i toksisitet mellom prøvene. Polymertypen var en signifikant prediktor for grunntoksisitet, med elastomerer som de giftigste materialene. Antall kjemiske komponenter forklarte ikke toksisiteten. Ballonger, bildekk og laboratoriehansker ble valgt for kronisk toksisitetstest, da de induserte en kraftig grunntoksisitet med henholdsvis EC_{20}-verdier på 4,6, 0,44 og 0,04 g plast L^{-1}. Migrantene fra bildekk og laboratoriehansker induserte betydelig akutt (5 d) og kronisk (21 d) dødelighet for *Capitella* spp. ved de høyeste testede konsentrasjonene (10 g L^{-1}, $p < 0{,}05$). I tillegg induserte migrantene fra laboratoriehansker en ikke-signifikant reduksjon i *Capitella* spp. lengde målt på dag 21 hos børstemark eksponert for middels konsentrasjon (1 g L^{-1}, $p = 0{,}08$). Det ble ikke observert effekt på dødelighet eller lengde fra ballongmigrant ved noen av de testete konsentrasjonene.

Disse resultatene indikerer at vanlige plastprodukter inneholder kjemikalier som kan migrere og forårsake toksisitet for marine organismer ved eksponering i høye konsentrasjoner. I tillegg indikerer de en høy variasjon i toksisitet mellom plastprodukter av forskjellige polymerer, men også mellom produkter fra samme polymertype. Fremtidig forskning er nødvendig for å forstå om disse kjemikaliene er biotilgjengelige for marine organismer og hvilke kilder som er mest relevante for eksponering, slik som vann, sediment og mikroplast. Det bør arbeides for å begrense produksjonen av plastprodukter med svært giftig kjemisk innhold, spesielt der sikrere alternativer kan brukes for å oppnå ønskede egenskaper.

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Abbreviations

MP Microplastic

BLT Bacterial Luminescent Toxicity

GC-MS Gas chromatography-mass spectrometry

PS Polystyrene

PE Polyethylene

PET Polyethylene terephthalate

PP Polypropylene

PA Polyamide

SD Standard deviation

ANOVA Analysis of Variance

Chapter 1

Introduction

1.1 Plastic pollution

Plastic materials are used for a wide range of applications due to their low cost and desired properties such as durability and low weight. Many plastic products have very short life spans, and a high amount of plastic ends up as waste. This, along with the slow degradation of plastic, causes its increasing and ubiquitous presence in the environment, and a large proportion of plastic waste becomes marine pollution. Jambeck et al. (2015) estimated that between 4.8–12.7 million metric tons of plastic entered the ocean from coastal countries in 2010 alone. Ocean plastics have been found to cause harmful effects to a large range of organisms by entanglement, ingestion, and suffocation (Gregory, 2009). More recently, however, the focus in the literature has shifted from macroplastics to microplastics (MP) and the harmful effects they might cause. MP are typically defined as particles below 5 mm in size, however, it was recently suggested to limit the definition to the micron range (1 $<$ 1000 μm) where MP are differentiated from nanoplastics (1 $<$ 1000 nm) and mesoplastic (1 $<$ 10 mm) (Hartmann et al., 2019). Microplastics are either intentionally produced in the micrometer size range from the manufacturer or are formed through the degradation of larger pieces of plastic (Andrady, 2017). The size of MP means that a range of marine animals may mistake them for food or ingest them unintentionally. Their small size also provides a greater surface area, which means chemicals associated with the plastic can more readily leach compared to larger plastic debris.

1.2 Plastic associated chemicals

Plastic materials are combined with a range of additives during manufacturing that provides certain functions and properties (Hahladakis et al., 2018). Among these are functional additives, such as plasticizers, stabilizers, antimicrobial agents, and flame retardants. Plasticizers are mainly used to improve flexibility and durability, while stabilizers prevent thermal and UV degradation. Other additives include colorants, fillers, and reinforcements. During manufacture, plastic polymers are created by chemical reactions of monomers, and unreacted residual monomers can remain in the final product. Along the same line, catalysts used during polymerization can remain in the plastic product (Hahladakis et al., 2018). In addition to these intentionally added chemicals, plastics may also contain non-intentionally added substances, including side products from polymerization, such as oligomers, as well as break-down products from additives and impurities from starting

monomers and additives (Muncke, 2009). Moreover, plastic debris may sorb organic chemicals from the environment, such as polychlorinated biphenyls, polycyclic aromatic hydrocarbons, and other persistent organic pollutants (Ogata et al., 2009). As emphasized here, plastic products contain a vast range of chemicals, many of which are unknown. Although plastic polymers are generally considered biochemically inert, the abovementioned plastic-associated chemicals may migrate to the surrounding media causing potential exposure to organisms in the environment.

Groh et al. (2019) presented an overview of known plastic-associated chemicals from food packaging and their hazards where they identified 906 chemicals likely associated with plastic packaging. Seven of these are classified as persistent, bioaccumulative, and toxic by the European Union, and 34 are reported to be endocrine-disrupting or potential endocrine-disrupting chemicals by the United Nations Environment Program. However, as noted by the authors, there is a lack of transparency and available information on the use and toxicity of many of the substances. For many of the chemicals in plastic products there is no data on potential hazards, and when adding lacking knowledge about the many unknown and unintentionally added chemicals and potential sorbed chemicals from the environment the complexity increases further.

Non-target chemical screening can reveal chemical content in plastic products, and previous studies have found hundreds to thousands of chemical features present in a single plastic product (Zimmermann et al., 2019, 2020). The vast number of chemicals, even if they could all be identified, makes hazard assessment based on single compounds impractical. Moreover, focusing on the effects of single chemicals would disregard the mixture effect caused by a diverse combination of chemicals. To assess possible hazards from these 'cocktails' of plastic chemicals, *in vitro* screening assays can be applied. Here, the chemical mixture present in the plastic product can be assessed for unspecific baseline toxicity or more specific modes of toxicity like genotoxicity or endocrine activity. An extensive review by Groh and Muncke (2017) has listed studies in which bioassays have been used to assess the toxicity of food contact materials. However, few of these studies included more than one type of plastic material, limiting their comparability. Furthermore, screening studies usually employ solvent extracts, which aim to extract all the chemical content from the plastic material. Solvent extracts might, therefore, contain more, and different, chemicals than those that would leach to the surrounding environment under more realistic conditions.

1.3 Leaching of plastic chemicals

Most plastic-associated chemicals are not chemically bound and may therefore migrate to the plastic surface where they can then leach into the surrounding environment (Hansen et al., 2013). However, the typology and quantity of leachates depend on various factors such as the concentration of the compound in the plastic material, molecular weight, temperature, and chemical nature of the surrounding media (Hansen et al., 2013; Messadi et al., 1983). The structure and pore size of the polymer also affect leachability (Teuten et al., 2007), as does weathering (Chen et al., 2019), and fragmentation (Luo et al., 2019). The term plastic leachate is often used to describe the fraction of plastic chemicals that leach to an aqueous media (Bridson et al., 2021), and several studies have prepared plastic leachates in the laboratory to examine the toxicity of bioavailable chemicals from plastics.

A recent review by Gunaalan et al. (2020) examined the impacts of plastic leachates on marine and freshwater organisms. Here, studies on marine microorganisms have reported reduced growth and changes in photosynthesis, gene expression, and germination after exposure to leachates from several plastic materials. As an example, Capolupo et al. (2020) found inhibition of growth on the marine microalga *Skeletonema costatum* after exposure to plastic leachates from four of five tested materials. For marine invertebrates and vertebrates, plastic leachates have been found to affect survival, growth, reproduction, and early development, as well as other endpoints. Many of these studies have been focused on sessile species, as they can be exposed due to their filter and deposit-feeding strategies. However, species like copepods (Bejgarn et al., 2015), seasnails (Ioakeimidis et al., 2019), fish (Hamlin et al., 2015), and seabirds (Tanaka et al., 2015) have also been studied. Although fewer studies focus on freshwater species, daphnia have been highly studied, and researchers have found that leachates from several types of plastic material induce immobilization or mortality (e.g., Lithner et al., 2009, 2012). Other studies on freshwater species include plants and bacteria (Schiavo et al., 2020), and fish (Boyle et al., 2020). These studies indicate clear negative effects from plastic leachates; however, effects occurring in the environment will be dependent on the presence of plastic-associated chemicals. And plastic additives have, indeed, been detected worldwide in marine waters and sediments, as well as in the tissues of numerous marine organisms (Hermabessiere et al., 2017), which highlights the risk for hazardous effects in the natural environment.

1.4 Exposure of plastics and associated chemicals to marine benthic species

The deep sea is a major sink for plastic debris (Woodall et al., 2014), meaning benthic species such as detritus- and deposit-feeding organisms are at a higher risk of exposure than organisms in other environmental compartments. Low-density macro- and microplastics are often buoyant when recently introduced to the ocean, but can accumulate an organic coating which then adsorbs sand and other debris causing them to sink to the sea floor (Corcoran, 2015; Goldberg, 1994). Additionally, incorporation in fecal pellets can move MP from the water column to the benthic environment (Cole et al., 2013) and enhance their uptake by bottom-dwelling species (Piarulli and Airoldi, 2020). MP can be resuspended from the sea floor (Martin et al., 2017); however, the sea floor is considered to be a major sink for most marine MP (Woodall et al., 2014). Once there, plastic debris can induce negative effects on benthic species (Goldberg, 1997). For macroplastics, this can happen by sediment coverage which can inhibit gas exchange from pore water, altering the benthic community (Goldberg, 1994). Also, non-selective and selective feeding of MP has been observed in several bottom-dwelling species (Bour et al., 2018; Wright et al., 2013a,b), and several studies have found them to have harmful effects (e.g., reviewed by Lusher, 2015). However, most studies are focused on the physical effects of plastic, and few studies investigate plastic-associated chemical exposure to this group of organisms.

Benthic organisms can be exposed to plastic-associated chemicals through natural pathways like water, food, and sediment, or by ingestion of MP (Hermabessiere et al., 2017). Polychaetes are sediment-dwelling marine worms and are a group of benthic species that are highly relevant for sediment ecotoxicology. They are continually and directly exposed to sediment-bound contaminants, especially so for species feeding via the sediment. They influence the sediment characteristics through bioturbation and tube building. Finally, as they are the primary food source for several fish species, they can be an important vector

for transferring contaminants to higher trophic levels (Lewis and Watson, 2011). Ingestion of MP by polychaetes has been documented (Bour et al., 2018; Knutsen et al., 2020; Nel and Froneman, 2018), and negative effects have been observed (Leung and Chan, 2018; Revel et al., 2018; Wright et al., 2013a,b). However, there are still large knowledge gaps regarding the effects of plastic exposure, especially regarding chemical toxicity. In lungworm, Browne et al. (2013) found that MP transferred additive chemicals and sorbed pollutants to the worms, where they then caused biological effects. Knutsen et al. (2020) found that MP were enriched in polychaetes compared to the sediment, implying poor excretion and prolonged retention which could lead to enhanced exposure to plastic associated chemicals. Moreover, MP were also found in the tubes the polychaetes dwelled in, which might suggest they favorably select MP particles when building their tubes. Together, these data suggest that benthic organisms, like polychaetes, are exposed to plastic chemicals. In this thesis, the polychaete *Capitella* spp. was, therefore, chosen to study the effect of plastic chemical leachates.

The *Capitella* genus consists of many similar species that are hard to differentiate based on morphological and life-history traits which often overlap (Silva et al., 2017). They are opportunistic marine deposit feeders, commonly found in areas rich in organic matter (Grassle and Grassle, 1976; Grassle, 1978). *Capitella* are very pollution resistant, and the genus has been used as an indicator of organic pollution due to its very high density in heavily polluted areas (Reish, 1957). However, different *Capitella* species tolerate toxicant stress to different degrees (Gamenick et al., 1998; Linke-Gamenick et al., 2000). In general, *Capitella* have short life cycles and continual reproduction (Tsutsumi, 1987), which makes them ideal for developmental and life cycle studies. The female deposits the eggs in a brooding tube made from sedimental particles, fecal matter, and mucus. Trochophores then hatch inside the brooding tube after 4-5 days and develop into metatrochophores within a few more days before finally leaving the brooding tube (Tsutsumi and Kikuchi, 1984). As polychaetes incorporate MP when building their tubes (Knutsen et al., 2020), this could introduce early exposure to plastic chemicals before juveniles leave their brooding tube.

1.5 Aims and hypotheses

This thesis has been conducted as a part of the MicroLEACH project funded by the Norwegian Research Council. The project aims to look at the long-term effects of plastic and associated chemicals on marine organisms and to distinguish the role of both MP and their additive chemicals on long-term effects on marine organisms. This thesis aimed to expand knowledge on the baseline toxicity of plastic chemicals and to relate this to polymer type, chemical content, and chronic toxicity to a benthic invertebrate. **I hypothesize that:**

1. Chemicals in plastic products induce baseline toxicity,
2. the polymer type and the number of chemicals detected in a sample predicts the baseline toxicity,
3. plastic products with high baseline toxicity will cause elevated mortality and reduction in growth of the polychaete *Capitella* spp.

Baseline toxicity assays can provide knowledge about unspecific toxicity and can, thus, be an aid in prioritizing plastic products with the potential for hazardous chemical effects.

Therefore, I tested 50 plastic products with a Bacterial Luminescence Toxicity (BLT) screen. From these, three materials with high but varying baseline toxicity were selected to test chronic toxicity *in vivo*. To enhance the understanding of the effect of plastic chemical toxicity on benthic species, this study included a 21-day, chronic toxicity test with the ecologically relevant polychaete *Capitella* spp., using seawater leachates from three selected materials. Additionally, this thesis compared results from toxicity tests with data from a non-target chemical screening conducted at SINTEF Ocean within the scope of the MicroLEACH project and assessed the relevance of this chemical screening for predicting baseline toxicity.

Chapter 2

Methods

2.1 Samples and chemical analysis

Within the MicroLEACH project, SINTEF Ocean selected 50 plastic products and performed an initial chemical screening (Tab. 2.1). The materials included consumer products, such as disposable utensils, children's toys, and single-use plastic gloves, as well as industrial products such as vinyl flooring. The products were extracted with methanol and then evaporated and redissolved in dimethyl sulfoxide (DMSO). A non-target chemical screening using gas chromatography-mass spectrometry (GC-MS) was done, and the number of chemical features (peaks) were recorded for each product. The polymer type was verified by Fourier-transform infrared spectroscopy. This work was used as a basis for material selection for the current study, and it was used to assess correlations with baseline toxicity.

Table 2.1: **Plastic products used for non-target chemical analysis and baseline toxicity testing.** The polymer type of each sample was confirmed using Fourier-transform infrared spectroscopy. Features denotes the number of unique features identified using GC-MS. Samples further selected for the *in vivo* study are highlighted in bold.

Sample ID	Product description	Polymer	Features
1	Vinyl flooring	Vinyl	67
2	Vinyl flooring	Vinyl	54
3	Vinyl flooring	Vinyl	54
4	Plant pots	Polystyrene (PS)	48
5	Hay bale net	Polystyrene (PS)	45
6	Disposable utensils	Polystyrene (PS)	35
7	Styrofoam packaging material	Polystyrene (PS)	51
8	BIC pen	Polystyrene (PS)	22
9	Cleaning sponges	Polystyrene (PS)	24
10	Paper file sheet	Polyethylene (PE)	29
11	Plastic Christmas Tree	Polyethylene (PE)	70
12	Cleaning sponges	Polyethylene (PE)	21
13	Seat Cushion	Polyethylene (PE)	22
14	Children's beach toys	Polyethylene (PE)	23
15	Wrapping	Polyethylene (PE)	63
16	Trash bag	Polyethylene (PE)	73
17	General tarpaulin	Polyethylene (PE)	71
18	Pipe insulation	Polyethylene (PE)	56

Table 2.1 continued

Sample ID	Product description	Polymer	Features
19	Plastic bag (e.g. KIWI)	Polyethylene (PE)	59
20	Freezer bags	Polyethylene (PE)	71
21	Microfiber wash cloth	Polyethylene terephthalate (PET)	10
22	Shower curtain	Polyethylene terephthalate (PET)	9
23	Fleece blankets	Polyethylene terephthalate (PET)	39
24	Curtains	Polyethylene terephthalate (PET)	2
25	Water bottle(s)	Polyethylene terephthalate (PET)	2
26	Plastic cups	Polypropylene (PP)	61
27	Plastic cups	Polypropylene (PP)	34
28	IKEA shopping bag (blue)	Polypropylene (PP)	53
29	Weed control fabric	Polypropylene (PP)	61
30	Disposable straws	Polypropylene (PP)	63
31	Reusable shopping bag	Polypropylene (PP)	102
32	Foam packaging material	Polypropylene (PP)	55
33	**Balloons**	**Elastomer**	**70**
34	Anti-slip mat	Elastomer	54
35	**Lab gloves**	**Elastomer**	**42**
36	**Car tire**	**Elastomer**	**89**
37	Sole of shoes/Shoes	Elastomer	51
38	Toy rubber duck (yellow)	Elastomer	51
39	Garden hose	Elastomer	64
40	Inflatable beach toy	Elastomer	102
41	Soccer ball	Elastomer	83
42	Dishwashing gloves	Elastomer	52
43	XPS mark-isolasjonsplate	Elastomer	41
44	Hay bale wrapping	Elastomer	25
45	Flame-retardant workwear	Elastomer	38
46	Bath sponge	polyester	19
47	Foam mattress	Polyether	23
48	Cigarette buds	Polyvinyl acetate	36
49	Phone case	unknown	25
50	Fishing line (nylon)	Polyamide (PA)	4

2.2 Baseline toxicity with the Bacterial Luminescence Toxicity (BLT) screen

As a baseline toxicity test, the BLT screen was performed with the methanolic extracts in DMSO from 50 plastic products. The BLT is a high-throughput assay in which up to four chemical extracts can be analyzed on the same plate, with only a 30-minute incubation time. The toxicity observed in the BLT screen can be categorized as baseline toxicity, a type of non-specific toxicity where the toxic mechanism is unknown. The goal of an unspecified assay such as the BLT, is to discover chemical toxicity which may act through a range of mechanisms (Groh and Muncke, 2017), and baseline toxicity has been shown to correlate well with toxicity to other aquatic organisms (Escher et al., 2008). These factors make the BLT an ideal initial screening of toxicity, and therefore an asset in material selection for plastic toxicity studies.

The BLT protocol was based on Van De Merwe and Leusch (2015) with minor adjustments. Samples, negative-, and positive control were added in duplicates to a 96 well plate in an eight-step serial dilution (1:2 in saline buffer) (Fig. 2.1). The concentrations corresponded to 0.4–150 g plastic l^{-1}. For positive controls, 3.5-dichlorophenol from a stock solution of 3.6 or 4.8 g L^{-1} was used. Cryopreserved aliquots of *Photobacterium leiognathi* were thawed on ice and diluted 1:30 in sterile growth media. After allowing the solution to approach room temperature for 10-30 minutes, 50 μL of the bacterial suspension was added to each well. The well plate was then covered with aluminum foil and incubated at room temperature for 30 minutes. After incubation, luminescence was recorded with the CYTATION 5 Imaging Reader by Biotek. Samples inducing a response above the measurable range were tested again after being further diluted in DMSO.

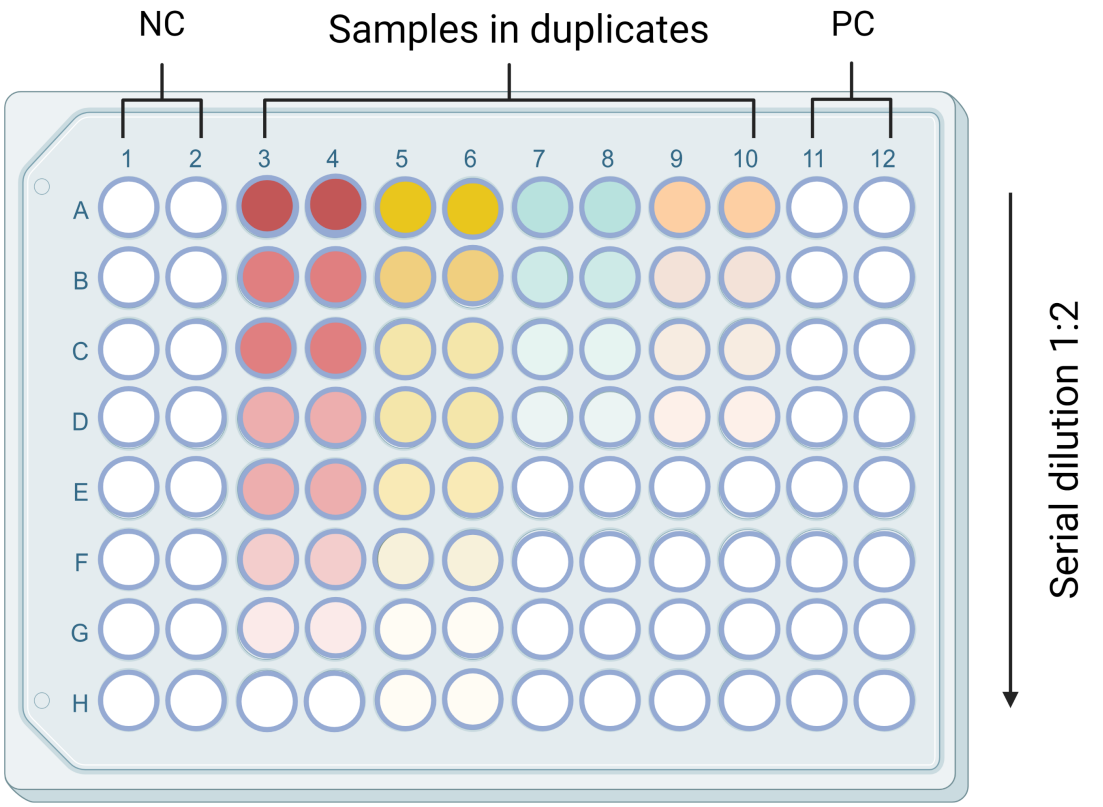

Figure 2.1: **Experimental setup for the BLT screen.** Each independently-run experiment included duplicates of four plastic extracts, negative controls (NC), and positive controls (PC) in serial dilutions (1:2).

Solvent blanks (methanol evaporated and dissolved in DMSO) and procedure blanks were included in the analysis, and none of the blanks were found to induce toxicity. The solvents are, thus, not contributing to the observed toxicity of the samples, and there is no sign of contamination during extraction and analysis.

2.3 *Capitella* chronic toxicity study

The sea floor is a highly relevant environment to explore effects of MP and plastic chemicals due to the levels of plastic debris, yet few studies focus on benthic species. Therefore,

the marine polychaete *Capitella* spp. was used to study chronic toxicity from seawater leachates from a selection of plastic products. The polychaetes were exposed for 21 days where mortality was recorded every second day and length was recorded at the end of the exposure as a measure of growth.

Animals, and parts of the material used for the toxicity test, were obtained from the national research infrastructure Norwegian Center for Plankton Technology (#245937/F50) hosted by SINTEF Ocean and NTNU.

Initially, the toxicity study was planned to include treatments with plastic particles in addition to their leachates. Therefore, an uptake study was attempted to determine the size range of particles ingestible by *Capitella* spp. Particle exposure was excluded from the study before finalizing method optimization, and results from the uptake study are therefore not included in the main results. The method and preliminary results from the uptake study can be found in the appendix A.

2.3.1 Study species and holding facility

For this study, *Capitella* spp. from an in-house culture at the Norwegian Center for Plankton Technology (#245937/F50) hosted by SINTEF Ocean and NTNU were used. The culture has been maintained at SINTEF Ocean since January 2019 on a diet consisting strictly of aquaculture sludge, supplied at rates of approximately 150 mg nitrogen m^{-2} day^{-1} (Tenore and Chesney Jr., 1985). The starting culture was originally obtained from a wild population caught in an area near Sandnessjøen (Nordland county, Norway). The culture has been maintained in cultivation trays (V=18 L) filled with a thin layer of crushed chamotte (1 cm depth, ceramic clay with grain size 0.5–2 mm, Alt for Keramikk AS, Norway) and supplied seawater (flow-through) collected from the Trondheim Fjord at 70 m depth (33–34 psu, 8–10 °C). For the toxicity study, larvae were collected from the cultures after they had left the maternal brooding tubes and reached the juvenile stage.

2.3.2 Leachate preparation

Seawater leachates were prepared by incubating the plastic material from balloons, lab gloves, and car tire (samples 33, 35, and 36) in seawater for 5 days (Fig. 2.2). To enhance the surface area, balloons and lab gloves were cut into fragments using scissors before incubation. The scissors were cleaned with ethanol before use. The car tire rubber was purchased in ground powder and did not need further fragmentation. The size distribution with standard diviation (SD) of fragments calculated based on a sample of 30 fragments randomly picked and measured with the imaging software ImageJ version 1.53j can be found in Table 2.2. Seawater collected from the Trondheim Fjord at 70 m of depth (33-–34 psu, 8–10 °C) was filtered using a Nalgene Rapid-Flow disposable sterile filtering unit utilizing a surfactant-free cellulose acetate membrane with a pore size of 0.2 μm, produced by Thermo Scientific. This was done to remove any microorganisms or other impurities present in the water. A total of 200 g of each material was suspended in 1 L filtered seawater. The material was divided to 40 g of material and 200 mL filtered seawater in five 250 mL glass bottles. These were placed in a bench top shaking incubator (IKA KS 4000 ic control) along with procedure controls, that is, glass bottles with filtered seawater, without plastic. The incubator was set to 125 rpm and 20 °C, and the bottles were left in the incubator for 5 days. The incubator was covered with aluminum foil to avoid UV degradation of sensitive compounds in the leachates. Minimum and maximum temperatures were recorded at 22.8

and 29.9 °C by an external thermometer which recorded temperature from a bottle with tap water inside the incubator throughout the incubation time. After the incubation period, the samples were filtered using the same setup as with fresh seawater and collected in 1 L glass bottles. Subsamples for chemical characterization were taken before the remaining leachates were frozen in 50 mL glass bottles at -18 °C to be thawed before use in the *Capitella* experiment.

Figure 2.2: **Plastic fragments from balloons (multiple colors), nitrile lab gloves (turquoise), and granulated car tire rubber (black).** The photos display (A) plastic fragments from the three materials used for measurements of size distribution; (B) fragments divided into five containers with 40 g each, and (C) fragments and seawater combined in glass bottles.

Table 2.2: **Size distribution of plastic material fragments used for leachate preparation.** Long and short side lengths were measured on 30 fragments for each material.

Material	Longest diameter (mm, mean ± SD)	Shortest diameter (mm, mean ± SD)
Car tire	0.42 ± 0.53	0.25 ± 0.30
Balloons	4.74 ± 2.18	2.47 ± 1.43
Lab gloves	7.31 ± 3.63	3.79 ± 2.23

2.3.3 Equipment and facility

For the preparation of leachate dilutions, solvent rinsed graduated glass pipettes were used (3× dichloromethane, 3× methanol 3× ultrapure water). Test vessels (50 mL glass beakers, 45 mm in diameter, and 30 mm in height) were washed in a dish washer, first using detergent, and then again without detergent. They were heat sterilized (800 °C) and then placed in an acid bath (10% nitric acid) before rinsing thrice with ultrapure water. During the experiment, the test vessels were covered with aluminum foil to prevent contamination and reduce evaporation. The foil was monitored daily for corrosion and exchanged if signs of corrosion were present. For handling of the polychaetes, heat sterilized (800 °C) pasture pipettes were used. The experiment was conducted in a climate-controlled room with the temperature kept at 17 ± 1 °C with a dark-light regime of 12:12 h.

2.3.4 Pilot study

A small pilot study was performed to evaluate an appropriate range of leachate concentrations for the exposure of *Capitella* spp. For this, 10 g L^{-1} was chosen as the high concentration based on concentrations of car tire leachates which caused significant

mortality in copepods (Halsband et al., 2020). In their study, concentrations of 5 and 15 g L^{-1} of plastic leachate both caused high mortality to marine copepods in an 18-day exposure experiment. For the pilot study, an additional, tenfold higher dilution (1 g L^{-1}) was included. Juvenile larvae were placed into beakers containing each sample at two concentrations (n=5) and monitored for mortality 2, 6, 24, and 48 h. after exposure. No mortality was observed in any of the treatments or in the control. Therefore, as the toxicity study was aimed to measure chronic toxicity including sublethal effects, the lack of mortality within 48 h was considered optimal and the concentrations were considered appropriate.

2.3.5 Setup and implementation

The toxicity test was designed as a full factorial study (Fig. 2.3). Leachates from the three materials were diluted with filtered seawater to three different concentrations corresponding to 0.1, 1.0, and 10 g plastic L^{-1} seawater, which are referred to as low, medium, and high concentration. These three concentrations were chosen based on the pilot study and were aimed to allow detection of differences between materials for mortality, and growth, which might be a more sensitive sub-lethal parameter. The lowest concentration was added to expand the concentration range. In addition, this concentration may be more similar to environmentally relevant concentrations.

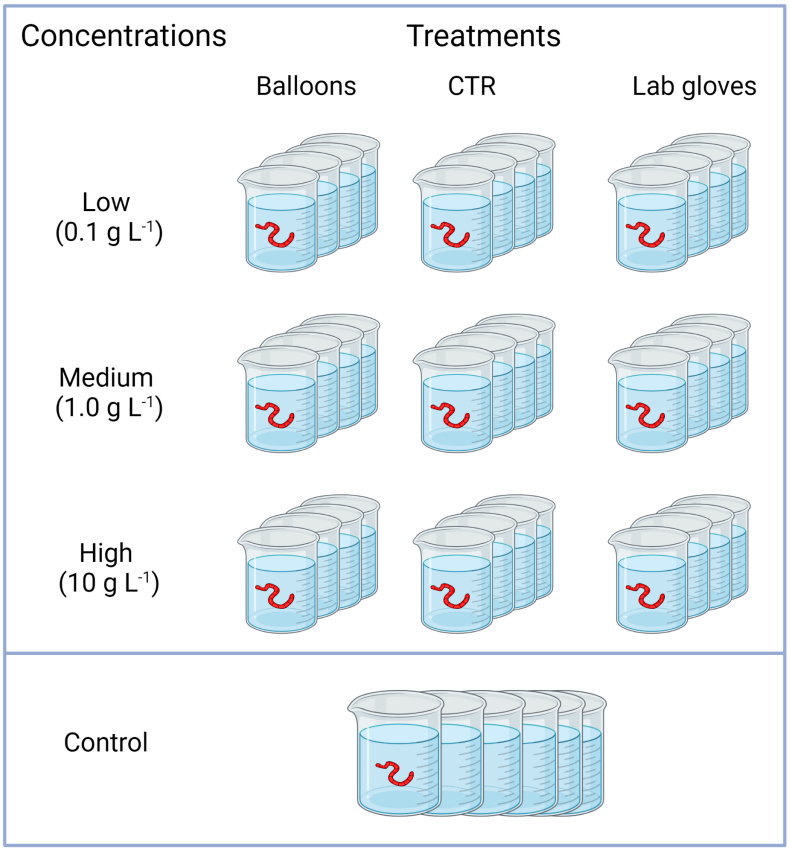

Figure 2.3: **Setup of *Capitella* toxicity experiment.** *Capitella* spp. were exposed to plastic leachate from balloons, car tire rubber (car tire), and nitrile lab gloves in three concentrations. Each of the nine treatments included four test vessels with ten larvae. As a control, the procedure blank was used in a dilution corresponding to the high concentration treatments. Here, six test chambers with ten larvae in each were used.

For each concentration, four test vessels, each with ten larvae, were set up. Additionally, six test vessels with ten larvae in each were included as a control. Here, the procedural blank from the leachate production was prepared in a dilution corresponding to the high concentration of the treatments. Individual larvae were randomly selected from the culture and allocated to each treatment and test vessel, and larvae were mixed before allocation to ensure genetic variance within the treatments. The larvae were picked out individually with a pipette and placed directly in a test vessel prefilled with the leachates or blanks. Pipettes were changed between treatments to avoid cross-contamination of leachates. Two to three drops of a solution of Tetra Pro, commercially available fish food flakes (25 g in 100 L filtered seawater) were added to each system before the beaker was covered with aluminum foil.

Mortality was recorded every 48 h, and this was done concurrently with leachate exchange, where larvae were transferred individually to clean test chambers with fresh leachate dilutions, and new feed was added. Water parameters were measured in the used test vessel after every water exchange, and the recorded water temperature, dissolved oxygen, salinity, and pH can be found in appendix B.

The final recording of mortality was done after 21 d of exposure. To record growth, 5–10 randomly selected larvae were transferred to a small glass beaker with seawater covering the bottom and a few drops of the sedative MS-222 (20 g L^{-1}). Pictures were taken of the individual larvae with a Nikon SMZ1000 microscope using an $\times 4$ magnification. The length was measured with the imaging software ImageJ version 1.53j, by measurement of a freehand line drawn from top to bottom along the center of the larvae.

2.4 Statistical analysis and visualization

Microsoft Excel MSO (16.0.14026.20202) was used for sorting of data and calculations, effect concentrations (ECs) of *in vitro* toxicity were calculated using the software GraphPad Prism 8, version 9.0.0, and statistical analysis and data visualization were performed in R, version 4.0.5 (R Development core team 2021) using the packages ggplot2 (Wickham, 2016), DescTools (Signorell, 2021), ggpubr (Kassambara, 2020), and multcomp (Hothorn et al., 2008). The significance level was set to $\alpha = 0.05$ for all statistical tests.

2.4.1 Effective concentrations (ECs) of *in vitro* toxicity

Due to high variation in luminescence in the first plate row, the baseline toxicity data for the highest concentration was considered unreliable and, thus, removed from the analyses. The highest concentration analyzed was therefore 75 g L^{-1} for most samples. Dose-response relationships were estimated from a four-parameter logistic model, with lower and upper constraints set to 0 and 100% inhibition, respectively. The mean value from two technical replicates, recorded as percent luminescence inhibition compared to negative control, was used to calculate each EC. The ECs presented are mean and SD from two or three independent experiments. When an EC_{20} could not be derived because of too low of an effect at the highest tested concentrations, an EC_{20} of 75 g plastic L^{-1} (i.e., the highest concentration tested) was used to visualize the data. In the cases where an EC_{20} was above the range of sample dilutions, the sample was tested again with higher dilutions. However, due to limited availability of cryopreserved bacterial culture, not all samples could be tested again in higher dilutions and are, therefore, presented as > 1.17

g plastic L^{-1}, which corresponds to the lowest tested concentration for most samples. For data visualization, all samples with an $EC_{20} > 1.17$ g plastic L^{-1}, were displayed as $>$ 1.17 g plastic L^{-1} for simplicity. Samples inducing 20% luminescence inhibition compared to control at the highest concentration tested (i.e., having $EC_{20} < 75$ g plastic L^{-1}) were considered active samples in this assay. This is a conservative value for minimal quantifiable response in the BLT screen (Van De Merwe and Leusch, 2015).

2.4.2 Statistical models

In addition to the calculation of ECs, the efficacy, or maximum response, of *in vitro* tested samples was estimated. A one-way Analysis of Variance (ANOVA) was performed on the relative luminescence inhibition of the highest concentration tested for each sample. This was followed by a Dunnett's post hoc test for comparison to control.

Linear regression was applied to determine the effect of the number of features on baseline toxicity, and a one-way ANOVA was used to compare baseline toxicity between polymer types. To determine the effect of treatments compared to control on *Capitella* spp. mortality, a one-way ANOVA was followed by a Dunnett's test. To determine the effect of different treatments compared to control on growth, a mixed-effects model was fitted with the length of each larva as observations and test chambers included as a random factor to account for the random variance introduced. A One-way ANOVA was then followed by a Dunnett's post hoc test. For all models, the normality of residuals was verified by visual inspection of Q-Q plots. For all post hoc tests, the p values reported are adjusted for multiple comparisons according to the Bonferroni correction. Tables with p-values for multiple comparison tests tests can be found in Appendix C.

2.4.3 Schematic figures

Schematic figures were created using the online software BioRender and Adobe Illustrator version 25.2.3.

Chapter 3

Results

3.1 Samples, polymer types, and chemical features

In this study, 50 plastic products were selected for a non-target chemical screening and baseline toxicity testing using the BLT screen (Tab. D.1). The non-target chemical screening using GC-MS found the number of unique chemical features present in the extracts for all 50 materials as well as for each individual material (Fig. 3.1). In total >3000 chemical features were found across the 50 different products, with a range between 2 and 102 features in the individual products.

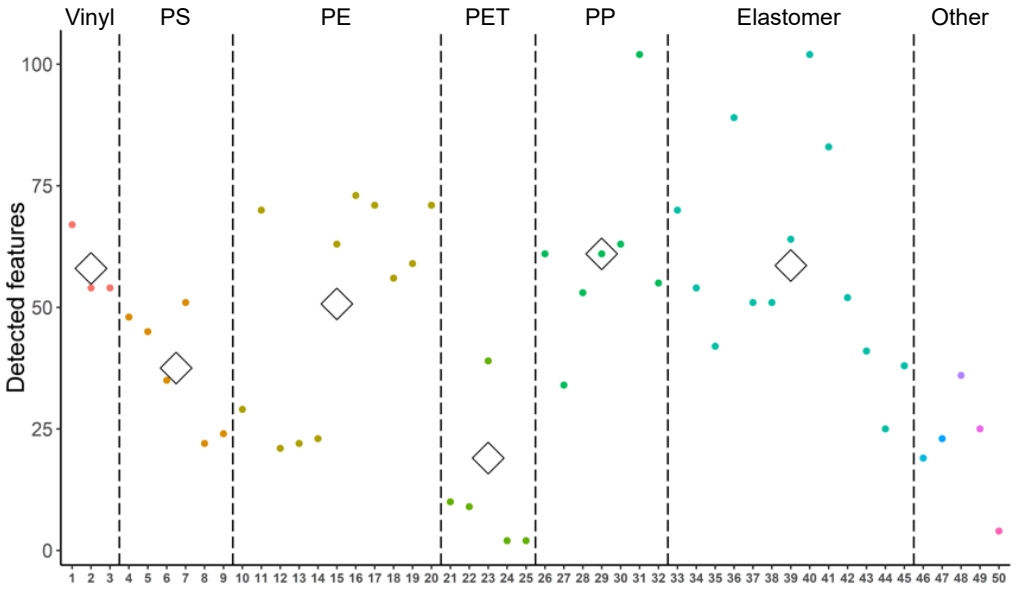

Figure 3.1: **Number of chemical features** detected in 50 plastic methanol extracts (dots) and mean for each polymer type (diamond).

3.2 Baseline toxicity

The BLT screen detects baseline toxicity as inhibition of luminescence in *P. leiognathi.* In the current experiment 46 of the 50 plastic extracts were active (i.e., having $EC_{20} < 75$ g plastic L^{-1}) (Fig 3.2). The toxic responses were variable, with a wide range of EC_{20} values for the different sample extracts. Five of the samples tested inhibited bioluminescence by more than 20% at the lowest concentration tested (1.17 g plastic L^{-1}), these are displayed as <1.17 g plastic L^{-1}.

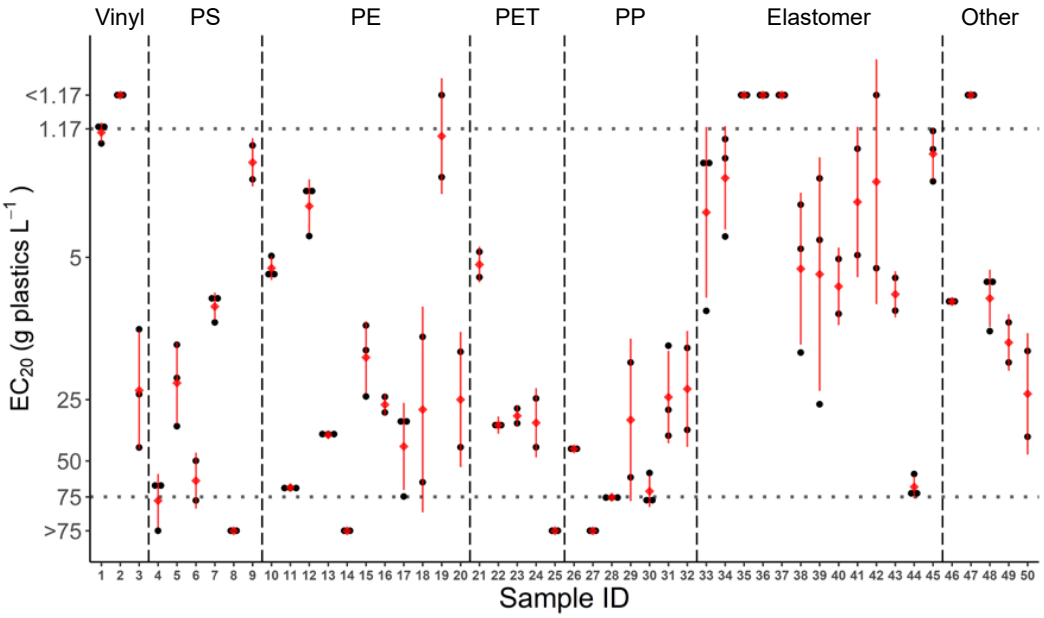

Figure 3.2: **Toxicity of plastic extracts in the BLT screen** after 30 min of exposure. Data is presented on a logarithmic scale with mean and SD (red shape and lines) of EC_{20} for luminescence inhibition from 2-3 independent experiments (black dots), each based on duplicates. Dotted lines represent the lowest (1.17) and highest (75) tested concentrations. Extracts that did not inhibit luminescence by 20% at the highest tested concentration are displayed as >75 g plastic L^{-1}, while extracts that inhibited the luminescence by more than 20% at the lowest tested concentration are displayed as <1.17 g plastic L^{-1}.

To add information about the efficacy of the samples, the maximum level of luminescence inhibition compared to control was investigated (Fig 3.3). The maximum luminescence displays a similar pattern to the EC_{20}s, and the two measures are strongly correlated (Pearson correlation $R^2 = 0.83$, $p < 0.001$, Fig. 3.4). Some samples display different patterns; however, the samples are not directly comparable due to differences in highest concentration tested for each sample. The samples EC_{20}, maximum luminescence inhibition, and their highest tested concentration can be found in Appendix D. Significant difference from control was found for 34 of 50 samples.

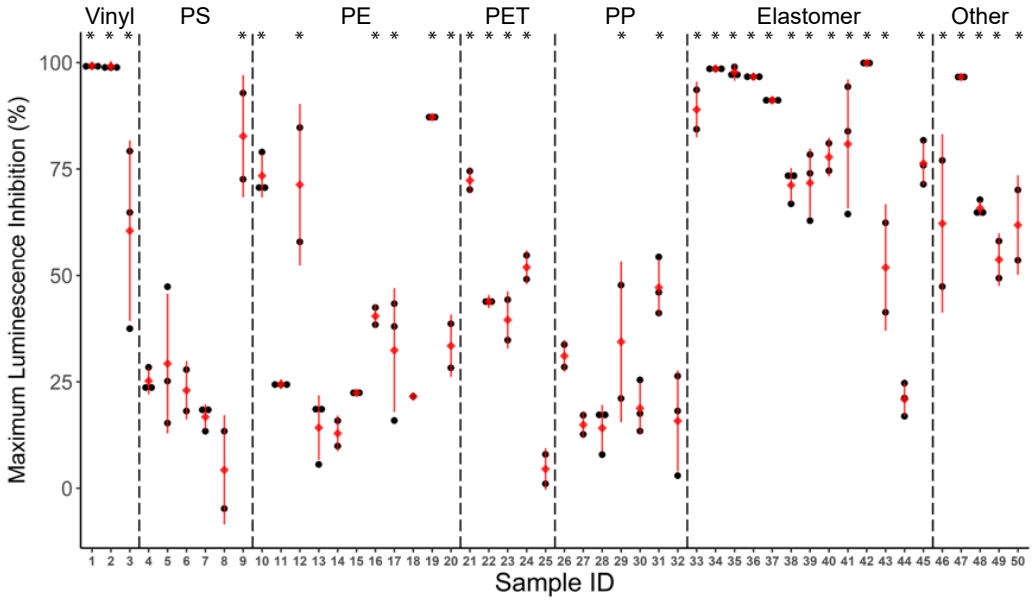

Figure 3.3: **Inhibition of luminescence of highest tested concentration.** Data is presented as mean and SD (red shape and lines) of luminescence inhibition from the highest tested concentration for each sample and are therefore not directly comparable. Black dots represent independent experiments based on duplicates. Asterisks denote significant difference from control ($p < 0.05$).

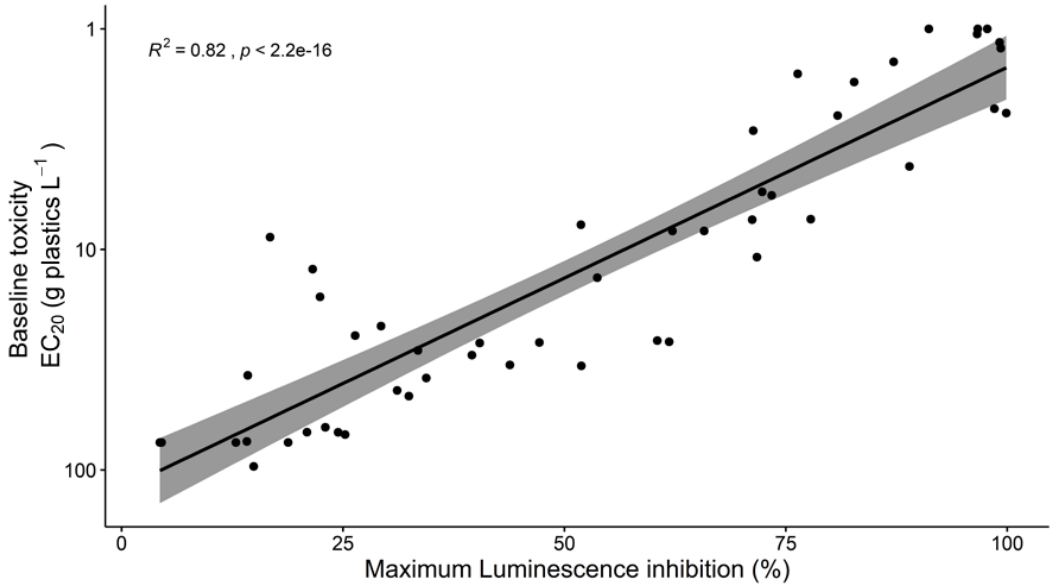

Figure 3.4: **Person correlation of EC_{20}s and maximum luminescence inhibition in the BLT screen**. The shaded area represents the 95% confidence interval.

3.2.1 Effect of polymer type on baseline toxicity

Polymer type was shown to be a significant predictor of baseline toxicity (EC_{20}) derived from the BLT screen ($p = 0.036$), with 36% of the variance in baseline toxicity explained

by the type of polymer ($R^2 = 0.36$). High toxicity was exerted by extracts from elastomers while relatively low toxicity was induced by PP and PET.

3.2.2 Prediction of baseline toxicity from number of chemical features

A weak trend indicates a positive relationship between number of chemical features detected from methanol extracts and baseline toxicity (EC_{20}) derived from the BLT screen (Fig. 3.5). However, this trend is not significant ($p = 0.21$) and explains very little of the observed variance ($R^2 = 0.013$).

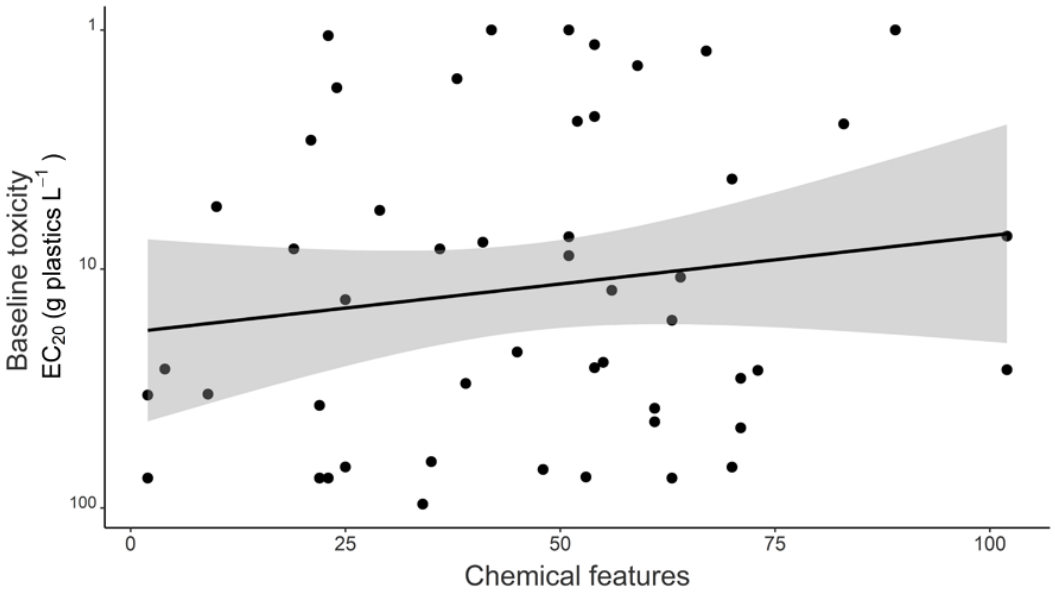

Figure 3.5: **Linear regression of baseline toxicity (EC_{20} in g plastic L^{-1})** determined in the BLT screen and number of chemical features detected from GC-MS analysis. The shaded area represents the 95% confidence interval. $p = 0.21$, $R^2 = 0.013$.

3.3 *In vivo* toxicity of seawater leachates to *Capitella* spp.

Balloons, lab gloves, and car tire (samples 33, 35 and 36) were selected for a leachate toxicity test on the polychaete *Capitella* spp. These materials all exhibited high toxicity in the BLT test with EC_{20} values of 4.2, 0.46, and 0.04 g plastic L^{-1}, respectively. Mortality was recorded every second day, and length was recorded at day 21 as a measure of growth.

3.3.1 *Capitella* mortality

High concentration leachates (1.0 g L^{-1}) from car tires and lab gloves, but not balloons, induced significant acute (96 h) and chronic (21 d) mortality in *Capitella* spp. compared to control (Fig. 3.6) ($p < 0.05$). No significant difference in mortality compared to control was found in the low and medium concentrations from any of the materials. A significant difference in mortality was first recorded after 96 h and became more profound during the

remaining time, with the high concentration of lab gloves causing 100% mortality at day 17. The experiment was continued until day 21 for all other treatments (Fig. 3.7).

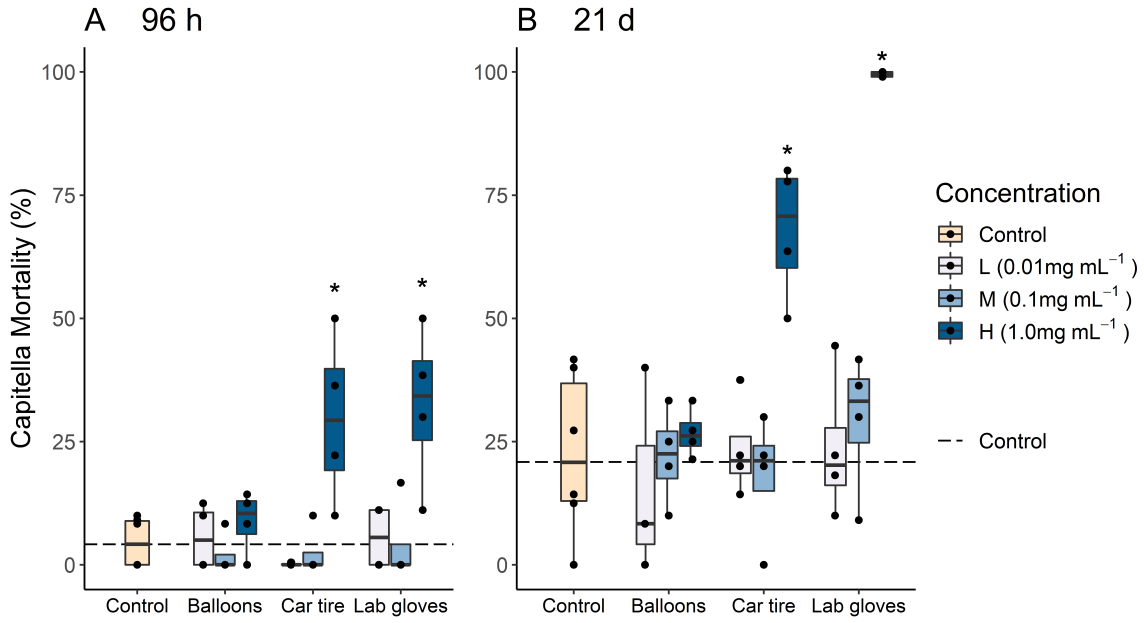

Figure 3.6: **Mortality of *Capitella* spp.** after exposure to plastic leachates of balloons, car tire, and lab gloves in three concentrations for 96 h (A) and 21 d (B). Data is displayed as median and quartiles of percentage mortality, with mortality of each replicate displayed as dots (n = 3-6). The median mortality of the control treatment is displayed as a dotted line. Treatments with a significant difference from control are indicated with an asterisk ($p < 0.05$).

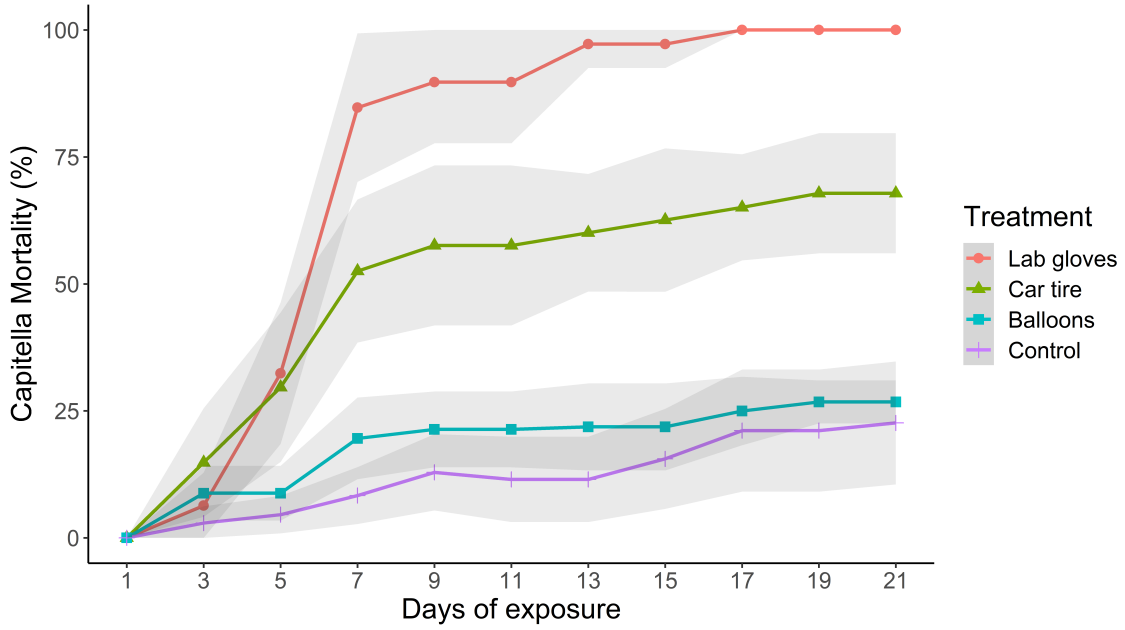

Figure 3.7: ***Capitella* spp. mortality during the 21-day exposure** with the highest concentration of each treatment in addition to the control treatment. Data is displayed as mean percentage mortality (n=4) for each measured time point, with a 95% confidence interval displayed as shaded area.

3.3.2 *Capitella* growth

As a measure of growth, the length of the larvae at day 21 was measured. None of the treatments displayed mean length significantly different to control. However, the medium concentration of lab glove leachate induced a non-significant reduction in length ($p = 0.08$) (Fig. 3.8).

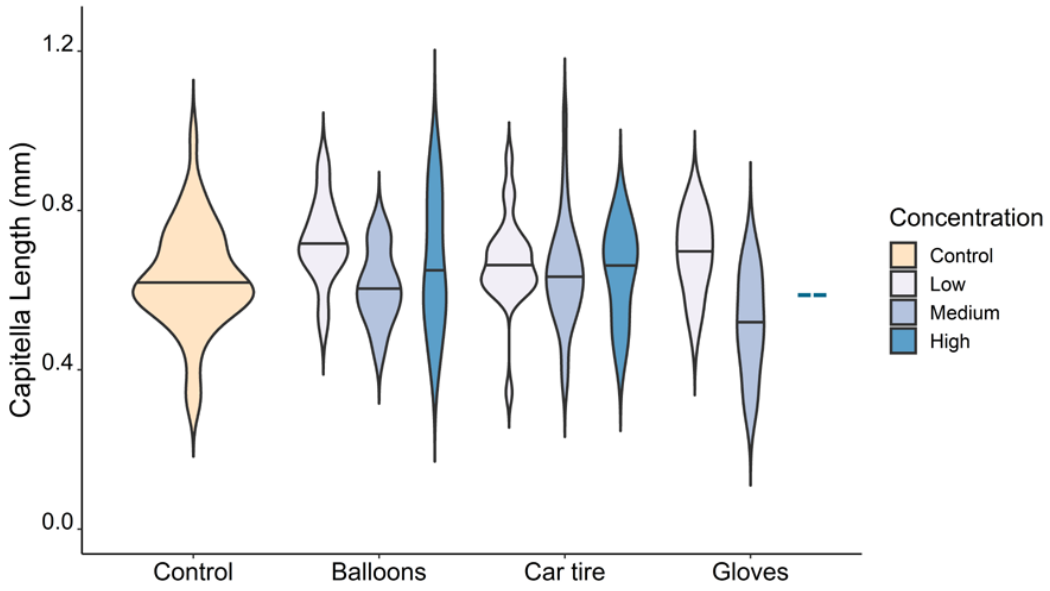

Figure 3.8: **Violin plot showing the distribution of *Capitella* spp. length of after 21 d of exposure**, including median as a black line (n = 16-40). No measure was included for the lab glove high treatment, due to 100% mortality in this group.

3.4 Comparison of baseline toxicity and *Capitella* mortality

Results from chemical analysis, baseline, and chronic toxicity testing of the materials selected for the *Capitella* study are displayed in Table 3.1, and the relationship between baseline toxicity and *Capitella* spp. mortality is shown in Figure 3.9.

Table 3.1: **Test results for materials selected for *Capitella* toxicity experiment.**

Material	Chemical features[a]	baseline toxicity[b]	*Capitella mortality*[c]
Control	-	-	22.6
Balloons	70	4.2	27
Car tires	89	0.04	68
Lab gloves	42	0.46	100

[a]Unique chemical features detected by GC-MS
[b]EC_{20} (g plastic L^{-1}) from BLT test
[c]Mean percent mortality after exposure to high concentration leachate (10 g L^{-1}) for 21 days.

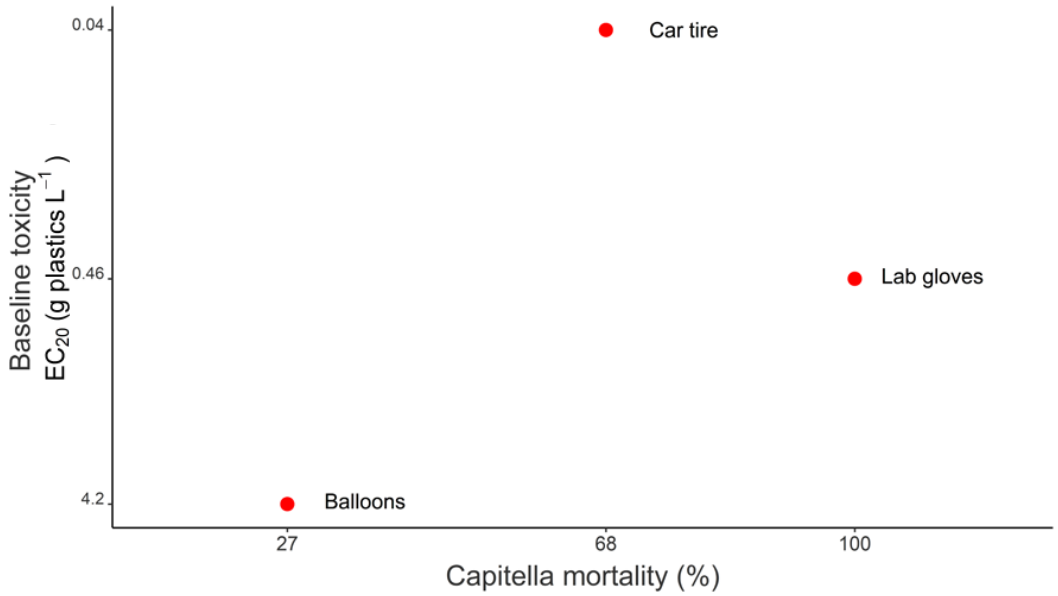

Figure 3.9: **Relationship between baseline toxicity (mean EC_{20} (g plastic L^{-1})) determined in the BLT screen and *Capitella* spp. mortality**, depicted as mean mortality (%) from the highest concentration of each material.

Chapter 4

Discussion

4.1 Plastic products contain a high number of chemical features, and the polymer type partly explains the number of features

Non-target chemical screening using GC-MS revealed a high number of chemical features in the selected plastic products, with half of the products containing > 50 features (Tab. 2.1). Few other studies report the total number of detected features from chemical screening. Furthermore, differences in detection method and analysis can make the comparison between studies difficult. In their 2019 study, Zimmerman and colleagues found a range of 0-194 features when they investigated 34 plastic products using GC-QTOF-MS, 15 of these contained > 40 features (Zimmermann et al., 2019), which is comparable to results in this thesis. However, in their more recent study, they were able to detect a total of 41,000 features in 42 plastic samples using UPLC-QTOF-MS/MS (Zimmermann et al., 2020). This shows differences in sensitivity between different analytical techniques and indicates that the number of chemicals in samples from this study could potentially be much higher than detected by GC-MS in this study. This highlights how complex the chemical profiles of plastics can be and indicates that assessing toxicity based on single chemicals can be a very limited approach.

There were significant differences in chemical composition between the polymer types, with a low number of chemical features present in PET and a relatively high number in vinyl and elastomer products (Fig. 3.1). The processing of polymers requires different additives depending on their material properties (Lithner et al., 2011). In addition, polymers of the same type are usually used for similar products, requiring separate sets of properties (Hahladakis et al., 2018). This can explain some of the observed differences in chemical composition between polymers. As an example, PET can be processed without the use of additives (Bach et al., 2012; Djapovic et al., 2021)(Bach et al., 2012, Djapovic et al., 2021), while the production of PVC requires high concentrations of plasticizers (Hahladakis et al., 2018). Despite the trends observed for polymer types, there was high variability between the individual products. As an example, chemical features ranged from 25–102 in elastomers. This shows that the chemical composition is not solely based on the polymer type. While it is difficult to generalize, the results presented here show that plastics have complex compositions and may contain large numbers of chemicals.

4.2 The majority of plastic extracts tested induced significant baseline toxicity

Testing methanol extracts from 50 plastic products with the BLT screen revealed that 47 samples inhibited luminescence compared to control (Fig. 3.2). In this strain of bacteria, light emittance is directly related to their metabolic activity, and a reduction in luminescence can be used as a measure of reduced activity (Van De Merwe and Leusch, 2015). This reduced luminescence can be referred to as baseline toxicity since it is a general and unspecific response that can be caused by multiple modes of action. Based on the results from this study, it is evident that most plastic products contain chemicals that induce baseline toxicity.

The polymer type was a significant predictor of baseline toxicity, and the clearest trends in this study were the relatively low toxicity of PE and PET and generally high toxicity of elastomers. The elastomer that induced the highest baseline toxicity in this study was car tires, which has been found to induce high toxicity in previous studies (Capolupo et al., 2020; Halsband et al., 2020). Car tire wear is the largest source of MP emission in Norway with an estimated annual release of 5000 metric tons (Mepex, 2014). Additionally, particles derived from tires used in artificial turf fields increase the total MP pollution by car tire further. The high toxicity induced by these materials is therefore important to evaluate. Most other studies find low chemical toxicity from PET products (reviewed in Groh and Muncke, 2017). However, there are also contradicting findings including a study that reported PET leachates to be the most hazardous to the echinoderm larvae *Paracentrotus lividus* out of 16 different food packaging leachates (Piccardo et al., 2021). Polymer type can partly help to explain toxicity, however, due to the large variation in toxicity within polymer groups, it cannot be used alone as a reliable predictor of toxicity.

4.3 Chemical composition was not a significant predictor of baseline toxicity

The number of chemical features detected in plastic extracts was not a good predictor of baseline toxicity in samples from this study, and samples with a high number of chemicals displayed both high and low baseline toxicity. As an example, sample 31, which is a reusable shopping bag, had the highest number of detected chemical features (102) but induced medium baseline toxicity (EC_{20} = 26.4). Similarly, Zimmermann et al. (2019) found that toxicity from multiple bioassays could not be predicted based on chemical complexity. This suggests that other aspects of the chemical content are more important regarding toxicity. Baseline toxicity could be more related to a few highly toxic chemicals present, and many studies focus mainly on few, well-known plastic chemicals, like bisphenols and phthalates, which are known to be highly toxic (Groh et al., 2019). However, it is more challenging to assess the toxic potential of unknown and unidentified chemicals as no chemical structures or analytical standards are available. Additionally, the concentration of compounds could be highly important regarding toxicity, and a low number of chemicals present at high concentrations in plastics could induce strong baseline toxicity, compared to plastics with a high number of chemicals present at low concentrations. Even chemicals with low potency can induce toxic effects at sufficiently high concentrations. This thesis used data from non-target chemical screening with GC-MS, which is a qualitative measure, and does not reveal the concentrations of the observed chemical features. Concentrations of chemicals present in the selected plastic products are therefore outside the scope of this thesis to

evaluate. In summary, the low correlation between the number of chemicals and baseline toxicity demonstrates that chemical aspects other than the total number of features are more important in determining the baseline toxicity of plastic extracts.

4.4 Two of three plastic leachates induced high mortality in *Capitella* spp.

To investigate whether samples with high baseline toxicity would induce mortality *in vivo*, leachates from three products were used for chronic exposure of *Capitella* spp. In the highest concentrations tested, both leachate from car tire and lab gloves significantly increased mortality to *Capitella* spp. larvae. This reveals that (1) harmful chemicals from these products have the potential to leach to seawater under realistic conditions, and (2) leached chemicals can cause elevated mortality *in vivo*. No other studies on the toxicity of plastic leachate to *Capitella* were available. However, studies on other polychaetes report that microplastics can cause negative effects, like reduction in regenerative capacity in *Perinereis aibuhitensis* after exposure to polystyrene particles (Leung and Chan, 2018) and immunotoxicity from MP exposure in *Hediste diversicolor* (Revel et al., 2018). *Capitella* are known to be highly resistant to pollutants (Reish, 1957) and a strong toxic response in this species might indeed be a warning sign for toxicity to other more sensitive species. However, it should be considered that the larval stage, which was chosen for this study, is considered the most vulnerable life stage (Mohammed, 2013). While chemicals leaching from two of three plastic materials induced strong mortality of *Capitella* spp. in this study, it remains uncertain if plastic chemicals pose a hazard to these species in environmental conditions. This will depend on the bioavailability of plastic-associated chemicals in a natural setting.

For the two treatments that induced mortality, the timing of the toxic response showed a similar pattern. The mortality was high during the first seven days of exposure, before leveling off. This pattern could be due to the developmental stage of the larvae: as earlier life stages are known to be more susceptible, exposure happening during early development (i.e., the first part of the experiment) could be more detrimental and therefore cause higher mortality. It could be related to individual differences, where the polychaetes that survived the first stage of the experiment were the fittest and were subsequently not susceptible to the leachate in the latter part of the experiment. A build-up of tolerance over time is another plausible cause, that would suggest the polychaetes might have acclimated to the exposure with unknown defensive mechanisms. For instance, this could happen by induction of detoxifying enzymes that can partly negate the chemical effects (Parkinson et al., 2019). This study does not evaluate the mechanisms of toxicity that occur and as such cannot determine the cause of the specific time pattern of when mortality was highest. However, this could be caused by higher resistance in more developed polychaetes, survival of the fittest, or a build-up of tolerance.

4.5 *Capitella* spp. growth was negatively affected by exposure to leachate from lab gloves

Despite the high mortality in animals exposed to car tire and lab gloves, the growth of *Capitella* spp. was only affected by exposure to leachate from lab gloves. The difference in length was not significant but will be discussed here, nonetheless. Due to the 100%

mortality of *Capitella* exposed to the high concentration of lab glove leachate, length could not be measured in this treatment. However, animals exposed to the medium concentration of the lab glove leachate were shorter at the end of the 21-d exposure, at which concentration no effects were observed on mortality. This indicates that effects on the growth of *Capitella* spp. can be observed at lower concentrations of leachate than effects on mortality. Mortality often correlates well with other sub-lethal endpoints, which can be more sensitive (Horie et al., 2017; Di Paolo et al., 2015). From the high concentration of car tire leachate there was high mortality in *Capitella* spp., but here there was no negative effect on the growth. It is possible that the mechanism of action of the two leachates differs, and that growth was affected to a higher degree than mortality by chemicals leaching from lab gloves. However, this difference could be an artifact biased by the selected sample in the car tire high treatment, where only 32% of the polychaetes survived until the end of the experiment. Variance in growth was large across all treatments, reflecting variation in development between individuals, and possibly variation in length introduced during selection before the start of the experiment. Although non-significantly, lab glove leachates seemed to negatively affect growth in *Capitella* spp., and growth was a more sensitive endpoint than mortality for lab glove leachate.

4.6 Baseline toxicity could aid in predicting toxicity *in vivo*

In this study, the number of chemical leachates tested on *Capitella* spp. (n=3) was too low to properly assess the correlation between baseline and in vivo toxicity. However, some comparisons were made (Fig. 3.9). Baseline toxicity assays can be predictive of toxicity in higher organisms as seen for algae and daphnids (Escher et al., 2008; Weltens et al., 2014). All three materials selected for the *Capitella* spp. experiment induced high baseline toxicity in the BLT screen. Although all were high, the range of the observed toxicity was large, with two orders of magnitude in difference between the highest and lowest EC_{20} value. The balloon sample induced the lowest baseline toxicity, with an EC_{20} of 4.2 g plastic L^{-1}, and this was the only sample to not induce any observed toxicity in *Capitella* spp. at the highest tested concentration. This could indicate that the baseline toxicity observed for the sample is below the threshold which would induce toxicity in *Capitella* spp. of the measured endpoints. Surprisingly, the chemicals leaching from lab gloves were the most toxic *in vivo* while car tire, which displayed the highest baseline toxicity, displayed lower toxicity *in vivo*. This rather large opposite observation in the two tests could have several explanations. One possibility may be different extraction efficiencies of the solvents used in the BLT and the *in vivo* toxicity tests (methanol and seawater). As an example, car tires contain metals like zinc and nickel, and the leachability of these metals could differ between sea water and methanol. In one study, Bocca et al. (2009) found that the leaching of metals from car tire was significantly higher in acidic media (pH = 5) than in deionized water. Alternatively, the mechanism of toxicity could differ between the organisms which could explain differences in toxicity between the materials. This study shows that plastic-associated chemicals that display high baseline toxicity *in vitro* are toxic to *Capitella* spp., perhaps, above a certain threshold.

4.7 Biological implications from the current results are complex to determine

This study demonstrated that chemicals present in 46 out of 50 samples trigger baseline toxicity, and two out of three plastic leachates induce profound mortality in the polychaete *Capitella* spp. However, the biological implications of these results in the natural environment are not obvious. When reaching the ocean, chemicals associated with plastic production can desorb from plastic debris. At the same time, other hydrophobic contaminants can sorb to plastics from the surrounding media, depending on the equilibrium state of the plastic item with the environment. These processes and the relative importance of MP as a vector for transferring hydrophobic contaminants were reviewed and reinterpreted by Koelmans et al. (2013a,b) Here, the authors find that most hydrophobic contaminants will be at equilibrium with MP within two years after release to the ocean. While this might take longer for larger plastic items, their model of the age distribution of plastic in the ocean shows that 80-90% of ocean plastic is older than 2-4 years. This indicates that most ocean plastics are close to, or at, sorption equilibrium regarding hydrophobic contaminants. The overall flux of hydrophobic contaminants from natural prey is also expected to be much higher than that of MP in most environments. Therefore, the authors state that ingested MP is not an important vector in transferring plastic-associated chemicals to marine organisms.

Even if MP or other plastic items may not be important vectors for exposing marine organisms directly, marine plastic pollution will transfer plastic-associated chemicals to the ocean, where marine organisms might be exposed to them through all relevant media (e.g., water, sediment, MP, and prey items). In a review of the occurrence of plastic additive chemicals, Hermabessiere et al. (2017) found that plastic additives have been detected in marine waters worldwide. Polybrominated diphenyl ethers and phthalates have been found in concentrations up to 10 ng L^{-1}, and nonylphenol and bisphenol A are also frequently detected. Furthermore, because most plastic chemicals are highly hydrophobic, their concentrations in sediments are much higher compared to the water column (Hermabessiere et al., 2017).

Heavily polluted areas and places with limited water exchange might contain concentrations of plastic-associated chemicals that are harmful to marine organisms. One study found high mortality in the marine fish orchid dottyback (*Pseudochromis fridmani*) after exposure to plastic leachate containing nonylphenol in concentrations of around 40 ug L-1 (Hamlin et al., 2015). This concentration is lower than what has been found in the vicinity of several sewage treatment plants and much lower than the highest concentration found in one study, where 644 ug L^{-1} was detected near a treatment plant (Solé et al., 2000). Moreover, Li et al. (2016) found plastic leachates to be acutely toxic to barnacles in similar concentrations to what the authors expect can be found in tidal pools containing plastic debris.

In the current study the chemical features present in plastic leachates were not identified or quantified and can therefore not be related to concentrations detected in the environment. However, the concentration of plastic used in the leachates might be relevant in constrained or heavily polluted environments. Moreover, as polychaetes retain MP in the gut and preferably use MP when building their tubes (Knutsen et al., 2020), the relative importance of MP in the transfer of plastic-associated chemicals might be higher than what was estimated by Koelmans et al. (2013a,b). Nonetheless, toxic effects might only be expected in heavily polluted areas. However, in future scenarios, the plastic load in the environment

might increase too much higher levels (Lebreton and Andrady, 2019), where these exposure scenarios would have greater relevance.

4.8 Strengths and limitations

The variety in chemical content and baseline toxicity between and within different polymer types is vast, and thus, analyzing the 50 plastic products selected in this study can only provide a limited part of the total picture. Nonetheless, this study is to my knowledge, the most comprehensive study in comparing chemical features and baseline toxicity. Most studies focus on single polymers, and the most comprehensive studies I have found to date are two studies by Zimmermann et al. (2019, 2020) where a total of 34, and 42 plastic products were examined, the latter focusing on bioplastic materials.

Analysis with GC-MS only reveals a certain fraction of the chemicals present in plastic extracts since this technique only detects semivolatile and nonpolar organic compounds. In addition, differences between analytical instruments, different data analysis strategies and solvents used, will affect which chemicals are detected (Zimmermann et al., 2020). Hence, the data presented here may only show a fraction of the total number of chemical features present in the plastic products. For the MicroLEACH project GC-MS was chosen as the optimal technique due to the comprehensive NIST libraries that are available for compound identification. For most toxicants, the toxicity to organisms is determined by the quantities the organism is exposed to. As this non-target method does not reveal the concentration of the chemical features present, the information about toxic potential is therefore more limited.

Both chemical analysis and baseline toxicity screening are based on methanol extracts. The use of solvents like methanol is expected to extract more chemicals than what would be extracted or leached into water, and methanol extracts may therefore contain chemicals and concentrations that would not be bioavailable from plastics in the environment. Yet, for screening larger amounts of plastics for toxic potential, methanol extracts are useful as they will contain most of the chemicals that might leach. In this way, they represent a 'worst case' scenario (Zimmermann et al., 2019).

The BLT assay is less widely used than the related Microtox assay that employs a similar setup using the bioluminescent bacteria *Aliivibrio fischeri*. However, it has some clear advantages, namely its low cost, high throughput, and high tolerance for varying temperatures during experimental setup (Van De Merwe and Leusch, 2015). In the current study some problems with the method occurred. Firstly, high variation of luminescence was evident within negative control on individual well plates. This variation differed between experiments, but some patterns remained the same, namely that the top row had significantly lower luminescence than the average. As this was the largest source of the variation, the removal of the data of the samples analyzed in this row (highest concentration) was necessary. Analysis including or excluding the first row did not affect the EC_{20} of highly toxic samples. However, samples with medium range toxicity had higher variation in calculated EC_{20}. The ranking of toxicity between these samples should therefore be considered with caution. Secondly, during the study a new batch of cryo-preserved bacterial culture (cryo-culture) was prepared by incubating a cryo-preserved aliquot with growth media. As the preparation of cryo-culture described by Van De Merwe and Leusch (2015) is based on reconstitution of freeze-dried culture, method optimization was required to find optimal incubation time, and procedure for centrifugation and reconstitution in

growth media with glycerol. After preparation of the new cryo-culture, it became apparent that the sensitivity differed between the cryo-culture batches, even when the same level of luminescence was measured in the negative control. Therefore, the new batch of cryo culture could not be used for experiments with comparability to experiments conducted with the old cryo-culture. Due to this, a third individual experiment could not be conducted for all samples as planned, and retesting at adjusted dilutions was not possible for all samples where this was required to calculate a valid EC_{20} value. The low cost, which is one of the advantages of the BLT screen, is partially based on the possibility of maintaining a supply of bacterial culture within individual laboratories by producing new batches of cryo-culture from aliquots, rather than being required to re-purchase freeze dried bacteria. A drawback of this approach is that this might produce grater variability between batches, as was experienced in this study. This makes comparability between studies more difficult but should not affect the validity of comparisons within a study using the same batch.

For the *in vivo* experiment, only three plastic products were examined, which limits comparisons of toxicity between plastic products and comparison of observed toxicity *in vitro* and *in vivo*. Rather than testing a wide variety of plastic products, this study prioritized to include several replicates and a range of three concentrations. The high number of polychaetes used, together with the inclusion of four exposure vessel replicates, lead to the robust nature of the results from this study where the likelihood of random mortality affecting the outcome is very low. Several concentrations was included to enable measurement of effects on mortality, as well as sublethal endpoints. Based on earlier experiments from SINTEF Ocean, it was estimated that the larvae would mature to adult polychaetes during the three-week experiment (unpublished data). This was not the case, and the polychaetes remained larvae until the end of the experiment, with low growth rate in both exposed and control polychaetes. Start length of larvae was not recorded to avoid causing unnecessary stress to experimental animals. Subsequently, this unrecorded individual variance might have become more important due to the low change in length and rendered it difficult to detect effects on growth. Moreover, in mature polychaetes it would additionally be possible to measure reproductive segments at the end of the experiment, which could be used to assess effects on the reproductive capacity, providing an additional endpoint to be measured. The reason for the slow maturation in this experiment is unknown, but it could be related to experimental conditions such as the beakers used as the exposure vessels, temperature, or water condition, or it could be related to biological variability, for example genetic variety or time of year. Nevertheless, with knowledge of the slow maturation, it could be deemed more useful to conduct a shorter acute toxicity study that would rather include several more material leachates.

All three materials used in the *in vivo* test were elastomers, making it impossible to make comparisons of *in vivo* toxicity between polymer types. Typically, when comparing toxicity between different materials, different polymer types are used, and conclusions are often made on which polymer type is more or less toxic to the study species. However, results from this study demonstrate that *in vivo* chemical toxicity can vary substantially between products from the same polymer type. Therefore, conclusions for a polymer type should not be made when only one or very few materials of each polymer type are used in the experiment.

The use of non-model species for toxicity testing is an important contribution to understanding the toxicity of a pollutant as the few model species commonly used cannot always give a representative picture of how a pollutant affects the ecosystem in general. However, using a non-model species has some operational drawbacks and challenges to be aware of. Since standardized protocols often do not exist, method optimization is required, which can

be time intensive. Additionally, for species not often studied, less information is available on their response to different laboratory conditions, and there are fewer studies on toxic responses to other environmental contaminants, preventing adequate comparison. In this study, the non-model *Capitella* spp. was used. This genus has previously been used in field studies, and there are some laboratory toxicity studies using *Capitella*, but these use adult polychaetes (Fang et al., 2018; Selck et al., 2003; Uc-Peraza and Delgado-Blas, 2015; Warren, 1976). As there is high genetic variability within the *Capitella* genus it is also difficult to compare responses and optimized methodology between different species. In this study, a relatively high mortality was observed in control (23% after 21 days). This mortality did not mask the clear toxic responses of car tire and lab glove leachate; however, it can make the test less sensitive to subtle changes, due to larger unexplained variance. This problem could be avoided somewhat if using a standard laboratory organism which has a high tolerance to laboratory conditions.

The small size and indistinguishable color of the *Capitella* spp. larvae introduced some challenges during the counting and water exchange. Dead larvae would disintegrate quite rapidly and could be mistaken for food particles and other debris. The mortality counts were therefore based on the number of surviving polychaetes found and transferred to the clean beaker with fresh leachate solution. This can potentially introduce bias during the counting of the larvae, and future studies should consider this limitation when choosing study species. In this study, the increased mortality in the toxic treatments was profound, and mistakes possibly introduced during counting are considered highly unlikely to have affected the results presented. However, some mistakes in the death count cannot be ruled out.

4.9 Conclusion and outlook

Plastic products are becoming more and more important in modern society and following the current trend, plastic production and subsequently plastic pollution will continue to increase. Therefore, it is important that plastic products that are produced are as safe as possible, especially products used as food contact materials, and products prone to end up in the environment, like single use items. This study demonstrates that there are profound differences in chemical toxicity of individual plastic products. Less toxic alternatives often exist for additive chemicals (Lithner et al., 2011), and products with similar function can have highly different toxicity (Hamlin et al., 2015). Efforts should therefore be made to implement safer alternatives, especially where already available. Additionally, since chemical toxicity is not solely based on known plastic additives, but also many unknown chemicals, *in vitro* toxicity assays should be conducted to screen for highly toxic plastic products.

This study demonstrated the toxicity of chemicals from a range of plastic products, however, the partitioning in environmental compartments and the bioavailability of these chemicals in realistic conditions are not well established. Further research should consider the difference in toxicity between plastic products due to different loads of associated chemicals and evaluate the relative importance of MP as a vector for these chemicals. For benthic species, the relative importance of the sediment as a vector of exposure should also be considered.

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Appendix

Appendix A

Capitella uptake study

A.1 Methods and remarks

To establish if the cultured *Capitella* spp. would consume plastic particles and which particle sizes were readily ingested an uptake study was attempted. There is no established method available for studying particle uptake in this species and several methods were tried for exposure and visualization of MP particles.

One method for quantification of particles ingested is exposure of the animal followed by digestion of the animal. Optimally this should leave only the plastic particles that have been ingested, which can then be visualized e.g., by staining with Nile Red. To test if this method could be viable for determining MP uptake in *Capitella* spp., several methods for digestion of the polychaete were attempted. A dedicated plastic-free lab room was used for the procedures. Here plastic equipment is limited to a minimum, and dedicated cotton lab coats are used. Lab personnel avoid wearing clothes made from plastic fibers inside the lab, and the door was kept close with sealing. Before any procedure, the surface areas were cleaned with filtered MQ water and then filtered ethanol, using soft tissue paper. Plastic lab gloves are only used when necessary due to health environment and safety measures. Filtration of ultrapure water and ethanol were done with glassware and a 7 um paper filter. All equipment used was rinsed with filtered MQ watered followed by filtered ethanol and again with filtered ultrapure water. First, a gentle digestion using 10% KOH with 10% Tween was tried. Polychaetes were rinsed and dried on a metal filter (10 µm). The filter was then transferred to a glass petri dish and KOH + Tween was added covering the filter. The dish was then covered with a lid and incubated at 50 °C overnight. Visual inspection determined that the polychaete was not fully digested. Further, digestion of the polychaete in a petri dish without the metal filter was tried. Digestion was also attempted using H2O2 alone and as an added step after KOH+Tween. None of these attempts were successful in digesting the complete polychaete. If deemed possible to distinguish polychaete remainders and plastic particles it could be an option to quantify particle uptake with partly digested polychaetes. Therefore, it was attempted to stain plastic particles; un-digested polychaetes; and digested polychaetes with NileRed and visualize these samples using blue-green UV light and an orange filter connected to a camera. Each sample was placed on a 10 um metal filter rinsed with ultrapure water and ethanol. 10 mL Nile Red (1% in ethanol) was added to the sample and left to incubate for exactly 30 minutes. The sample was then rinsed with ultrapure water and ethanol and let dry before the filter could be placed in the camera station for visualization. Visualization of the samples proved that the microplastic particles

could not easily be distinguished from stained polychaete fragments. As some fragments in the digested polychaete sample appeared as possible plastic fragments the procedure was attempted again after a stricter cleaning routine to rule out the possibility of plastic contamination. However, the fragments observed were still not easily distinguishable from the plastic particle sample. As staining after partial digestion was ruled out, exposure of the polychaetes to fluorescent particles was tried. Nile Red staining is likely to also leak fluorescence possibly staining part of the polychaete. Therefore, fluorescent spherical beads (10 um) were used. The polychaetes were exposed for fluorescent beads in different concentrations, for several exposure times (1, 3, 5, and 24 h), and with and without added food. The polychaetes were then rinsed with clean seawater to remove any particles stuck on the outside of the polychaete and then visualized using the imaging station. Particles were observed through the body cavity, which were believed to be ingested by the animal. However, autofluorescence in the polychaete made it difficult to quantify this. Also, it could not be ruled out that some particles could still be attached to the outside of the polychaete.

Quantification of uptake can be done by analysis of fecal matter after exposure. This method can revile the fraction of MP ingested that is then excreted by the organism. Since little MP is believed to be retained by the organism this method is often applied as a preferred method as it can revile the total amount ingested and egested within a certain period, rather than the 'snapshot' captured if only analyzing the number of particles within the organism at a given time. To determine appropriate exposure time, polychaetes were exposed to MP for different amounts of time (1, 2, 3, 7, and 24 h). The polychaetes were then rinsed and transferred to clean seawater and monitored regularly to determine the appropriate amount of time to allow the polychaetes to egest. As polychaetes can ingest their own fecal matter finding the optimal egestion time when the fecal matter has been excreted, but before a substantial amount can be re-ingested is important. Production of fecal pallets was overall very limited. Most individuals produced none or very few fecal pellets. For most polychaetes, some form of fecal matter could be observed, mainly in the form of unassembled matter or slime. It became apparent that access to feed was necessary for pellet production, preferably not just during exposure, but also after transfer to clean seawater. Examination of the few fecal pallets that were observed reviled that MP in the pellets were not possible to observe directly. This was partly because of the hardness of the pellet, that could not be demolished easily and partly due to the fact that the fecal matter was semi-fluorescent making distinguishing of MP more difficult even if they appeared on the surface of the pellet. Therefore, fecal pellets from control and exposed polychaetes were digested in H2O2, filtered and photographed in the imaging station. Apparent luminescent particles from each filter where then counted. More suspected MP particles were identified in the exposed group, but the difference was not large enough that this method was though optimal.

A.2 Preliminary results and conclusions

Due to delays in delivery of milled plastic material and limitations in time we decided to exclude MP exposure as part of the *Capitella* spp. toxicity test, and further efforts in method optimalization of the uptake study were therefore discontinued. The initial efforts did not provide any quantifiable results; however, ingestion of some MP was observed through pictures of polychaetes (Fig. A.1,A.2). The conclusion is therefore that *Capitella* spp. can ingest MP in the size range of 10 um and may as a result be exposed to plastic-associated chemicals through MP ingestion.

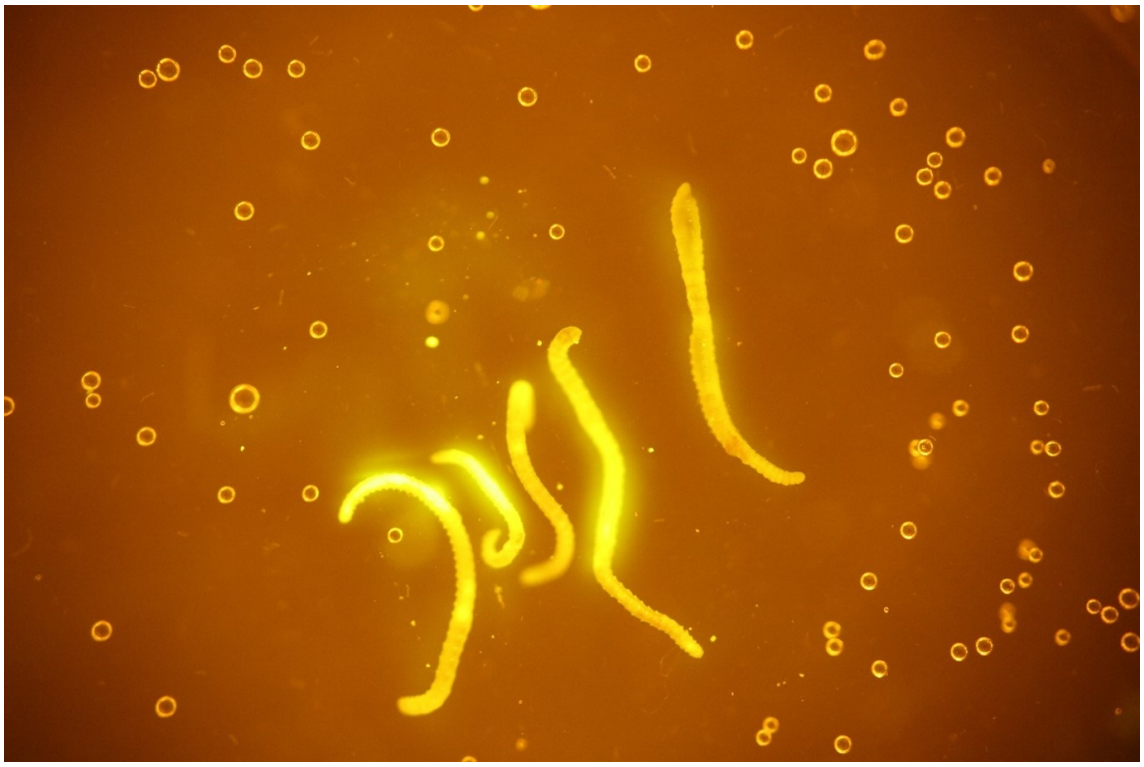


Figure A.1: **Polychaetes after 24h exposure with leaked luminescence from luminescent MP beads evident from the body cavity**

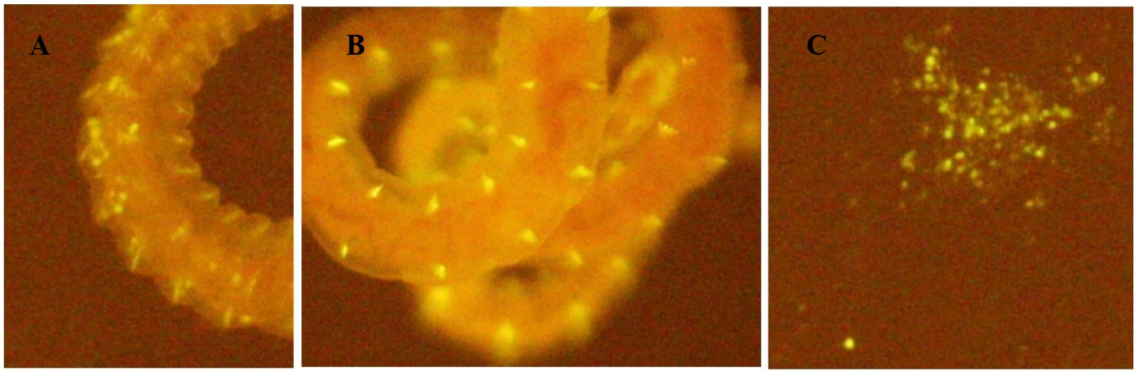


Figure A.2: **Fluorescence pictures of polychates** (A) Part of polychaete with fluorescent particles visible through body cavity. (B) Part of polychaete with natural fluorescent brush structures, and (C) fecal matter with fluorescent particles visible.

Appendix B

Water parameters leachates

Table B.1: **Water parameters for seawater leachates used for *Capitella* spp. exposure** measured after water exchange every second day during the 21 day experiment

Date	Treatment	Temp C	DO	Sal	pH
24-Feb	Control		97.4		
	Car tire Low		97		
	Car tire Medium		96		
	Car tire high		95.2		
	Lab gloves Low		95.7		
	Lab gloves Medium		97.4		
	Lab gloves High		94.1		
	Ballons Low		94.8		
	Ballons Medium		94.9		
	Ballons High		88.1		
26-Feb	Control		98.7		7.88
	Car tire Low		99.2		7.89
	Car tire Medium	15.5	99.2	34	7.88
	Car tire high	15.5	97.7	34	7.92
	Lab gloves Low	16	98.2	34	7.93
	Lab gloves Medium	15.6	98.2	34	7.92
	Lab gloves High	15.8	97.8	34	7.92
	Ballons Low	15.9	97.8	35.4	7.89
	Ballons Medium	15.6	92.4	35	7.94
	Ballons High	16.2	97.2	34	7.87
2-Mar	Control	16	100	35.9	7.75
	Car tire Low	16.1	99.5	35.8	7.8
	Car tire Medium	15.8	99.4	36.7	8
	Car tire high	15.6	98.7	38.7	8
	Lab gloves Low	15.7	99.2	41.2	8
	Lab gloves Medium	15.9	99.4	38	8
	Lab gloves High	15.9	98.7	35.9	8
	Ballons Low	16.1	99.5	52	7.8
	Ballons Medium	16	98.8	40.9	8
	Ballons High	16.2	96.9	38.4	8

Table B.2: **Table B1 continued**

Date	Treatment	Temp C	DO	Sal	pH
4-Mar	Control	15.2	99	35	7.5
	Car tire Low	15.3	99.3	36	7.7
	Car tire Medium	15.3	99.2	35	7.8
	Car tire high	15.1	99.2	36.5	7.8
	Lab gloves Low	15.5	98.9	35.7	7.8
	Lab gloves Medium	15.4	98.5	35.5	7.9
	Lab gloves High	15.5	98.1	34.7	7.9
	Ballons Low	15.6	98.2	39	7.9
	Ballons Medium	15.6	98.7	38	7.9
	Ballons High	15.6	92.8	38	7.9
8-Mar	Control	15.4	99	36	7.8
	Car tire Low	14.9	99.1	36.5	7.9
	Car tire Medium	14.8	98.9	36.3	8
	Car tire high	14.8	98.3	35.1	8
	Lab gloves Low	14.9	98.6	35.9	8
	Lab gloves Medium	14.9	98.1	35.6	8
	Lab gloves High				
	Ballons Low	14.9	97.7	35.5	8
	Ballons Medium	15	97	34.2	8
	Ballons High	15.2	94	33.8	8
10-Mar	Control	15.1	97.9	34.6	7.5
	Car tire Low	14.9	97.2	34.7	7.7
	Car tire Medium	14.9	96.8	34.9	7.8
	Car tire high	14.9	96	34.6	7.8
	Lab gloves Low	14.9	96.4	35	7.9
	Lab gloves Medium	15.2	96.1	34.1	7.9
	Lab gloves High				
	Ballons Low	14.9	96.2	34.7	7.9
	Ballons Medium	15	95.7	33.9	8
	Ballons High	15.2	93	33.5	8
12-Mar	Control	15.2	94.2	34	7.9
	Car tire Low	14.8	94.4	34.8	8
	Car tire Medium	14.7	94.4	34.7	8
	Car tire high	14.8	93.6	34.5	8
	Lab gloves Low	14.9	93.6	35.2	8
	Lab gloves Medium	15.1	93.8	34.3	8
	Lab gloves High				
	Ballons Low	14.9	94	34.6	8.1
	Ballons Medium	14.8	93.8	34.2	8.1
	Ballons High	14.9	91.5	33.7	8.1

Appendix C

P values from statistical tests

Table C.1: **P values of difference in mortality compared to control at 96 hours**, significant p values are bold

Treatment	p values
Car tire Low	>0.1
Car tire Medium	>0.1
Car tire High	**<0.001**
Lab gloves Low	>0.1
Lab gloves Medium	>0.1
Lab gloves High	**<0.001**
Balloons Low	>0.1
Balloons Medium	>0.1
Balloons High	>0.1

Table C.2: **P values of difference in mortality compared to control at 21 days**, significant p values are bold

Treatment	p values
Car tire Low	>0.1
Car tire Medium	>0.1
Car tire High	**<0.001**
Lab gloves Low	>0.1
Lab gloves Medium	>0.1
Lab gloves High	**<0.001**
Balloons Low	>0.1
Balloons Medium	>0.1
Balloons High	>0.1

Table C.3: **P values of difference in length compared to control at 21 days.**

Predictors	p values
Car tire Low	>0.1
Car tire Medium	>0.1
Car tire High	>0.1
Lab gloves Low	>0.1
Lab gloves Medium	0.083
Lab gloves High	-
Balloons Low	>0.1
Balloons Medium	>0.1
Balloons High	>0.1

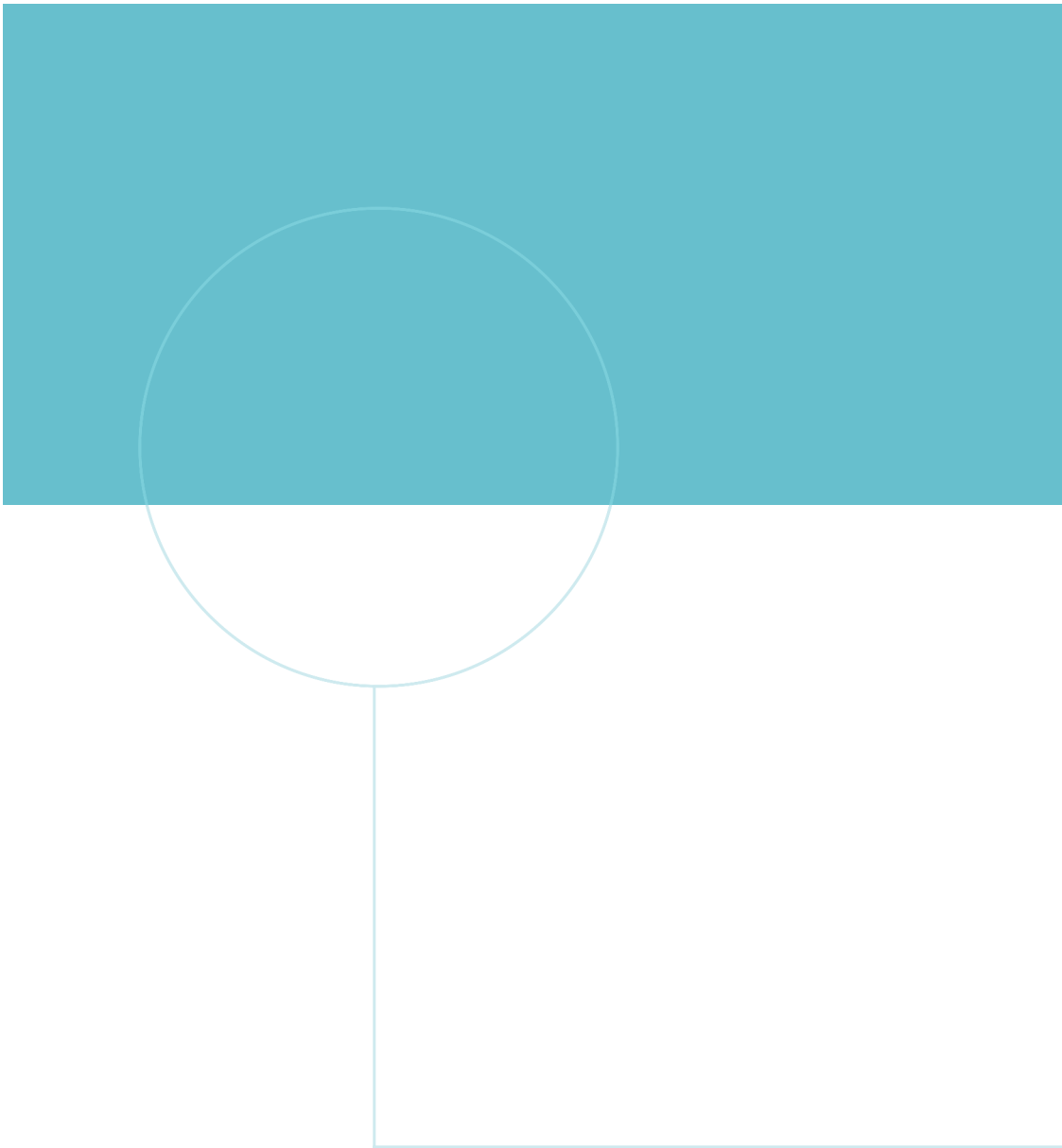
Appendix D

BLT results

Table D.1: **Results and highest tested concentration for samples tested in the BLT screen**.Max. lum. inhibition denotes the inhibition of luminescence (%) at the highest tested concentration. P values explain difference from control for maximum luminescence inhibition, and significant p values are bold.

ID	EC_{20}(g plastic L^{-1})	Highest tested conc.	Max. lum. inhibition	p value
1	1.22	75.25	99.27	**<0.001**
2	1.15	75.00	99.13	**<0.001**
3	25.90	75.50	60.49	**<0.001**
4	69.05	75.00	25.24	>0.1
5	22.25	71.00	29.28	0.065
6	64.02	75.00	23.00	>0.1
7	8.79	31.00	16.76	>0.1
8	>75	75.00	4.32	>0.1
9	1.74	59.00	82.71	**<0.001**
10	5.68	73.00	73.40	**<0.001**
11	67.50	75.00	24.45	>0.1
12	2.89	69.00	71.31	**<0.001**
13	37.24	71.43	14.24	>0.1
14	>75	75.25	12.90	>0.1
15	16.40	51.00	22.41	>0.1
16	26.56	70.75	40.43	**0.006**
17	46.23	73.75	32.43	**0.031**
18	12.28	50.00	21.58	>0.1
19	1.41	53.50	87.16	**<0.001**
20	28.68	75.50	33.47	**0.038**
21	5.48	72.00	72.33	**<0.001**
22	33.41	75.50	43.85	**0.0022**
23	30.13	71.50	39.55	**0.0081**
24	33.72	75.00	51.90	**<0.001**
25	>75	75.75	4.52	>0.1
26	43.59	75.50	31.10	0.065
27	96.33	75.00	14.92	>0.1
28	74.20	70.75	14.14	>0.1
29	38.27	61.67	34.40	**0.03**
30	75.00	72.65	18.81	>0.1

31	26.40	75.25	47.17	**<0.001**
32	24.57	51.50	26.37	>0.1
33	4.20	76.00	88.96	**<0.001**
34	2.30	76.25	98.55	**<0.001**
35	<1.17	75.00	97.74	**<0.001**
36	<1.17	75.50	96.67	**<0.001**
37	<1.17	76.00	91.15	**<0.001**
38	7.33	75.75	71.20	**<0.001**
39	10.83	75.25	71.73	**<0.001**
40	7.29	76.00	77.80	**<0.001**
41	2.47	75.50	80.86	**<0.001**
42	2.41	76.50	99.89	**<0.001**
43	7.73	52.00	51.86	**<0.001**
44	67.37	73.00	20.93	>0.1
45	1.60	75.00	76.33	**<0.001**
46	8.25	71.00	62.20	**<0.001**
47	1.06	61.00	96.60	**<0.001**
48	8.24	76.25	65.77	**<0.001**
49	13.44	76.50	53.71	**<0.001**
50	26.22	69.50	61.83	**<0.001**



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