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Exposure of marine invertebrates to car tire rubber: Uptake of rubber particles and related organic chemicals

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Abstract

Car tire rubber particles (CTRPs) are a major source of microplastic pollution to the environment, originating from abrasion during driving or the use of crumb rubber granulates made of discarded tires. However, large knowledge gaps remain regarding the distribution and presence of CTRPs in marine environments and interaction with marine organisms. The aims of this thesis were to investigate the uptake of CTRPs and associated organic chemicals in marine invertebrates exposed to car tire rubber (CTR), and to assess the use of these chemicals as markers of CTRP exposure in their environment. To do so, we used blue mussels (*Mytilus edulis*), shore crabs (*Carcinus maenas)* and edible crabs (*Cancer pagurus*) exposed to CTRPs. First, blue mussels (n = 60) were experimentally exposed for seven days to low or high concentrations of a CTRP mix (with a tenfold increase), followed by a depuration period of seven days and sampled throughout the experiment. In addition, crabs were sampled *in situ* in reference sites (Kristiansand) and in a highly contaminated site, by Norway's largest car tire shredding facility in Frierfjorden (Southern Norway) were investigated for ingested rubber particles in stomachs (n = 49). Tissues of mussels and crab gills (n = 33) were analyzed for targeted CTRP-related compounds, specifically 6PPD and its transformation product 6PPD-quinone using gas chromatography/mass spectroscopy (GC/MS). Seven CTRP-related chemicals (6PPD, CPPD, DPPD, TPPD, DTPD, $C_{21}H_{26}N_2$ and $C_{23}H_{26}N_2$) were found in all experimentally exposed blue mussels, indicating that all of them ingested CTRPs and/or took up leachates from their surrounding water. We reported uptake of all associated chemicals in highly exposed individuals, except for 6PPD-Q. Moreover, our data indicated the accumulation of some chemicals during exposure (TPPD and (DTPD), as well as a linear relation between the exposure dose and the 6PPD uptake in mussel tissue. DTPD was detected in high concentrations and decreased slowly in mussels post-depuration and appears as a promising candidate marker in blue mussels for CTRPs in surrounding environments. While no large CTRPs (> 0.3 mm) were detected in wild crabs, 6PPD was detected at similar concentrations in gill tissue of both species from all sites, further highlighting gills to be a useful tissue to detect 6PPD. Overall, our study emphasizes that blue mussels and both crab species can be prime monitoring candidates for exposure to CTRP contamination in the environment. Their use as biomonitors of CTRP exposure is enhanced by their ecological characteristics such as the sessility of blue mussels and the robustness of crabs towards heavily polluted waters in addition to being both of relevance for human consumption.

Abbreviations

ACN	Acetonitrile
AIC	Akaike´s Information Criterion
ANOVA	Analysis of variance
CEMP	OSPAR´s Coordinated Environmental Monitoring Program
CPPD	N-Cyclohexyl-N´-phenyl-p-phenylenediamine
CR	Crumb rubber
CRG	Crumb rubber granules
CTR	Car tire rubber
CTRP	Car tire rubber particle
DPPD	N,N´-Diphenyl-p-phenylenediaminie
DTPD	N,N´-Di-o-tolyl-p-phenylenediamine
ELT	End of life tire
GC-MS	Gas chromatography mass spectroscopy
ISTD	Internal standard
IUCN	Internationally Union for Conservation of Nature and Natural Resources
LOD	Limit of detection
Log K_{OW}	*n*-octanol–water partition coefficient
LOQ	Limit of quantification
MED POL	The Programme for the Assessment and Control of Marine Pollution in the Mediterranean
MILKYS	Miljøgifter i kystområdene
MP	Microplastic
n.d.	Not detected
NP	Nanoplastic

PPD	*p*-Phenylenediamine
RAS	Recirculating aquaculture system
RSTD	Recovery standard
SD	Standard deviation
TRWP	Tire and road wear particles
TPPD	N-o-Tolyl-N'-Phenyl- p-phenylenediamine
TWP	Tire wear particles
URMS	Urban runoff mortality syndrome
Ww	Wet weight
6PPD	N-(1,3-dimethylbutyl)-N′-phenyl-p-phenylenediamine
6PPD-Q	N-(1,3-dimethylbutyl)-N′-phenyl-p-phenylenediamine -quinone

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1 Introduction

During previous decades, plastic production has increased rapidly, with a simultaneous mismanagement of plastic waste (Derraik, 2002). Estimates predict that 19 - 23 million metric tons of plastic are annually (2016) emitted to aquatic ecosystems (Borrelle et al., 2020). Large plastic items (macroplastics) are a significant environmental concern due to their slow degradation and accumulation in the ocean and on shorelines. Their negative impact on marine life, including entanglement and ingestion has made them a focus in environmental research. Lately, the smaller size fractions of microplastics (MPs) and nanoplastics (NPs) have raised concern. MPs have been found attached to external and internal organs in a range of species throughout the water column, in invertebrates, fish, sea birds and mammals, including commercially exploited species intended for human consumption. (Botterell et al., 2022; Courtene-Jones et al., 2017; Devriese et al., 2015; Li et al., 2015; Murray & Cowie, 2011; Smith et al., 2018). Harmful effects caused by uptake of MPs and leached chemicals have been shown in marine organisms from both experimental studies and in the wild (Wright et al., 2013a). Due to a great variety in the sources and properties of MP particles, there are still large knowledge gaps on the effects of MPs on marine ecosystems.

1.1 Microplastic in marine environments

Microplastics (MP) are defined as plastic particles < 5 mm and result from primary- and secondary sources (Cole et al., 2011). While primary MP are manufactured microscopic particles made intentionally for applications in cosmetics, personal care products, medical drugs or in other industrial activity (Auta et al., 2017; Cole et al., 2011), secondary MP originate through the degradation from larger plastic items by a various of chemical, physical, and/or biological processes. For example, weathering or photooxidation of plastic structures can result in fragmentation to MP or NP particles (Sundt et al., 2014). Around 15 – 31% of all MPs in the oceans are estimated to originate from primary sources, while the remaining fraction originates from secondary sources (Boucher & Friot, 2017). As such, land-based activities are significant contributors to MPs pollution to the world's oceans (Sundt et al., 2014) with the largest contributors being fibers from synthetic textiles and particulate from car tire rubber (CTR) (Boucher & Friot, 2017). It is noteworthy that primary MPs have also been defined as fibers released from synthetic clothes or car tire rubber particles (CTRPs) have also been defined as primary MPs (Boucher & Friot, 2017; Kershaw & Rochman, 2015). Accordingly, CTRPs are being considered as primary MP in this thesis. In general, MP particles come in a variety of

shapes, sizes, and polymers with varying additive compositions resulting in properties leading to differences in physiological and biochemical toxicity (Choi et al., 2018).

Yet, after MPs reach the marine environment, their movement and distribution are not fully understood. Different plastic polymers have different densities resulting in particles being distributed throughout the water column according to their physical properties, with the largest fraction ending up at the sea floor (Pham et al., 2014). MPs exposed to natural seawater are subject to biofouling, which can increase their density and accelerate their sinking rate towards the seafloor (Halsband, 2021, p.6). Biofilm formation of natural microbes on the surface of MPs can make them more attractive as food items, increasing the ingestion rates (Vroom et al., 2017). Ingested and excreted MPs will also be transported to the seafloor through the food chain, in fecal pellets or within the bodies of sinking dead organisms (Cole et al., 2016). Particles tend to accumulate in certain sediment layers mainly determined by their density properties suggesting availability for several benthic species (Courtene-Jones et al., 2017; Devriese et al., 2015; Graham & Thompson, 2009; Li et al., 2015; Murray & Cowie, 2011; Thompson et al., 2004).

1.1.1 MPs as vectors of chemicals

Plastic additives are chemical compounds added to the plastic polymers during manufacturing to provide a broad range of desired characteristics. Additives are mainly used as pigments, antioxidants, plasticizers, flame retardants and stabilizers (Rochman et al., 2019; Teuten et al., 2009). In most case, additives are not chemically bound to the polymer and can therefore potentially leach out to the environment (Hahladakis et al., 2018). The different physicochemical properties of additives as well as the properties in the surrounding environment (e.g. pH, temperature and salinity) will influence the timing and intensity of leakage (Kwan & Takada, 2017, p.54). The environmental impact on plastic additives has raised concerns since many of them are known to be hazardous substances (Mato et al., 2001; Teuten et al., 2009). These substances/chemicals can reach aquatic organisms through two main routes: direct exposure through uptake of particles and indirect exposure through aqueous media contaminated by chemicals leached from plastics. MPs have also been reported to absorb surrounding contaminants and metals on their surface, providing an additional route for chemicals to enter marine organisms upon uptake (Hirai et al., 2011; Mato et al., 2001).

1.1.2 Interaction with marine biota

Microplastics have been showen to interact with organisms through respiration and ingestion with species throughout the water column, including the deep benthic habitat (Galloway et al., 2017; Taylor et al., 2016). Namely, MPs have been found attached on the gill surface in crabs and fish (Białowąs et al., 2022; Watts et al., 2014; Yin et al., 2018). Due to their small size resembling that of plankton and other suspended material, the particles are easily mistaken as food by suspension feeders, filter feeders, detrivorous, and predators (Wright et al., 2013a), especially when being masked in biofilm (Fabra et al., 2021). Both field studies and exposure experiments have reported ingestion of microplastics in marine organisms with findings in the digestive system (Botterell et al., 2022; Browne et al., 2008; Murray & Cowie, 2011; Wright et al., 2013b; Yin et al., 2018). After being consumed, MPs may remain in the organism's digestive tract, be egested in fecal matter, or be translocated to different tissues. In a study conducted by Farrell & Nelson (2013), MPs were introduced to crabs through polluted blue mussels (*Mytilus edulis*) as their food source. The study found relocation of particles from the stomach of crabs to hepatopancreas and ovaries and demonstrated trophic transfer of MPs through the food web. Another study by Brown et al. (2008) observed MPs (~ 10 μm) to be distributed in the circulatory system of blue mussels after ingestion, with a retention time of over 48 days. A study which investigated the uptake of MPs (8 – 10 μm) in shore crab (*Carcinus maenas*) by comparing the retention time between the digestive tract and gills (Watts et al., 2014), showed that MPs remained in the gills for a longer period (21 days) compared to the stomach (14 days). This suggests that the gills also represent a route for uptake of MPs.

1.1.3 Effects in marine organisms

Upon uptake, MPs can cause negative physical effects on marine organisms by clogging gills, feeding apparatus and digestive tracts (Murray & Cowie, 2011; Taylor et al., 2016; Wright et al., 2013a). The presence of plastic items in the digestive systems of organisms can lead to injuries on organs and have been shown to reduce feeding capacity and proper digestion (Cole et al., 2015; Wright et al., 2013a). Most effect studies have been performed in controlled laboratory experiments. For example, Wright et al., (2013b) showed that the uptake of MPs in lugworm (*Arenicola marina*) lead to suppressed feeding, inflammation, and depleted energy reserves. Other studies have observed several negative health effects such as oxidative stress, reduced reproduction, and cellular damage (Browne et al., 2013; Wright et al., 2013b). A study by Watts et al., (2016) reported some effects from MPs interaction with gills, specifically reduced branchial function, although these effects were transient.

As shown in section 1.1.1., particles can act as vectors for harmful chemicals attached on the surface or from leaching additives (Auta et al., 2017; Hirai et al., 2011; Rochman et al., 2014; Teuten et al., 2009). Several of these chemicals are hazardous substances including toxic organic chemicals, persistent organic pollutants (POPs), polyaromatic hydrocarbons (PAHs), and heavy metals (Hirai et al., 2011; Rochman et al., 2014). The leached additives can interfere with biological processes and cause toxic effects, including disruption of the immune – and endocrine systems, development, reproduction, or cause carcinogenesis (Cole et al., 2011; Lithner et al., 2011). The effects can be acute, depending on the chemical and the species (Brinkmann et al., 2022; Tian et al., 2021), while some pollutants can accumulate and persist within organisms over time. Through trophic transfer, persistent pollutants can potentially biomagnify in the food web, reaching high level in top predators, including humans where such chemicals can pose severe health risks (Li et al., 2021; Smith et al., 2018).

1.2 Car tires

1.2.1 Composition

Car tires are made of a complex mixture of a synthetic polymer-based material combined with a range of additives and chemical residues. The formula differs between tire-models as they are designed for different vehicles, weather conditions, producer, and quality. Tire surfaces are made of treads, where the largest fraction consists of an elastomer material which is commonly based on Styrene Butadiene Rubber (SBR), combined with natural rubber, polyisoprene rubber and butadiene rubber (Capolupo et al., 2020; Rogge et al., 1993; Sundt et al., 2014). The second largest fraction is commonly represented by fillers (carbon black and silica) used to reinforce the rubber, vulcanization agents (e.g. sulphur, zinc and stearic acid), protective agents (antioxidants (e.g. 6PPD, CPPD and DPPD)), and processing aids (e.g. plasticizers, softeners and peptizers) (Halsband et al., 2020; Rogge et al., 1993; Sundt et al., 2014; Wik & Dave, 2009).

1.2.2 Sources of CTRPs

Car tire rubber particles (CTRPs) are one of the major contributors to worldwide MP pollution, estimated to reach the marine environment at an annual rate of 500 000 tons (Boucher & Friot, 2017; Hann et al., 2018). In Norway, CTRPs are also estimated to be the largest contributor to MP pollution, with emissions exceeding 7000 tons per year (Sundt et al., 2021). CTRPs include tire-wear particles (TWP) and crumb rubber (CR). TWPs are generated through abrasion from

frictional interaction between tires and the road surface, are small in size (10 nm to several 100 μm) and exhibit elongate shapes (Kole et al., 2017; Rogge et al., 1993). CR granulates are made from end-of-life tires (ELTs) which give rise to another pathway of CTRP to the environment, as the downcycled material is shredded to pieces and commonly used as granulate infill or composite in playgrounds, as fill on football fields and running tracks, and on boat and ferry docks (size up to several mm)(Simon, 2010). The contribution of CR granulates (CRG) from football fields to the total MPs pollution in Norway was estimated in 2020 to be approximately 30% of the total MPs emission of 19 000 tons (Sundt et al., 2021). In 2021, over 60 000 tons of ELTs were collected in Norway, most of which were further shipped to Ragn-Sells (Skjerkøya/Bamle), the main facility collecting used car tires in the country (Dekkretur, 2021).

1.2.3 Transport to the marine environment

There are large knowledge gaps regarding the transfer of tire rubber particles once they have reached the environment. Rubber particles can be transported by wind and water and are found in snow, rivers, sediment, air and the ocean (Rødland et al., 2022; Sommer et al., 2018; Sundt et al., 2021; Vogelsang et al., 2019). Tire wear and tear contribute to 5-10% of the total amount amount of plastics ending up in the ocean, making it a significant contributor to the overall pollution (Kole et al., 2017). Factors such as infrastructure, geographical location of the pollution source or roads will affect the proportion of CTR that will reach the marine environments. Tire rubber has a density of approximately 1.2 g/cm^3 (Degaffe & Turner, 2011) and particles from highway runoffs and detention basins have been found to have a density ranging between 1.5 – 2.2 g/cm^3 (Kayhanian et al., 2012), suggesting that particles reaching the ocean will sink and potentially reach the benthic habitat.

1.2.4 CTRPs in biota

Bråte et al. (2020) found rubbery fragments to be the most prevalent type of microplastic in blue mussels at 16 out of 100 sites in Nordic waters, suggesting that these fragments originated from tire rubber wear particles (TRWP). Laboratory studies have also demonstrated the uptake of tire particles in other invertebrates (Hägg, 2022; Redondo-Hasselerharm et al., 2018, Siddiqui et al., 2022). Siddiqui et al.(2022) demonstrated negative adverse effects by exposing early life stage mysid shrimp (*Americamysis bahia*) and Inland Silverside (*Menidia beryllina*) to different concentrations of tire particles at micro and nano size and to leachates across a salinity gradient. Although few studies have examined the interaction between CTR and marine biota, existing research indicates that marine organisms can take up CTR and associated

chemicals. In a recent study by Hägg (2022), adult lumpfish (*Cyclopterus lumpus*) were exposed to crumb rubber granulates (CRG) mixed with food. The study documented the ingestion of CRG by lumpfish and the uptake of several related organic chemicals in their blood. It is however unclear whether these chemicals can accumulate in organisms and can be transferred to higher trophic levels.

1.2.5 CTR leachates

In a recent study by Müller et al. (2022), tires were found to contain 214 organic chemicals, of which 145 were identified as leachable. About 60% were found to be mobile compounds, which suggest that they can be easily released and spread to the environment. Another study by Halsband et al. (2020) revealed large numbers of rubber leachates in sea water from crumb rubber granulates, which included benzothiazole, zinc, PAHs and several heavy metals. Additionally, other studies have reported leachates of para-phenylenediamines (PPDs), such as N-(1,3-dimethylbutyl)-N′-phenyl-p-phenylenediamine (6PPD) and phenolic compounds. Some of these tire-derived chemicals are known to be toxic for marine organisms, when exposed at both low and high concentrations (Capolupo et al., 2020; Halsband et al., 2020; Tian et al., 2021).

1.2.6 Identification of MP particles

Several analytical techniques are available for identifying MPs, including Fourier transform infrared spectroscopy (FTIR), Raman spectroscopy and gas- or liquid chromatography mass spectrometry. However, there is lack of standardized methods and the complex mixture of MP particles often requires the use of various analytical methods to identify the different types of MPs. For example, TWPs cannot be identified by Raman spectroscopy due to an unspecific spectrum (Wagner et al., 2018) nor with FTIR due to the interlinked polymeric structure, neither resulting in any IR absorption bands. A common approach to identify TWPs/CTRPs involves a combination of visual identification of potential particles with a stereo microscope. However, this method is limited by its inability to identify small size fractions and to distinguishing black synthetic particles from natural ones. To address this, other detection methods have been investigated to identify chemical markers of TWP/CTRPs concentrations in the surrounding environment (Klöckner et al., 2019). While chemical markers such as zinc have shown some success (Vogelsang et al., 2019; Wagner et al., 2018), zinc has not only many other sources in addition to CTR but is also regulated in organisms. An alternative promising marker candidate

for CTRPs is the antioxidant 6PPD, which has been suggested as an interesting option (Wagner et al., 2018), see section 1.2.3.1. for more details on 6PPD.

1.2.7 Highlighted rubber additives

p-phenylenediamines (PPDs) are commonly used as antiozonants in manufacturing of car tires and other rubber products, primarily due to their high reactivity with ozone. Antiozonants work as protective agents, extending the lifetime of tires by preventing wear-out and cracking. N-(1,3-dimethylbutyl)-N′-phenyl-p-phenylenediamine (6PPD) is one antiozonant based on PPD and is commonly added in tires at levels of approximately 0.4 - 2% during manufacturing (Babbit, 2010, as cited in Foldvik et al., 2022). As tires undergo attrition, the concentration of 6PPD decreases over time as it migrates towards the surface, potentially leading to its leaching to the surrounding environment.

Environmental and human health concerns have been raised regarding PPDs, including 6PPD, N-cyclohexyl-N'-phenyl-p-phenylenediamine (CPPD), Diphenyl-p-phenylenediamine (DPPD) and N,N′-Di-o-tolyl-p-phenylenediamine (DTPD), which have been reported in e.g. airborne particles, dust, sediment, surface water or wastewater (Huang et al., 2021; Huntink et al., 2004). Toxicity testing of DPPD in rats has shown negatively effects on reproduction and development (Matsumoto et al., 2013). Additionally, 6PPD has been shown to induce acute lethality at high concentrations in several fish species (*such as Danio rerio, Gobiocypris rarus* and *Oryzias latipes)(*Di et al., 2022; Hiki et al., 2021; Varshney et al., 2022). Although the uptake and accumulation of the chemical in various species remains poorly studied, Hägg (2022) documented the uptake of 6PPD in tissues and other CTR-related organic chemicals from crumb rubber (CR) particle ingestion in lumpfish.

In 2002, 6PPD was added to the OSPAR List of Chemicals for Priority Action and registered under REACH regulations (OSPAR, 2006). Although 6PPD has raised high concern, it was not prioritized to be included in the REACH restricted chemical list (ECHA, 2023).

The reaction between 6PPD and ozone as well as metabolic processes can convert the compound to a quinone, 6PPD-quinone (Lattimer et al., 1983) (Figure 1). The Wild Coho salmon (*Oncorhynchus kisutch*) population in the USA is experiencing higher mortality during rain events causing high runoffs and stormwater also known as urban runoff mortality syndrome (URMS; Chow et al., 2019). High urban runoffs enter the stream and ocean, exposing salmons

to particles from car tire abrasion and a cocktail of chemicals. Namely, 6PPD-quinone was identified as the toxic chemical causing the mortality within the population (Chow et al., 2019; Tian et al., 2021). The acute toxicity of 6PPD-Q revealed high inter-species differences in vulnerability. Namely, coho salmon showed the highest 24 h LC50 (0.79 ug/L) compared to three other species within the same Salmonidae family (rainbow trout, brook trout, arctic char) and the white sturgeon (*Acipenser transmontanus*). While Arctic char and white sturgeon did not suffer any negative effects from exposure to high levels of 6PPD-Q, increased mortality occurred for rainbow- and brook trout at environmentally relevant concentrations (Brinkmann et al., 2022). In addition, other studies have reported sensitivity to 6PPD-Q in other aquatic organisms (Hiki et al., 2021; Varshney et al*.,* 2022). With a predicted log Kow of 5 – 5.5, 6PPD-Q has been suspected to accumulate in sediments and biomagnify in the food chain (Hiki et al., 2021; Tian et al*.,* 2021), even though this was not observed in a study in Hägg (2022). However, the mode of action and the level of toxicity of 6PPD and 6PPD-quinone in marine systems are still broadly unknown.

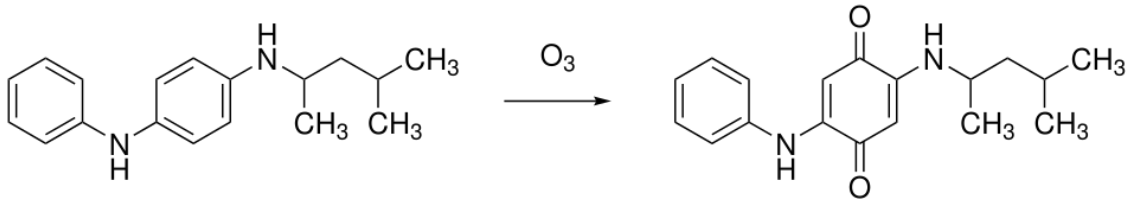


Figure 1 - The conversion from 6PPD to 6PPD-quinone by reaction with ozone (O3). From "6PPD oxidation" [Figure], by Gimli21, 2022, Openverse (https://openverse.org/image/6ad245c5-b3d7-4194-97be-d5c188bb0011?q=6ppd). CC BY-SA 4.0

1.2.8 Monitoring of MPs in marine environments

Monitoring of MPs in the marine environment contributes to the understanding of distribution and abundance of particles. By identifying sources and pathways of contamination, the knowledge can be utilized by authorities to reduce pollution to the environment (Jensen et al., 2022; Li et al., 2016). As such, studies reporting MP uptake, accumulation and toxicity can contribute to increased attention and action regarding the problematic materials. Several monitoring programs (CEMP, MEDPOL and MILKYS) have used blue mussels as a bioindicator as they have been shown to uptake and accumulate anthropogenic pollutants, including MPs (Beyer et al., 2017; Farrington et al., 2016; Mathalon & Hill, 2014; Van Cauwenberghe & Janssen, 2014). In addition, mussels have a high tolerance for environmental parameters (oxygen, temperature, salinity, and food availability), a broad global distribution (Gosling, 2003, p.49) and are sessile, long-lived organisms that can form large beds of individuals easily collected in shallow waters (Beyer et al*.,* 2017; Gosling, 2021, p.417). As

filter-feeders, they transport water over their gills and can accumulate pollutants which reflect their surrounding environment (Gosling, 2021, p.417). They serve essential ecological services by cleansing water and are important prey for many organisms. Finally, mussels are easily grown in the laboratory, making them an ideal study species.

While experimental MP exposure experiments provide valuable knowledge, transferring this knowledge to the environment is more complex as MPs come in a variety of sizes, shapes, and different combinations of polymers and additives. Laboratory concentrations often rely on specific size fractions and plastic types and can exceed environmental concentrations (Watts et al., 2014; Wright et al., 2013a). The duration of experiments is often restricted, and long-term effects may not be captured. In addition, while experimental organisms are often exposed to single stressors (i.e., MPs), wild organisms are exposed to a variety of abiotic and biotic factors, which are difficult to mimic in laboratory facilities. To address these knowledge gaps, it is important to also investigate MP exposure and related chemicals in wild organisms to provide a comprehensive understanding of the impact of MPs role in marine ecosystems.

1.3 Research questions

The aim with this study was to investigate ingestion and the uptake and accumulation of CTR-related organic chemicals in marine invertebrates. First, we investigated if blue mussels (*Mytilus edulis*) ingest CTRPs and accumulate associated organic chemicals in an exposure experiment. Secondly, we collected *in situ* shore crabs (*Carcinus maenas*) and edible crabs (*Cancer pagurus*) from a polluted site of CTR and reference sites to investigate ingestion of rubber particles in stomachs and chemicals in gill tissue. We performed analyzes for target CTRP compounds, specifically N-(1,3-dimethylbutyl)-N′-phenyl-p-phenylenediamine (6PPD) and the transformation product 6PPD-quinone using gas chromatography/ mass spectroscopy (GC/MS). Finally, we discussed the potential of the identified chemicals in the organisms as markers or proxies for the presence of CTRPs in the surrounding environments.

To answer the questions of whether **marine invertebrates take up CTRPs and related organic chemicals**, the following points were investigated in this thesis:

1) Do marine organisms ingest CTRPs?

2) To what extent do marine organisms absorb and accumulate CTRP-related chemicals? Additionally, which specific chemicals among the ones considered can serve as reliable proxies for the presence of CTRPs in the environment?

3) Are mussel body tissue and crab gills useful matrices for the detection of CTRP-related chemicals?

2 Materials and methods

This section gives an overview of the studied species, sites they were collected from, collection methodology, laboratory procedures including method development and the choices of statistical analyses/models.

2.1 Study species

The chosen species are all commercially exploited and serve as ecological sentinels of the marine benthic habitat. They are all generalist feeders and have a broad geographical distribution.

2.1.1 Blue mussels - *Mytilus edulis*

Blue mussel *(Mytilus edulis,* Linnaeus 1758*) (*Mollusca) is a commercial and much farmed species. The mussel appears to have the widest distribution within the genus *Mytilus,* being found from subtropical areas to the Arctic (Gosling, 2003, p.49). They extend from high intertidal to subtidal zones, from sheltered to highly exposed locations, found in both brackish and saline waters (Bayne, 1976; Gosling, 2003, p.66). In addition to human consumption, the mussels are important prey for marine organisms including echinoderms, crustaceans, flatworms, fish, sea birds, walrus, seals and sea otters (Gosling, 2003, p.96-97). The mussels filter large volumes of water daily over ciliated gills which are functioning both in respiration and feeding (Gosling, 2021, p.417). Phytoplankton, bacteria, and detritus are its main sources of nutrition, and as a selective filter feeder it can ingest particles with a certain size and shape (Ward & Shumway, 2004). From the incoming water current, particles are taken up in mucus covered cilia on the gills surface and transported to the mussel's foraging apparatus, the labial palps and further into the stomach. The labial palps are equipped with structures producing pseudofaeces which can eliminate unwanted particles, as MPs, before ingestion.

2.1.2 Shore crab - *Carcinus maenas*

The shore crab (*Carcinus maenas*, Linnaeus 1758) (Arthropoda) is found from the North coast of Africa to Northern Norway, and sporadically in USA, The Red Sea, Madagascar, Sri Lanka and in Australia after being introduced (Ingle, 1997, p.150; Van der Meeren et al*.,* 2022). In Norway, the crab is found at shallow waters, mainly above 5-7 meters, but occurs downwards to 30 meters on all types of substrates. The adult crabs have a high tolerance for both temperature and salinity ranges (Klassen & Locke, 2007; Zanders, 1980). The crab is a generalist feeder, having a diet regarding on food availability, but mainly predates on mollusks,

annelids and other crustaceans (Scherer & Reise, 1981, as cited in Van der Meeren et al., 2022). They can be predators, grazors, scavengers, acting as carnivorous, herbivorious or omnivours (Scherer & Reise, 1981, as cited in Van der Meeren et al., 2022). As a scavenger, the crab plays an important role for the benthic ecosystem and is important prey for a variety of other crustaceans, fish, mammals and birds.

2.1.3 Edible crab - *Cancer pagurus*

The edible crab (*Cancer pagurus*, Linnaeus 1758) (Arthropoda) is a widely distributed specie in the north-east Atlantic Ocean. It is found sporadically in Europe from southern waters by the Canary Islands, to north in Norway. The highest abundance of the crab is in the North Sea, British Isles, the coast of France and by the Norwegian coast. (Brattegard, 2011; González et al., 2016). It inhabits a wide range of benthic habitats from soft to hard bottom, and is usually between intertidal zone, down to 100 m depth, (Neal & Wilson, 2008, p.3) but tend to search to warm waters during wintertime down to depths of 300-400m (Moen & Svensen, 2020, p.290). The crab is an active predator with a broad diet with mollusks and crustaceans being preferred preys. It tends to predate on smaller crabs from its own specie as well as on a variety of other crab species including the shore crab (*Carcinus maenas*) (Lawton, 1989; Mascaron & Seed, 2001). The edible crab is a highly commercial species, as in 2020 the global capture was above 40 400 tons (live weight) (FAO, 2022). Concerns are raised by consumptions of edible crab, especially of "crab butter" consisting of the hepatopancreas and ovaries. As the hepatopancreas is contributing to detoxifying (Štrus et al., 2019), this organ has the potential to accumulate toxins and is known to contain concentrations of heavy metals and dioxins above EUs recommendations. Intake of edible crab can exceed levels of tolerance of toxins.

Crabs' respiratory and digestive system

Crabs use their gills to ventilate water from its surroundings, mainly for respirational function. Water is drawn into their bodies through the base of their limbs and over gills, before expelling it through their mouth (Taylor, 1976). In addition, Watts et al. (2014) demonstrated that gills can serve as a route of uptake for MPs by exposing shore crabs to polystyrene microspheres (8-10 μm).

A crab's gut is divided into the fore-, mid-, and hindgut. After food is cut and crushed by mandibles in the mouthpart it passes through the esophagus into the foregut, where it is ground up by the gastric mill, which is equipped with tooth-like structures (Štrus et al., 2019). Between

the foregut and the midgut, ingested food particles sorted by size, and particles smaller than 100 nm are transferred to the hepatopancreas for further intracellular digestion (McGaw & Curtis, 2013 and references therein). The remaining material digested in the midgut and is then processed in the hindgut before being excreted as fecal pellets (McGaw & Curtis, 2013).

Environmental factors can influence the contraction rates of the foregut muscles and the gastric mill, however the typical food retention time for food in the gut of shore crabs is around 12 hours, with fecal pellets being fully evacuated from the digestive system after 12-48 hours (Hopkin & Nott, 1980 in Watts et al., 2014). Other species of the genus *Cancer* (except *Cancer pagurus*) have gastric clearance rates ranging between 15.5 – 36 hours (MacGaw & Curtis, 2013 and references therein.)

2.2 Blue mussel exposure experiment setup

The experiment included exposure of blue mussels (*Mytilus edulis)* to tire wear rubber and was conducted at the facilities of the Institute for Agricultural, Fisheries and Food Research (ILVO) in Belgium. Blue mussels with a body length of 4.9 ± 3 cm and sea water were collected on the 23rd of February 2022 on Coastbusters site, at the Belgian part of the North sea (Map of sampling sites: Appendix B). Seawater was stored on an ILVO storage tank and purification procedures were conducted from 23rd of February to the 14rd of March 2022, with 520 L of filtered water (1 μm and UV) filled into a recirculating aquaculture system (RAS). The mussels were fed with an algae mixture during the acclimatization procedure, but not during the experiment. Exposure experiments started on the 17th. A total of 156 acclimatized blue mussels were divided into three tanks (made of polypropylene and filled with 25L of seawater): a control group, and two treatment groups. A tire tread mix (TTmix), made of car tires from 20 different tires was prepared with a cryo mill using pure sea sand (SiO_2 to) a diameter of 23.7 ± 16.7 μm (volume: 76.4 ± 40.7 μm). Low and high concentrations were prepared with 0.05g/L of sandmix (0.01g/L of TTmix) and 0.5g/L of sandmix (0.1g/L of TTmix), respectively, and one tank without tire rubber served as a control. The low treatment was designed to mimic environmental conditions while the high treatment was 10 orders of magnitude higher to better identify concentration trends. Through the experiment, a pump system was active to ensure a constant flow of water in the tanks.

The experiment lasted for a total of 14 days during which the blue mussels in treatment tanks were exposed to TTmix the first 7 days. This was followed by a 7-day depuration period, during which the blue mussels were transferred to new aquariums with fresh seawater and were not exposed to any external sources of tire rubber. From this exposure experiment, this study utilized 60 mussels (out of 156; i.e., 20 per treatments). Blue mussels (n = 5) and water (10 mL) from each tank were sampled at days 1, 3, 7, and 14. On each sampling day, the width, length and total weight of each individual was recorded. Each individual was hereafter dissected, and the soft tissue was weighed and transferred to a glass vial (with a teflon (PTFE)/aluminum foil cap) and both mussel and water samples were stored in a freezer at -20°C. During the initial sampling day, five mussels from the low treatment tank and three from the control tank were found dead but no additional mortality was observed until day seven after which only one mussel from each tank was found dead. The dead mussels were handled and sampled in the same manner as the rest of the mussels.

The setup and sampling of water and mussels are shown in Figure 2. Table 7 in Appendix F shows the water parameters which were measured on each sampling day in each tank. Both the temperature and salinity were kept stable at 12°C and 33 ppt, respectively. The pH was highest in the high treatment aquarium on all sampling days.

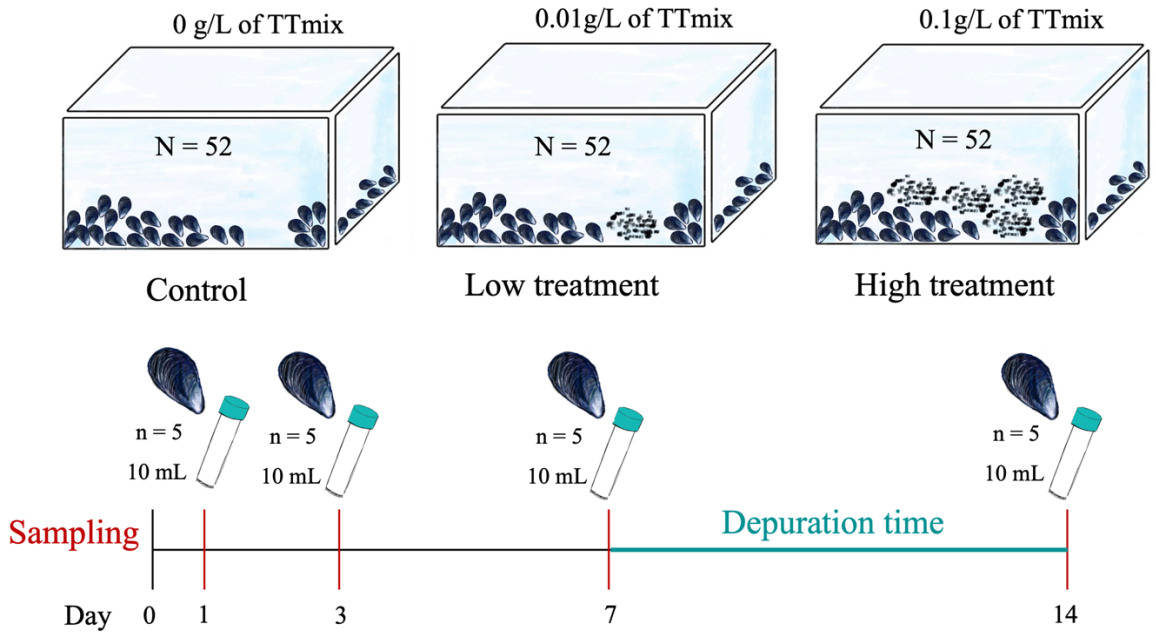


Figure 2 - Experimental setup of the blue mussel (Mytilus edulis) in 3 tanks with 7 days of exposure followed by 7 days of depuration time in clean tanks. One control tank with only sea water (0 g/L of TTmix), and one low exposure (0.01 g/L of TTmix) and high exposed (0.1 g/L of TTmix) tank with TTmix added to the waters. Sampling of mussels (n = 5) and water (10 mL) from the tanks were done on day 1, 3, 7, and 14 during the experiment.

2.3 A seabed polluted by car tire rubber

2.3.1 The Grenland fjords in Norway

The Greenland fjords in Southern Norway have had a great industrial activity and ship traffic for several decades, leading to heavily polluted waters (Fagerli et al., 2016). The Norwegian food authority has given recommendations to limit the intake of certain foods caught in these fjords. The recommendations are based on measurements high levels of toxicants in biota exceeding EUs limits of contaminants and heavy metals, especially mercury, dioxins and furans in seafood (Miljø og helse grenland, 2021).

2.3.2 Pollution from shredded car tires by Ragn-Sells in Frierfjorden

The recycling company Ragn-Sells is located on Skjerkøya, which is South-West in the 14 km long fjord Frierfjorden, in Grenland, Telemark (Lundbo, 2022). Ragn-Sells receives Norway's largest volume of end-of-life tires (ELTs) and has collected, stored and scraped tires since year 2000 (O. Paulsen, personal communication, February 16, 2023). At Ragn-Sells, ELTs are shredded to fragments with a diameter of 8-30 cm, stored at a barge until they are loaded and shipped for further treatment and usage (Figure 3). Satellite photographs from 2004 until today show activity and storage around the facility on a poorly secured barge and ramp (Appendix A, Figure 18) which has caused tons of rubber to spill into the fjord during operations. In 2019, the company carried out a clean up after a mapping of the seabed around the facility. An excavator on deck collected around 30 tons of shredded car tires during the cleanup. However, findings of eel (*Anguilla anguilla*) in the matter, being an endangered species, led to a stop in the cleanup activity (Fylkesmannen I Vestfold og Telemark, 2020).

Figure 3 - Picture showing Ragn-Sells car tire shredding facility on Skjerkøya and storage of shredded car tires at their barge (Google, n.d.).

In September 2020, The County Governor (Statsforvaltaren) in Vestfold and Telemark carried out an inspection of the waters from the barge at Ragn-Sells facility and up to approximately 40 meters away, using an underwater drone (Fylkesmannen i Vestfold og Telemark, 2020). The survey reported large amounts of shredded car tires at the seabed and Ragn-Sells was reported for violation of the law of pollution resulting in a fine of 1.5 million NOK and a requirement to conduct a cleanup of the seabed by 15th March 2021 (Øverbø & Hansen, 2022).

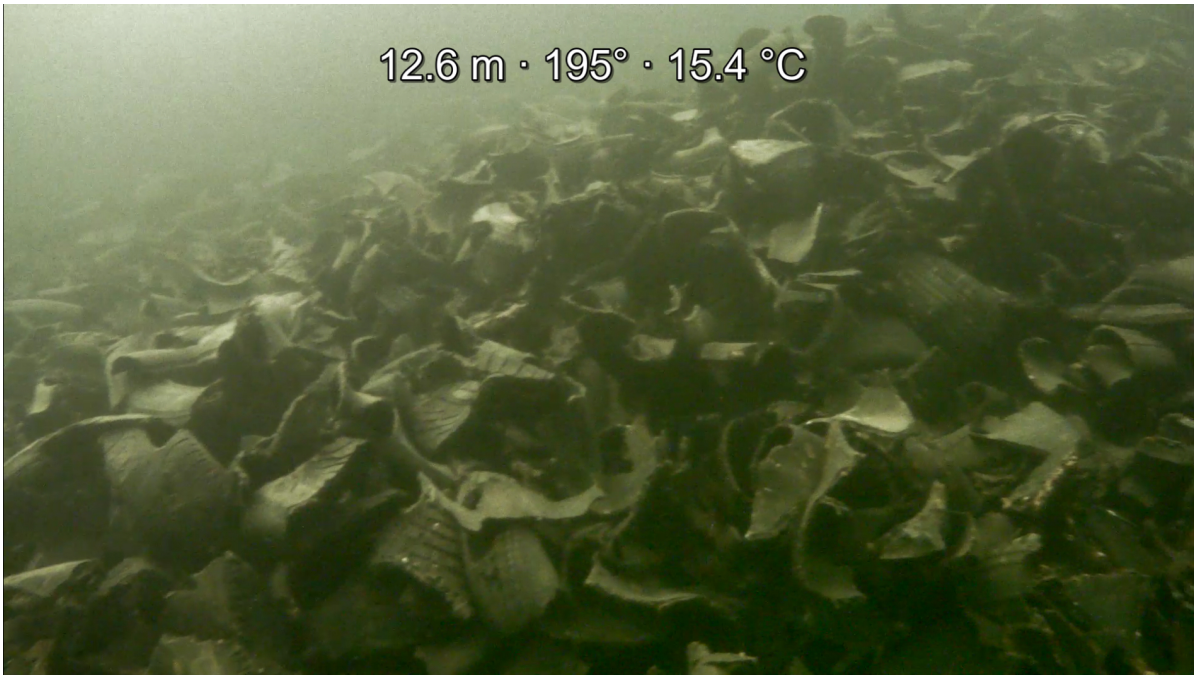


Figure 4 - A picture taken in October 2020 during an inspection by the County Governor in Vestfold and Telemark using a remote operated underwater vehicle (ROV) of the seabed near Ragn-Sells' facility in Frierfjorden. The picture shows the seabed covered with shredded car tires. (Statsforvalteren i Vestfold og Telemark, 2020). Reproduced with permission.

A cleanup of 5000 m^3 of seabed around the facility was completed during May-June 2021 (Koltsova & Breivik, 2023). The clean-up process involved the use of an excavator, airlift and manual labor. However, during the cleanup, Ragn-Sells discovered more rubber underneath their barge, which was later cleaned up during March-May 2022 (O. Paulsen, 2023).

A new inspection of the seabed was conducted by The County Governor in December 2022, revealing individual pieces of car tire rubber still present in the sediments. To prevent any new pieces from emerging, divers will clean the seabed twice a year. As of December 2022, approximately 100 – 120 tons of rubber has been removed from the seabed surrounding the facility and under the barge according to rough estimates (Koltsova & Breivik, 2023). Additionally, Ragn-Sells has improved the security of their barge and loading processes for shredded car tires (O. Paulsen, 2023).

2.3.3 Collection of marine organisms naturally exposed to CTR in Frierfjorden

In October 2021, marine organisms were collected by Akvaplan-niva nearby Skjerkøya (Figure 5). The aim was to investigate naturally exposed organisms to CTR and compare concentrations of related organic chemicals with the laboratory exposed blue mussels from section 2.2. Figure 6 shows Ragn-Sells facility at Skjerkøya on the day of sampling (12.10.2021). No blue mussels were found around Skjerkøya, and overall few marine organisms except for shore crabs (*Carcinus maenas)*, edible crabs (*Cancer pagurus)*, one squat lobster (*Galathea strigosa*) and one small unidentified starfish. For this project, we have therefore decided to focus on crabs.

Figure 5 - Organisms sampled in Frierfjorden. From the left: squat lobster (Galathea strigosa), edible crabs (Cancer pagurus) and the shore crab (Carcinus maenas). Frierfjorden, 12.*October 2021.*

Figure 6 – Picture taken of Ragn-Sells car tire shredding facility. From the left: Car tires and shredded car tires on land and on the dock. Frierfjorden 12.October.2021. (Christensen, G., 2021). Reproduced with permission.

2.4 *In situ* sampling of crabs

First, 36 crabs (22 shore crabs and 14 edible crabs) were caught around Skjerkøya in Frierfjorden (59.0597244° N, 9.6439396° E) in October 2021 in prior to the completed clean-up process (see chapter 2.3.4). In addition, in October 2022, 13 crabs were caught around Dvergsnestangen (6 shore crabs at 58.121068° N, 8.062831° E) and Terneholmen (7 edible crabs at 58.105616° N, 8.063942° E)(See chapter 2.4.2. and Figure 7 for a map of the sampling sites) and served as control individuals based on the assumption that these fjords were much less polluted than Frierfjorden. Crab traps were set four days and one day in advance of collection of crabs in Kristiansand and Frierfjorden, respectively.

At all locations, crabs were caught using traps baited with fish meat (cod or trout meat). Both crab species were killed according to the Norwegian Food Authority with a stroke on top of the shell, above the nervous center, wrapped in aluminum foil and frozen at -20°C. The crabs were flown to Tromsø and stored in a freezer at Akvaplan-niva at -20°C until further treatment.

Throughout this thesis, for the sake of clarity, the two sampling sites, Frierfjorden and Kristiansand will be referred to as the “polluted site” and the “reference site”, respectively. A map of the sampling sites can be found in Figure 7 with the abbreviations F1, K1 and K2 for sampling sites of crabs in Frierfjorden and shore crabs and edible crabs from different sites in Kristiansand, respectively.

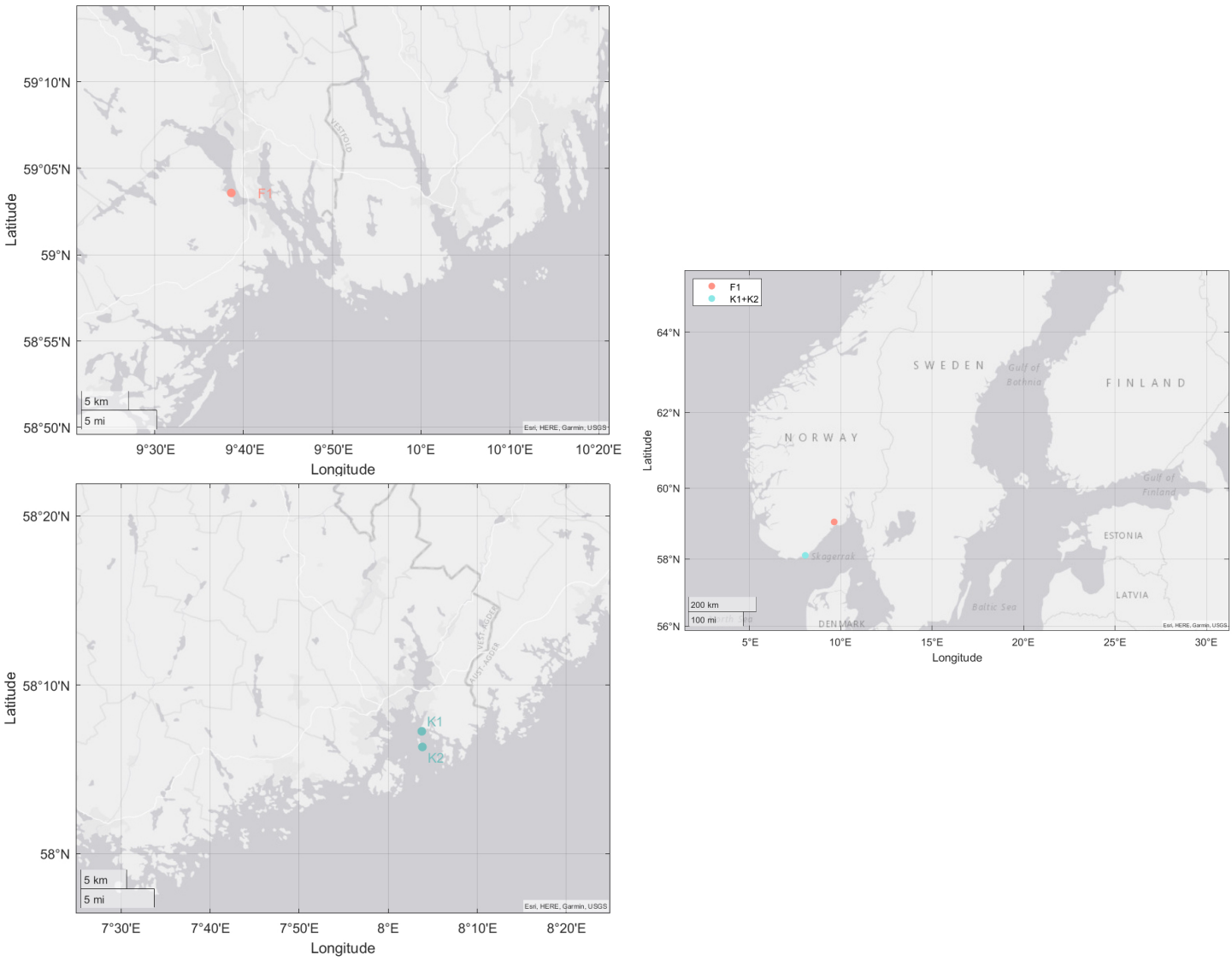


Figure 7 - Maps of the sampling sites in southern-Norway of edible- and shore crabs from the polluted site (F1, Frierfjorden, coral color) and shore crabs (K1) and edible crabs (K2) from the reference sites (Kristiansand, turquoise color).

2.5 Treatment of crab samples

Dissection and stomach investigation of crabs (n = 49) were carried out at the laboratory facilities of Akvaplan-niva in Tromsø. The analyses of organic chemicals and CTR particle analysis were performed in the laboratory at The Norwegian Institute for Air Research (NILU) in Tromsø.

2.5.1 Dissection and sample preparation of crabs

Glass vials and aluminum foil were burned at 450°C for 8 hours in prior to dissection. To avoid any cross-contamination during dissection, working surfaces were covered with new aluminum foil and all equipment was rinsed with tap water and ethanol between each sample. The crabs

were thawed at room temperature, approximately 1 day before start of dissection. The following sections describes the specific procedures for each species.

Weight, width, and length were recorded for each crab. Laying dorsally, the sex was identified. The crabs were cut open from dorsal side by cutting the shell open with a knife and scissors. The mass filling of the crab was noted, and the gills, hepatopancreas, gonads and stomach were individually sampled. In addition, were the claws opened with scissors and the muscles were sampled on glass vials. For only the shore crabs, various entrails were also sampled. All samples were frozen at -20°C until further analysis.

When cut open, a lot of body fluid leaked out of the crabs. Parts of this fluid, when possible, were collected with the sampled hepatopancreas. It appeared that some of the gill tissue had absorbed some of the body fluid, as it came in contact with various organs during thawing and dissection. Also, when both species of crabs were thawed, they leaked body fluid in the plastic bag and on the cutting board, so that the weight is not representative for the live weight of the crab.

2.5.2 Crab stomach investigation

A total of 49 stomachs from shore- and edible crabs were investigated for crumb rubber particles using a stereo microscope (Leica MDG41 (Camera: Leica DFC 3000 G)) with magnification from 8 x– 180 x. The stomachs were placed on a glass petri dish covered with a new layer of burned aluminum between each stomach. Scissors and a needle were used to open the stomachs to investigate them for rubber particles. Salt water (Milli-Q + NaCl) was added to the petri dish when stomach tissue dried up under the microscope to continue the investigation. Potential CTR particles were measured, and their pictures taken with a Leica DFC 3000 G camera in addition to the software program Leica LAS X. The particles were then wrapped in aluminum foil and stored on glass vials for further polymer identification and the stomachs were kept on glass and frozen for further analysis.

Several of the stomachs contained small grids of sand which made it difficult to distinguish potentially small CTR fragments from the grain. Small particles could not be confirmed through visual identification nor with FTIR. It is thus reasonable to assume that the number of particles were highly underestimated. Only a few particles found in the crabs' stomachs were evaluated as potentially originating form CTR. These particles were further analyzed with Fourier transform infrared spectroscopy (FTIR).

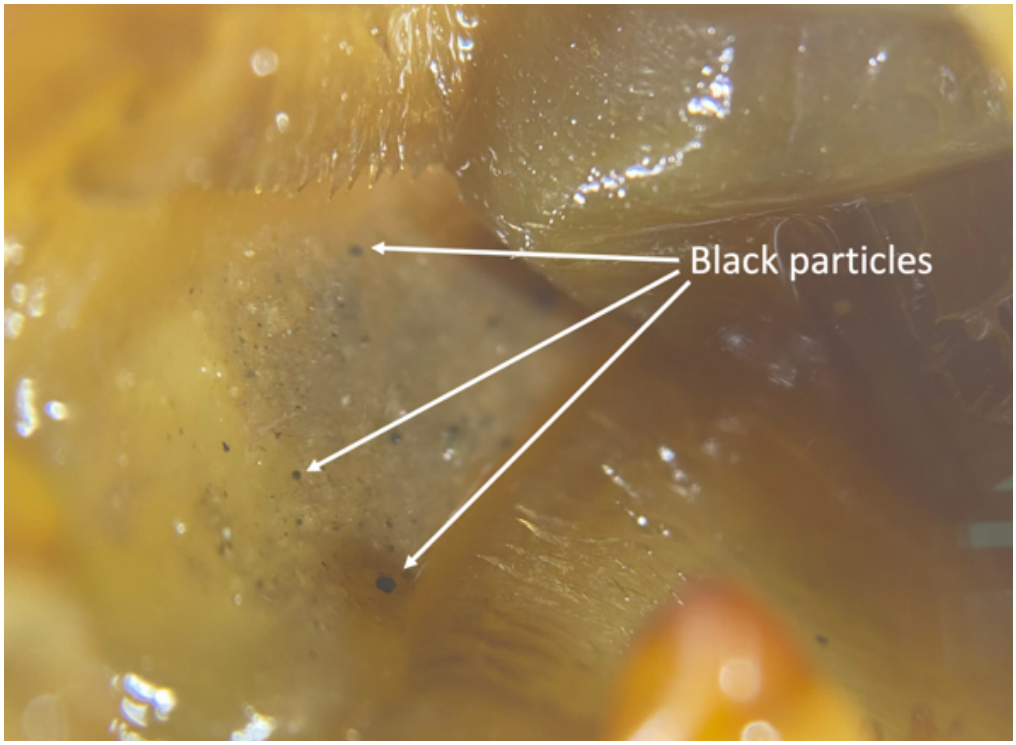


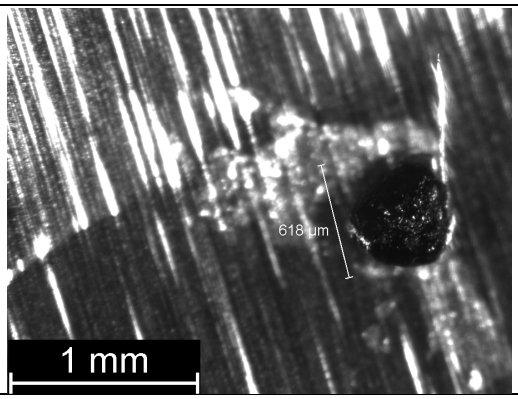
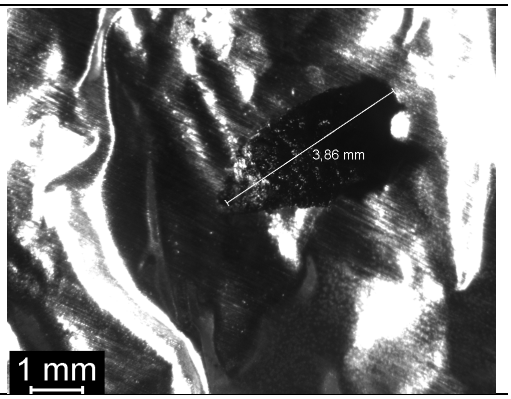
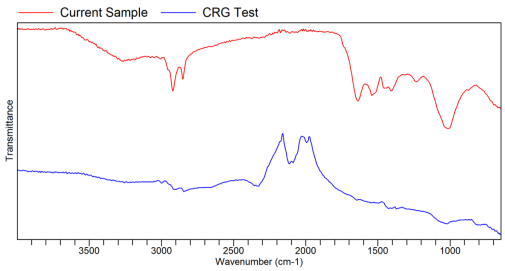
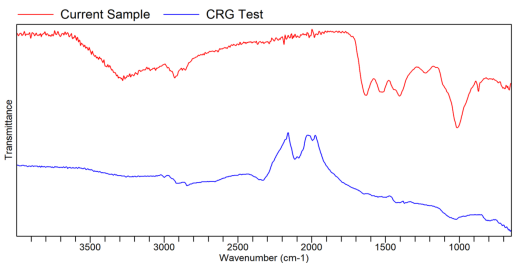
Figure 8 - Picture taken during stomach dissection of stomach content in an edible crab, showing grits of sand and black particles.

2.5.2.1 Fourier transform infrared spectroscopy (FTIR) analyses

Potential rubber particles (detection limit > 0.3 mm) were analyzed by a FTIR (Table 1). A car tire rubber granulate from Ragn-Sells was analyzed and used as a reference to compare transmittance with wavenumber with particles found in crab stomachs. However, black particles smaller than those analyzed by the FTIR were observed in the crabs' stomachs which could potentially originate from car tires but were not analyzed due to difficulties in distinguishing them from other sand particles, difficult to collect and such small particles are not identifiable with the FTIR utilized in this study. In addition, CTRPs are generally difficult to identify using FTIR (more details in section 1.2.5.).

No particles found in crab stomachs were identified as particles originating from CTRPs.

Table 1 – Pictures and measurements (mm) taken with a Leica LAS X software program through a stereo microscope of potentially car tire rubber (CTR) particles found in two crab stomachs from edible crabs (Cancer pagurus), one from each sampling site. Analyzes of the potential particles was conducted using a Fourier transform infrared spectroscopy (FTIR). The transmittance of the unidentified particle (red line) is compared to a known car tire rubber granulate (CRG test, blue line).

	Sampling site	
	Polluted site	**Reference site**
Diameter of particle	~ 0.6 mm	~ 3.9 mm
LAS X pictures		
FTIR		

2.6 Organic chemical analysis

Initially, crab tissue samples from the stomach, muscle, and gills were analyzed for organic chemicals using precellys tubes (7 mL) in the extraction method. Due to contamination issues caused by the O-ring in the tubes (see FTIR analyses of the O-ring in appendix E), a new extraction method was implemented using glass tubes (method 2.6.3.1.), but only extraction of crab gill tissue was repeated due to time constraints.

Analyses were performed at the laboratory at NILU in Tromsø, in gills from both shore- (n = 16) and edible crabs (n = 17), and blue mussels (n = 60) and water samples (n = 12) from the exposure experiment. The samples were extracted and analyzed for target compounds,

specifically N-(1,3-dimethylbutyl)-N´-phenyl-p-phenylenediamine (6PPD) and the transformation product 6PPD-quinone using gas chromatography/ mass spectroscopy (GC/MS). The selection of these chemicals was based on their use in tire manufacturing, making them potential indicators of CTRPs. In addition, have they gained attention in research communities recently due to their demonstrated toxicity towards several marine species (see section 1.2.5 and 1.2.6.).

2.6.1 Quality control

Na_2SO_4 was burned at 600°C for 8 hours and all glass wear and ceramic beads were rinsed and burned at 450°C for 8 hours and covered with aluminum foil in prior to laboratory work to avoid contamination. Ceramic beads were rinsed with tap water and alkine rinse before burning. Metal equipment was rinsed according to the standard of the laboratory (NILU) followed by an ultrasonic bath in n-hexane for 10 minutes. The 100 µL glass pipettes used in method 2.5.6. were not burned due to the risk of changing the unit of measure caused by heat damage to pipette markings.

To ensure extraction of both polar and non-polar organic chemicals from the tissues, solvents such as acetonitrile (non-polar) and n-hexane (polar) were utilized. An internal standard was added to the samples to detect and quantify organic chemicals related to CTR, combined with a recovery standard in prior to GC/MS analyses, additionally to control potential loss of samples during the laboratory procedure.

2.6.2 Testing the organic chemical extraction method

Three blue mussels others than those used in the experiment were used to test the new method (2.6.3.1) with glass centrifuge tubes. These blue mussels were collected in the wild near a harbour in Tromsø (69.642285° N, 18.949197° E) where snow is dumped during wintertime (Figure 9) as snow has been shown to contain tire wear and road particles (TWRP) (Rødland et al., 2022). 6PPD (ng/g ww) was detected in all 3 individuals with an average of 17.5 ± 6.65 ng/g ww.

Figure 9 – Pictures showing snow and ice at the seasurface of dumped snow collected in Tromsø. The red arrow shows the sampling site where test mussels were collected in September 2022 for testing the organic chemical extraction method. Tromsø 20.March 2023.

2.6.3 Extraction of organic chemicals

2.6.3.1 Blue mussels

5 mussels from each treatment and sampling day (n = 60) were analyzed for organic chemicals.

Lab blanks were prepared with tap water (1 mL) in glass centrifuge tubes (15 mL). Frozen mussels were dissolved using a spatula and scalpel and tissue (1 gram) was transferred to glass centrifuge tubes (15 mL). Further, the samples were spiked with internal standard (50 µL) (0.4 ng/uL ACN 6PPD-D5, 6PPD-Q-D5) before adding ceramic beads (3 large and 5 small), acetonitrile (1.5 mL), n-Hexane (1.5 mL) and Na_2SO_4 (0.5 g). Homogenization was performed with a vortex shaker for 15-20 seconds before the samples were transferred to a sonication bath for 15 minutes (temperature: < 30°C). The vortex- and sonication steps were repeated once. After the tubes were shaken in a horizontal shaker for 25 minutes, with a turning of the tubes halfway during the process (12.5 minutes) before centrifugation (1500 rpm, 10 minutes). The hexane- and acetonitrile layers were separated and stored in glass vials (2 mL) at -20°C. To prepare the samples for instrumental analysis, recovery standard ($^{13}C_6$ – 6PPD Quinone, 0.1ng / µL in EA) (20 µL) was added with 100 µL of organic hexane layer to LC/MS vials (0.3 mL). The vials were vortex shaken (5 seconds).

Figure 10 – Car tire rubber particles (CTRPs) observed in blue mussel tissue of a high exposed mussel during laboratory work of extracting organic chemicals.

When mussels were manually dissolved in their glass tubes, black CTRPs were visible in the tissue from both low- and high exposed mussels (Figure 10).

2.6.3.2 Water samples

Water samples (n = 12) were thawed at room temperature before 2 mL of n-hexane were added and further spiked with 50 µL of internal standard (6PPD-D_5, 6PPD-Q-D_5, 0.4 ng/µL in acetonitrile). The bottles were shaken by hand and settled over night before the top layer of the sample (a bubbled hexane layer) was transferred to centrifuge glass tubes. The samples were centrifuged for 2 x 10 minutes at 1500 rpm followed by 20 minutes at 2000 rpm. The samples were put in a freezer overnight and the hexane layer was transferred to a glass vial (2 mL) the next day. Finally, the hexane layers were analyzed in a GC/MS.

2.6.3.3 Crab gills

From the polluted site, gill samples were analyzed from 10 crabs from each species, as well as samples from 6 shore crabs and 7 edible crabs from the reference site. The same method as described in 2.6.3.1. was used to extract organic chemicals from gill tissue, but with smaller sample sizes and solvent volumes (~ 0.5 g sample, 0.5 mL tap water, 1.5 mL of hexane and 1 mL of acetonitrile).

2.6.4 Preparation of samples prior to GC/MS

100 µL of hexane layer from extracted samples were transferred to GC/MS vials (0.3 mL) and spiked with 20 µL of recovery standard ($^{13}C_6$ – 6PPD-Quinone, 0.1ng/µL in ethyl acetate). Each vial was vortex shaken for 5 second before they were run on the gas chromatography mass spectroscopy (GC/MS, Orbitrap).

2.6.5 Instrumental analysis – Gas chromatography mass spectroscopy

The organic chemical extracts from crab gills, water and blue mussels were analyzed by a Q Exactive GC (Orbitrap GC/MS) at the laboratory of NILU. The samples were analyzed for target CTR compounds, and specifically 6PPD and 6PPD-Q.

2.6.6 Quantification and quality assurance

The standards added during extraction (6PPD-D_5, 6PPD-Q-D_5) were used to demine 6PPD concentrations. The other chemicals which were detected were estimated based on the concentrations of 6PPD assuming that the peak area (response) for the detected diamines were identical or highly similar.

When no chemicals were detected in the blanks, the limit of detection (LOD) which were used for calculations was set to LOD/2. If a chemical was detected in the blanks, *Equation 1* was used to calculate a new LOD, which was set to half of the recalculated value.

$$(LOD_1 + LOD_2 + \ldots + LOD_n / n) + 3 * SD$$

Equation 1

2.7 Statistical analysis

Statistical analysis was performed using R-studio, R version 4.2.2 (2022-10-31).

Blue mussels

The data of organic chemical concentrations detected in blue mussels from the exposure experiment were rank transformed to meet the assumptions for normality and homoscedasticity to conduct a parametric two-way ANOVA. The two-way ANOVA was computed to evaluate if the chemical concentrations were affected by treatments at different sampling days. Statistical significance was accepted for p-values < 0.05.

Boxplots, using the package "ggplot" were used to present the chemical concentrations from each treatment groups at each sampling day. The Wilcoxon rank-sum test was used to determine if there was a significant difference between concentrations of chemicals in the independent groups at each sampling day. In addition, the Wilcoxon rank-sum test was used to test the difference in chemical concentrations between the start (day 1) and end (day 7) of the exposure of TTmix and the start (day 7) and the end of the depuration time (day 14) within the low- and high treatment groups to evaluate if the chemicals were accumulating during exposure.

Crabs

To determine the most suitable model for explaining the variability in the concentrations of 6PPD in the crabs, two different combinations of models were tested (Appendix K). The evaluation was based on an AIC (Akaike Information Criterion) estimating the quality of each model. The model which resulted in the lowest AIC value, was considered as the best fit. The different models tested included species (shore- and edible crab), location (the polluted site or the reference site), and carapace width. The score of the models was calculated and corrected for a small sample size using AICc.

Due to a significant amount of body fluid lost from the crabs during thawing, their weight was not included as a parameter in the models, as it does not represent the true weight of the crabs due to variations in the rate of fluid loss. In addition, sex was excluded as a variate in the models due to a low number of each sex represented in the crab samples.

To test the difference in mean concentrations of chemicals within crab species between the sampling locations the non-parametric Wilcoxon rank test was chosen. The test does not assume normality of equal variances, and is suitable for small and uneven sample sizes, as observed between the polluted site (n = 10) and the reference site (shore crab: n = 6, edible crab: n =7).

3 Results

In this study, seven CTR-related organic chemicals were detected in water and mussel samples from the exposure experiment, five different *p*-Phenylenediamines (PPDs), specifically N-(1,3-dimethylbutyl)-N′-phenyl-p-phenylenediamine (6PPD), N-Cyclohexyl-N′-phenyl-p-phenylenediamine (CPPD), N,N′-Diphenyl-p-phenylenediaminie (DPPD), N-o-Tolyl-N'-Phenyl- p-phenylenediamine (TPPD), N,N′-Di-o-tolyl-p-phenylenediamine (DTPD) and the two compounds $C_{21}H_{26}N_2$ and $C_{23}H_{26}N_2$ (See Appendix D, Table 6 for chemical structures). $C_{21}H_{26}N_2$ and $C_{23}H_{26}N_2$ are only identified on the basis of their molecular composition and not chemical structure, due to the lack of commercially available chemical standards. In crab gills, the only detected compounds were 6PPD and $C_{23}H_{26}N_2$.

Even if present in the TTmix itself, the oxidation product of 6PPD, i.e., 6PPD-quinone was not detected in any of the samples and will therefore not be further presented in the result section. The ingestion of rubber particles in the study species will not be further presented, as CTRP>0.3 mm were not detected in any of crab stomachs (Section 2.5.2.), and although particles in mussel tissues were observed during the extraction processes from the low and high treatment groups (Figure 10), they were not further investigated for particles due to instrumental constraints (particle size).

3.1 Exposure experiment

3.1.1 Organic chemicals in tank water

The concentrations measured in the 12 water samples are presented as nanograms per liter (ng/L) of water in Table 2. Figure 11 illustrates the patterns of chemicals in relation to other compounds in low and high treatment tanks.

In the high treatment tank, all chemicals were detected during exposure (days 1-7), except for $C_{21}H_{26}N_2$. On sampling day 1, several chemicals were found at high concentrations in the high treatment tank on sampling day 1, especially 6PPD (952 ng/L), TPPD (590 ng/L), DPPD (285 ng/L), DTPD (109 ng/L) and $C_{23}H_{26}N_2$ (109 ng/L) (Table 2). 6PPD was detected in both the low- and high treatment tanks across all exposure days (Figure 11, Table 2), but not in the control treatment. In contrast, $C_{23}H_{26}N_2$ was detected in low concentrations throughout the experiment in both control and low treatment tanks while its concentration was high during exposure in the high treatment tank. On all days, the proportion of 6PPD was higher in the high

treatment tank, compared to the low treatment. In contrast, the relative proportion of DTPD was higher in the low treatment tank compared to the high treatment tank (Figure 11).

After the depuration time (day 14), $C_{23}H_{26}N_2$ was detected in all tanks, including the control. In the high treatment tank 6PPD, TPPD and DTPD were also present (Figure 11, Table 2).

Table 2 - Concentrations (ng/L) of 6PPD, CPPD, DPPD, TPPD, DTPD, $C_{21}H_{26}N_2$ and $C_{23}H_{26}N_2$ in tank water samples (10 mL) from the control, low- and high treatment tanks collected at 4 sampling times (day 1, 3, 7 and 14) during the experiment. Values presented as «< 0.x» indicate the limit of detection (LOD) ng/sample.

		Compounds (ng/L)						
Sampling day	**Sample (n = 1 per treatment)**	**6PPD**	**CPPD**	**DPPD**	**TPPD**	**DTPD**	**$C_{21}H_{26}N_2$**	**$C_{23}H_{26}N_2$**
1	Control	< 0.3	< 0.5	< 0.2	< 0.5	< 0.5	1.35	0.582
	Low	4.12	< 0.5	1.97	5.79	4.47	< 0.5	4.25
	High	952	9.53	285	590	336	< 0.5	109
3	Control	< 0.3	< 0.5	< 0.2	< 0.5	< 0.5	< 0.5	2.72
	Low	20.0	< 0.5	4.43	25.4	29.8	< 0.5	12.8
	High	331	2.24	114	317	184	< 0.5	46.7
7	Control	< 0.3	< 0.5	< 0.2	< 0.5	< 0.5	< 0.5	1.11
	Low	7.04	< 0.5	1.62	12.9	13.9	< 0.5	7.30
	High	255	1.98	73.3	257	153	< 0.5	43.8
14	Control	< 0.3	< 0.5	< 0.2	< 0.5	< 0.5	< 0.5	2.23
	Low	< 0.3	< 0.5	< 0.2	< 0.5	< 0.5	< 0.5	2.70
	High	1.68	< 0.5	< 0.2	5.36	3.17	< 0.5	2.30

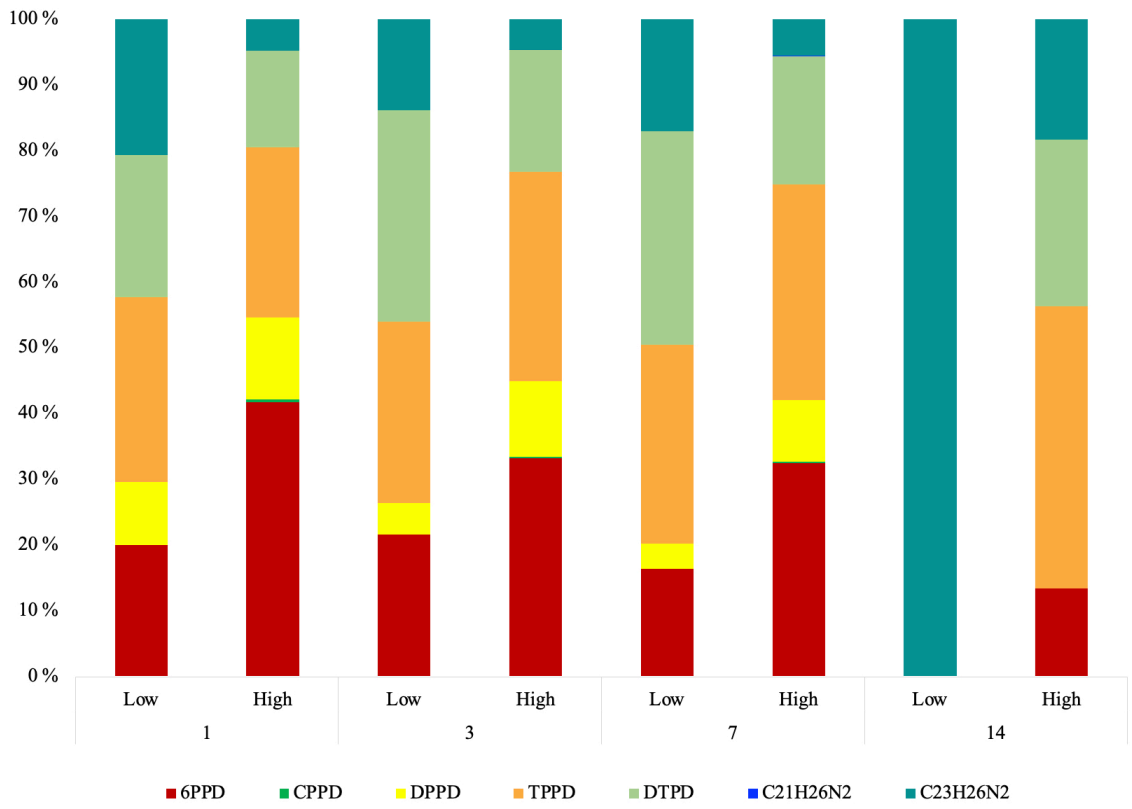


Figure 11 - A stacked bar plot displaying the relative proportion of chemicals (6PPD, CPPD, DPPD, TPPD, DTPD, $C_{21}H_{26}N_2$ and $C_{23}H_{26}N_2$) in water samples in the low and high treatment tanks, sampled at day 1, 3, 7, and 14. (Sample, n= 1 per treatment)

3.1.2 CTRP-related chemicals in blue mussels

The mean tissue concentrations (nanograms per gram of wet weight (ww)), standard deviation (SD), median and range (min and max values) of all 7 organic compounds of interest are listed in Table 3. Figure 12 illustrates the patterns of chemicals in relation to other compounds detected in the low and high exposed mussels from day 7 and 14, and in the TTmix. In Figure 12 the TTmix was analyzed and compared with the relative proportion of the same chemicals found in the low and high treatment mussels from day 7 and 14. The relative composition in the mussel tissues does not vary much both between day 7 and day 14 as well as between treatments. In contrast, the TTmix shows considerable amounts of additional 6PPD-Q, CPPD and DPPD, not present in mussel tissue to a large degree.

Table 3 - Mean and standard deviation (ng/g) of 6PPD, CPPD, DPPD, TPPD, DTPD, $C_{21}H26N_2$ and $C_{23}H_{26}N_2$ analyzed in 60 blue mussels from treatment low and high and the control group on 4 sampling dates (day 1, 3, 7 and 14). Values presented as " <0.x" indicate the LOD ng/sample.

		Compounds (ng/g ww)																			
		6PPD			**CPPD**			**DPPD**			**TPPD**			**DTPD**			**$C_{21}H_{26}N_2$**			**$C_{23}H_{26}N_2$**		
Sampling day	Treatment (n = 5 per treatment)	Mean ± SD	Median	Range	Mean ± SD	Median	Range	Mean ± SD	Median	Range	Mean ± SD	Median	Range	Mean ± SD	Median	Range	Mean ± SD	Median	Range	Mean ± SD	Median	Range
1	Control	< 1	< 1	-	< 1	< 1	-	< 1	< 1	-	< 1	< 1	-	< 1	< 1	-	< 1	< 1	-	12.0 ± 3.22	12.5	8.31 – 16.1
	Low	30.1 ± 16.3	26.4	17.5 – 57.9	< 1	< 1	-	2.07 ± 0.870	1.94	1.21 – 3.38	10.4 ± 4.52	9.72	5.94 - 17.9	13.1 ± 8.62	10.9	4.14 – 25.9	2.57 ± 1.00	2.13	1.67 – 3.99	22.1 ± 7.13	22.2	13.6 – 29.6
	High	195 ± 139	168	56.4 – 407	2.68 ± 1.83	2.53	< 1 – 5.49	8.61 ± 4.17	9.51	4.18 – 14.1	48.6 ± 27.1	44.7	14.6 – 82.3	44.7 ± 28.0	31.6	15.9 – 78.8	14.2 ± 6.96	11.0	9.83 – 26.4	38.6 ± 13.9	30.3	28.1 – 59.7
3	Control	< 1	< 1	-	< 1	< 1	-	< 1	< 1	-	< 1	< 1	-	< 1	< 1	-	< 1	< 1	-	18.5 ± 19.9	14.9	< 1 – 52.3
	Low	21.9 ± 11.3	18.0	11.3 – 41.0	< 1	< 1	-	0.646 ± 0.321	< 1	< 1– 1.22	19.5 ± 5.6	18.7	12.1 – 26.6	21.7 ± 8.73	18.9	10.5 – 33.2	2.70 ± 0.737	2.29	2.09 – 3.68	26.3 ± 4.90	23.9	22.7 – 34.5
	High	207 ± 109	184	104 – 361	2.64 ± 1.19	2.37	1.37 – 4.44	9.74 ± 4.81	8.20	4.74 – 16.0	98.6 ± 33.2	116	46.9 – 127	109 ± 44.5	110	42.6 – 158	12.8 ± 2.01	12.4	10.7 – 15.8	41.9 ± 7.49	41.9	32.1 – 51.2
7	Control	< 1	< 1	-	< 1	< 1	-	< 1	< 1	-	< 1	< 1	-	< 1	< 1	-	< 1	< 1	-	10.9 ± 6.82	14.1	< 1 – 16.3
	Low	17.0 ± 10.6	13.6	7.7 – 32.3	< 1	< 1	-	< 1	< 1	< 1– 0.650	19.4 ± 6.18	18.4	14.0 – 29.7	33.0 ± 9.78	30.7	25.0 – 49.3	1.70 ± 0.57	1.90	0.84 – 2.27	21.2 ± 3.55	22.5	15.0 – 23.4
	High	138 ± 52.4	136	63.0 – 205	1.96 ± 1.07	1.93	< 1– 3.44	7.46 ± 3.18	7.35	3.74 – 12.5	181 ± 59.2	184	102 – 256	200 ± 73.2	210	103 – 301	12.5 ± 3.47	13.0	8.62 – 17.6	37.5 ± 8.22	35.0	29.3 – 50.8
14	Control	< 1	< 1	-	< 1	< 1	-	< 1	< 1	-	< 1	< 1	-	< 1	< 1	-	< 1	< 1	-	21.4 ± 6.94	17.9	14.8 – 32.1
	Low	6.63 ± 3.06	6.52	3.35 – 11.6	< 1	< 1	-	< 1	< 1	-	7.71 ± 3.44	6.62	4.59 – 13.5	25.1 ± 10.4	21.2	13.6 – 40.6	0.764 ± 0.363	0.510	< 1– 1.21	22.5 ± 7.68	20.4	15.6 – 35.2
	High	56.8 ± 24.8	54.8	26.9 – 95.6	0.769 ± 0.593	< 1	< 1 – 1.83	1.24 ± 0.271	1.13	1.10 – 1.72	85.2 ± 26.8	78.5	62.7 – 130	140 ± 61.2	122	91.1 – 245	1.86 ± 0.387	1.74	1.59 – 2.54	33.2 ± 2.99	32.4	30.5 – 38.2

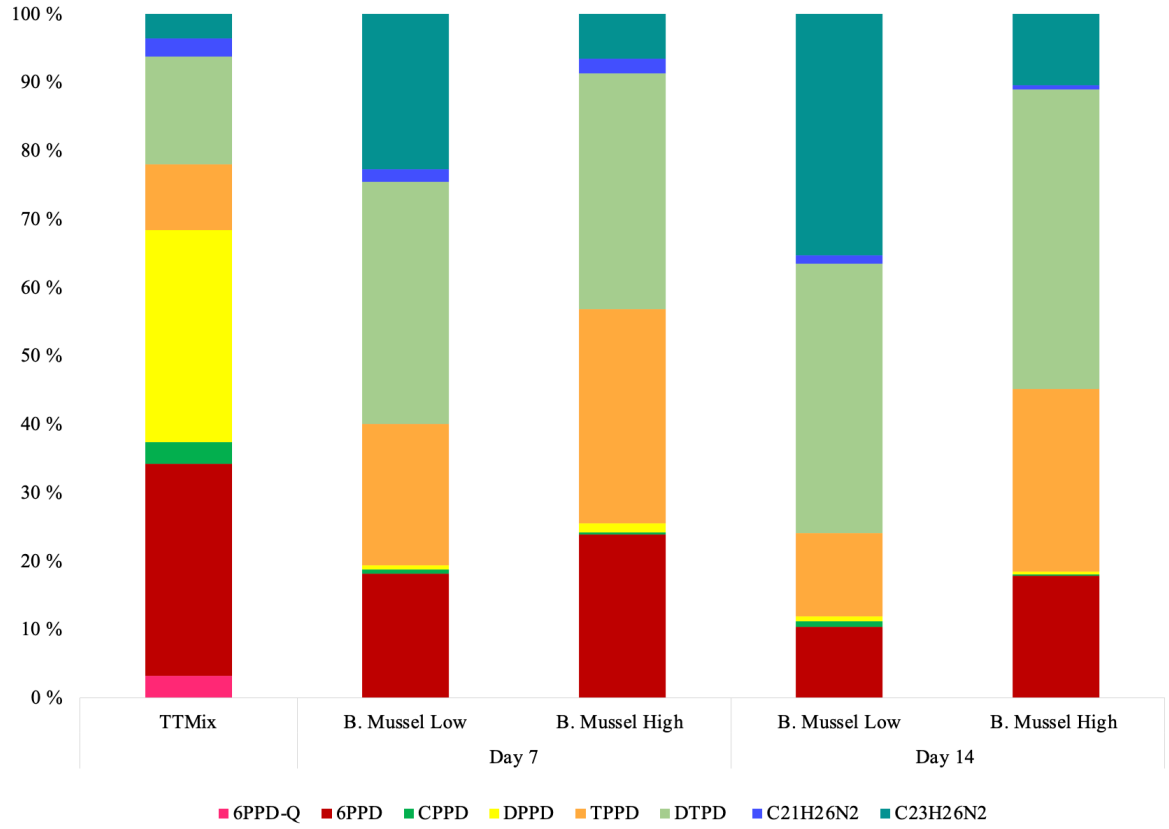


Figure 12 - A stacked bar plot displaying the relative proportion of chemicals (6PPD-Q,6PPD, CPPD, DPPD, TPPD, DTPD , $C_{21}H_{26}N_2$ and $C_{23}H_{26}N_2$) extracted from TTmix and in mussel samples from the low and high treatment tanks, sampled at day 7 and 14. The bars are based on the mean values from table 3.

3.1.2.1 6PPD

6PPD was detected in mussels from both high and low treatments on all sampling dates although the mean concentration was significantly higher in the high treatment group on all sampling days compared to the low and control groups (Figure 13, Wilcoxon tests, $p < 0.05$). The highest mean (207 ± 109 ng/g ww) and median (184 ng/g ww) were reached for the high treatment on day 3, and on day 1 for the low treatment (mean = 30.1 ng/g ww ± 16.3, median = 26.4 ng/g ww) (Table 3). The high exposure had a notably high SD (± 139) and wide range (56.4 – 407 ng/g ww) of concentrations of 6PPD on day 1 (Table 3). The proportion of 6PPD was higher in the high treatment group during exposure on day 7, compared to the low treatment (Figure 12).

On the last day of depuration (day 14), concentrations of 6PPD were still detected but showed decreases of 73% and 78% for the high and low treatments, respectively compared to their highest concentrations (Table 3). Over the exposure period, the mussels did not show any

significant difference in concentrations of 6PPD within neither of the treatment groups (low: p = 0.22, high: p = 0.69, Wilcoxon tests, Figure 14). Between the end of exposure (day 7) and the end of the depuration time (day 14) concentrations of 6PPD decreased significantly within each treatment groups (low: p = 0.032, high: p = 0.016, Wilcoxon tests) (Figure 14).

6PPD concentrations detected during exposure (day 1-7) and depuration (day 14) were approximately 8 times higher in the high treatment compared to the low treatment at both periods.

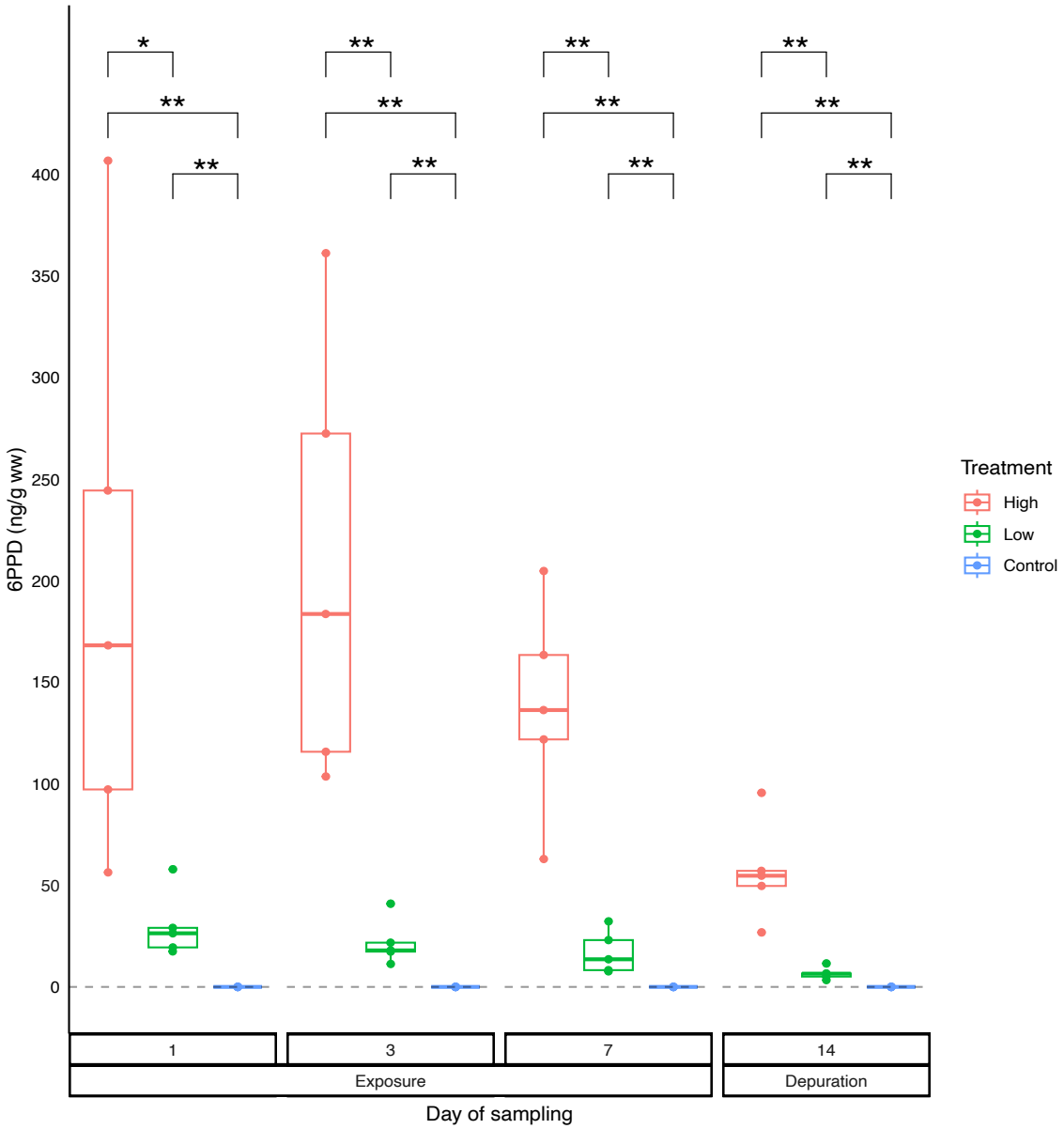


Figure 13 – The boxplot displays the concentrations of 6PPD (ng/g ww) detected in blue mussels (Mytilus edulis)(n= 60) from control, low and high treatment groups from exposure to car tire rubber particles (CTRPs; diameter of ~23.7 ± 16.7 µm). The exposure lasted for 7 days, followed by a depuration time of 7 days, starting on day 7. The boxes show the interquartile range (IQR) of the data, with median indicated by the horizontal line inside each box. The whiskers extend to the minimum and maximum values within 1.5 times the IQR. Outliers beyond this range are shown as individual points. Asterisks (*) indicate statistically significant differences in 6PPD (ng/g ww) according to a Wilcoxon rank test between the treatment groups from each sampling day (, p<0.05, **, p<0.01).*

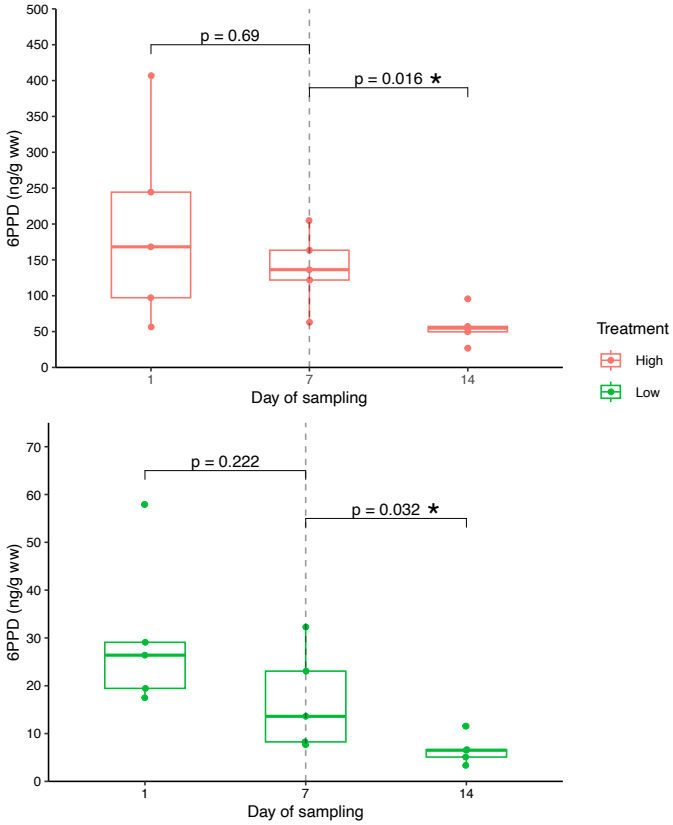


Figure 14 – Concentrations of 6PPD (ng/g ww) in blue mussels between the first (day 1) and the last day (day 7) of exposure, and between the first (day 7) and last day of depuration (day 14) for both the low- and high treatment. Each box corresponds to a sample size of n = 5. Asterisks (*) indicate statistically significant differences in 6PPD (ng/g ww) according to Wilcoxon tests between sampling day 1 and 7, and 7 and 14 (: p<0.05). The y-axis represent different ranges for the respective treatment groups. The vertical dashed line on day 7 marks the start of the depuration period.*

3.1.2.2 TPPD and DTPD

The uptake of TPPD and DTPD in mussels were similar with concentrations increasing through the exposure period in the high treatment group with the highest mean and median detected on the last day of exposure (day 7) (Table 3).

TPPD and DTPD were detected in the low treatment group on all sampling days. Both chemicals differed statistically between the start (day 1) and the end of exposure (day 7) in both treatment groups (TPPD: low: p = 0.032, high: p =0.008; DTPD: low: p = 0.016, high: p = 0.008, Wilcoxon tests). Over the depuration period, TPPD concentrations significantly decreased within each treatment group (TPPD: low: p = 0.008, high: p =0.016, Wilcoxon tests) but not for DTPD (low: p = 0.22, high: p = 0.31, Wilcoxon tests)(Figure 15 & 16).

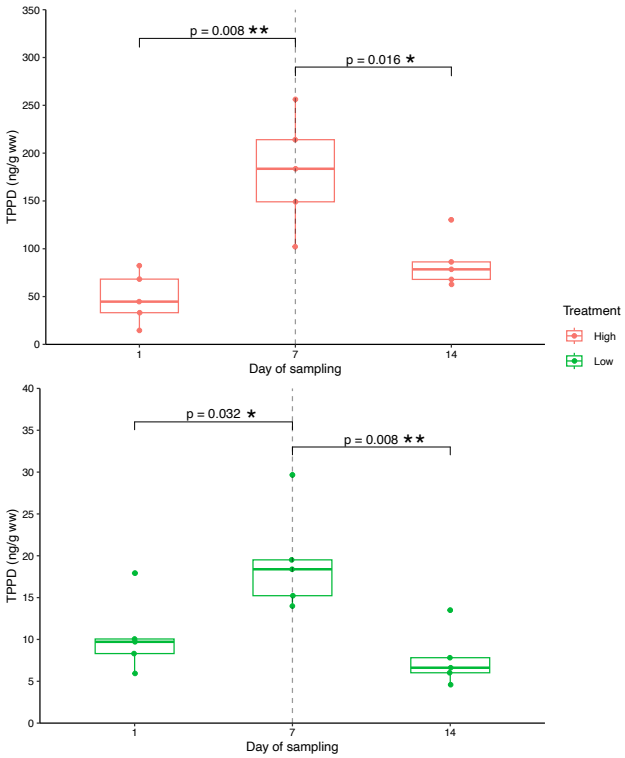


Figure 15 – Concentrations of TPPD (ng/g ww) detected in blue mussels between the first (day 1) and the last day (day 7) of exposure, and between the first (day 7) and last day of depuration (day 14) for both the low- and high treatment. Each box corresponds to a sample size of n = 5. Asterisks (*) indicate statistically significant differences in 6PPD (ng/g ww) according to Wilcoxon tests between sampling day 1 and 7, and 7 and 14 (: $p < 0.05$, **: $p < 0.01$). The y-axis represent different ranges for the respective treatment groups. The vertical dashed line on day 7 marks the start of the depuration period.*

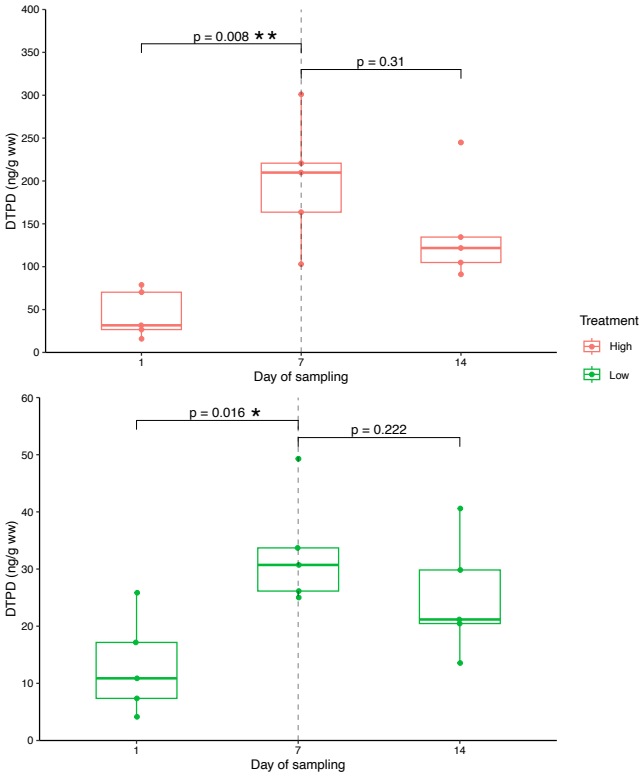


Figure 16 - Concentrations of DTPD (ng/g ww) detected in blue mussels between the first (day 1) and the last day (day 7) of exposure, and between the first (day 7) and last day of depuration (day 14) for both the low- and high treatment. Each box corresponds to a sample size of n = 5. Asterisks (*) indicate statistically significant differences in 6PPD (ng/g ww) according to Wilcoxon tests between sampling day 1 and 7, and 7 and 14 (: $p < 0.05$, **: $p < 0.01$). The y-axis represent different ranges for the respective treatment groups. The vertical dashed line on day 7 marks the start of the depuration period.*

3.1.2.3 $C_{23}H_{26}N_2$

$C_{23}H_{26}N_2$ was detected on all sampling days in both treatment groups and the control although at higher concentrations in the high and low treatment groups (Table 3). The highest concentrations in the high treatment group were detected on day 3. Concentrations of $C_{23}H_{26}N_2$ did not significantly differ between the low treatment and the control groups on day 3 and on day 14 ($p = 0.15$, $p = 0.84$, respectively, Wilcoxon tests) (Appendix H, Figure 27).

3.1.2.4 Other chemicals

The uptake of CPPD, DPPD, and $C_{21}H_{26}N_2$ (ng/g ww) by mussels in the high treatment group followed a similar pattern to 6PPD, albeit at lower concentrations (< 30 ng/g ww). They were detectable on all sampling days, but the concentrations did not statistically differ between day 1 and 7 during the exposure (Table 3). Similar to 6PPD, the three chemicals decreased significantly during the depuration time between day 7 and 14 (Table 3).

In the low exposure group, CPPD was not detected at all, while DPPD was detected in all mussels sampled on the first day (1.21 – 3.38 ng/g ww), but only in one mussel on the third sampling day. After that, DPPD was no longer detected in any individuals throughout the remainder of the experiment (Table 3).

3.2 *In situ* sampled crabs

3.2.1 Organic chemicals in crab gills

6PPD (< 0.02 - 0.830 ng/g ww) was detected in both crab species from all sites. Among the other chemicals analyzed (i.e., 6PPD-Q, CPPD, DPPD, TPPD, DTPD, $C_{21}H_{26}N_2$, $C_{23}H_{26}N_2$), $C_{23}H_{26}N_2$ was the only one detected, and was only found in one edible crab from the reference site (mean: 3.12 ng/g ww), and in one shore crab from the polluted site (mean: 3.43 ng/g ww) (Table 4).

Concentrations of 6PPD did not significantly differ between crab species within each site (polluted site: $p = 1$, reference site: $p = 0.14$, Wilcoxon tests). At the polluted site, concentrations of 6PPD were higher in both crab species (edible crab: mean = 0.299 ± 0.298 ng/g ww, shore crab: mean = 0.236 ± 0.162 ng/g ww) compared to the reference site (edible crab: mean = 0.128 ± 0.244 ng/g ww, shore crab: mean = 0.175 ± 0.079 ng/g ww, Table 4) although, the differences were only marginally significant in edible crabs ($p = 0.055$) and not

significant for shore crabs (p = 56, Wilcoxon tests, Figure 17). Additionally, 6PPD was detected in a greater number of crabs at the polluted site (edible crabs: n = 7/10, shore crabs: n = 8/10), compared to the reference site (edible crabs: n = 2/7, shore crabs: n = 4/6).

There was a significant size difference in carapace width between the shore crabs from the two sites (polluted site: mean = 45.7 ± 8.3 mm, reference site: mean = 64.3 ± 4.2 mm, p = 0.0014, Wilcoxon test, Appendix J, Table 8), but not between the edible crabs (p = 0.26, Wilcoxon test). However, the observed variability in the concentrations of 6PPD was not statistically driven by carapace width but by location (Appendix K).

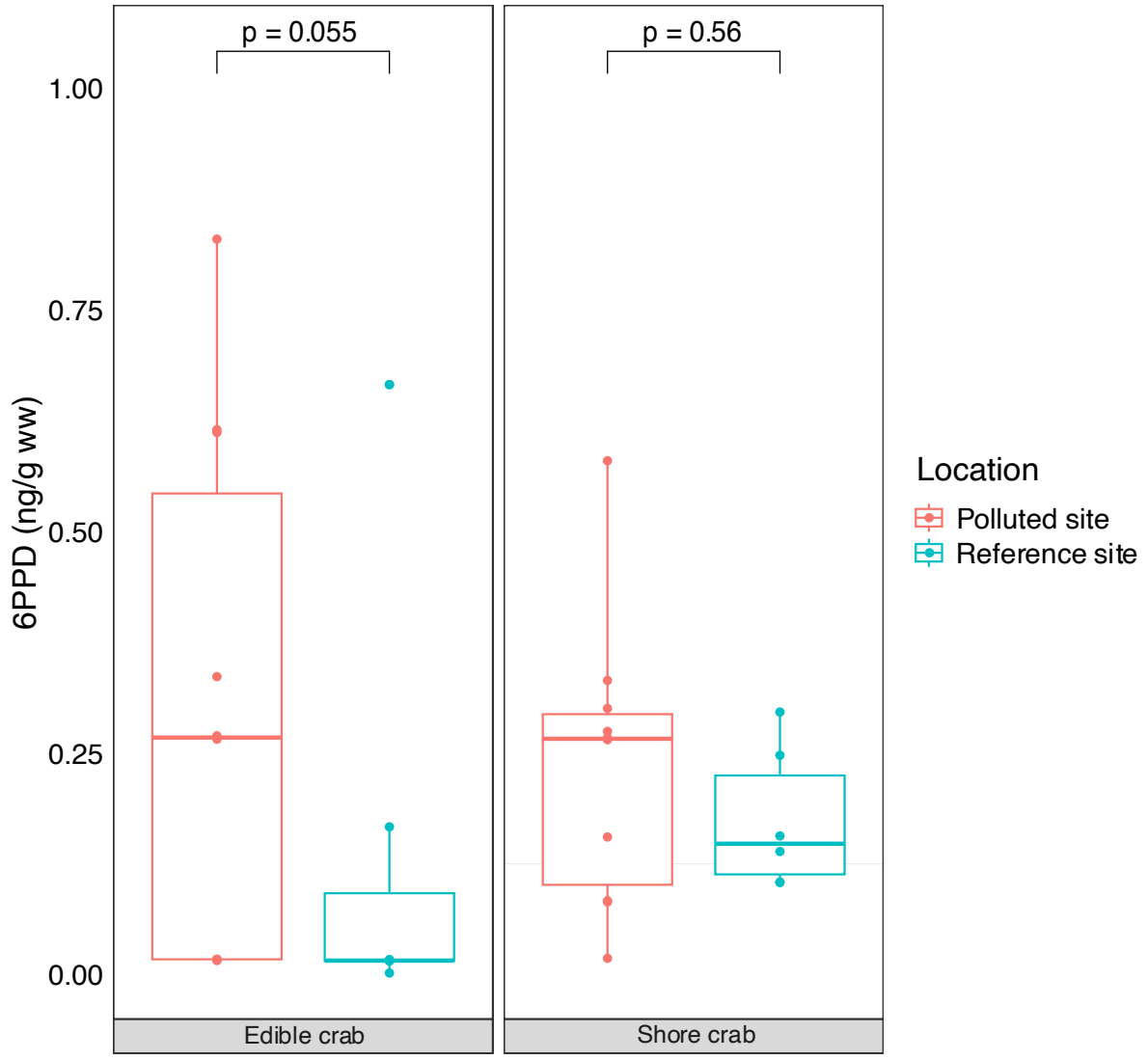


Figure 17 - Concentrations (ng/g ww) of 6PPD detected in gill tissue from both edible crab (Cancer pagurus)(n = 17) and shore crab (Carcinus maenas)(n = 16) from the polluted site (Frierfjorden) and the reference sites (Kristiansand). The boxes show the interquartile range (IQR) of the data, with median indicated by the horizontal line inside each box. The whiskers extend to the minimum and maximum values within 1.5 times the IQR. Outliers beyond this range are shown as individual points. The bracket above each species shows a p-value from Wilcoxon tests of statistically significant differences in 6PPD (ng/g ww) within each species between the sampling sites.

Table 4 - Concentrations (ng/g ww) of 6PPD, CPPD, DPPD, TPPD, DTPD, $C_{21}H_{26}N_2$ and $C_{23}H_{26}N_2$ detected in gill tissue from edible crabs (Cancer pagurus)(n = 17) and shore crabs (Carcinus maenas)(n = 16) from the polluted site (Frierfjorden) and at the reference site (Kristiansand). Presented with mean, standard deviation (SD), median and range.

	Edible crab						**Shore crab**					
	Polluted site			**Reference site**			**Polluted site**			**Reference site**		
Chemical	Mean ± SD	Median	Range	Mean ± SD	Median	Range	Mean ± SD	Median	Range	Mean ± SD	Median	Range
6PPD	0.299 ± 0.298	0.267	<0.02 - 0.830	0.128 ± 0.244	< 0.02	< 0.02 – 0.665	0.236 ± 0.162	0.266	< 0.02 – 0.580	0.175 ± 0.079	< 0.2	< 0.2 – 0.296
CPPD	< 0.2	< 0.2	-	< 0.2	< 0.2	-	< 0.2	< 0.2	-	< 0.2	< 0.2	-
DPPD	< 0.2	< 0.2	-	< 0.2		-	< 0.2	< 0.2	-	< 0.2	< 0.2	-
TPPD	< 0.2	< 0.2	-	< 0.2	< 0.2	-	< 0.2	< 0.2	-	< 0.2	< 0.2	-
DTPD	< 0.2	< 0.2	-	< 0.2	< 0.2	-	< 0.2	< 0.2	-	< 0.2	< 0.2	-
$C_{21}H_{26}N_2$	< 0.2	< 0.2	-	< 0.2	< 0.2	-	< 0.2	< 0.2	-	< 0.2	< 0.2	-
$C_{23}H_{26}N_2$	< 0.2	< 0.2	-	< 0.2	< 0.2	< 0.2 – 3.12	< 0.2	< 0.2	< 0.2 – 3.43	< 0.2	< 0.2	-

4 Discussion

This study investigated the uptake of car tire rubber and related chemicals in marine invertebrates (filter-feeding mussels and particle feeding crabs) using experimental and observational approaches. First, blue mussels were experimentally exposed to car tire rubber particles (CTRPs) representing 20 different tires (TTmix). In addition, *in situ* shore- and edible crabs were collected from a CTR polluted site and reference sites. These study species all inhabit coastal systems that may receive CTR from various sources, such as runoff of TWP, e.g. with road dust or contaminated snow dumped into the water, release of crumb rubber granulates from football fields through wastewater or directly through runoff (Rødland et al., 2022; Simon, 2010). As sessile species filtering large amounts of water daily (Gosling, 2021, p.417), blue mussels can potentially uptake small CTRPs and leachates (i.e., additives). In contrast, crabs, being larger, mobile and opportunistic feeders, may ingest larger rubber particles and can also be exposed to particles and leachates in the water through respiration via the gills (Watts et al. 2014). The main objectives of this thesis were to investigate if CTRPs were ingested by blue mussels and crabs and if rubber-related chemicals accumulated in their tissues to evaluate their use as biomonitors of CTRP exposure. More specifically, five different *p*-Phenylenediamines (PPDs) and two unidentified compounds (by structure) ($C_{21}H_{26}N_2$ and $C_{23}H_{26}N$) were analyzed in water, whole mussel tissues and crab gills. PPDs (e.g. 6PPD) are widely used as antiozonants in tire manufacturing, making them characteristic components of CTRPs (Babbit, 2010 as cited in Foldvik et al., 2022). The selection of these PPDs was based on several studies reporting detection of these compounds in various environmental samples, including in surface waters, soil, water (Cao et al., 2022; Huang et al., 202) and in marine organisms upon ingestion of particles (Hägg, 2022). Recent studies have also demonstrated that transformation products of PPDs, such as 6PPD-Q, are toxic to several aquatic species (Brinkman et al., 2022; Chow et al., 2019). Given our three study species' roles as important food sources for various organisms and humans, investigating these compounds in these species is of great relevance. Finally, this study investigated if any of the selected chemicals could serve as markers or proxies for the presence of CTRPs in the surrounding environment, addressing the limitations of current methods and exploring alternative chemical markers.

4.1 Detection of CTRP-related additives

The low exposure treatment was designed to mimic environmental conditions while the high treatment TTmix concentration was 10 times higher to better identify concentration trends. *In-situ* sampled mussels showed 6PPD concentrations (17.5 ± 6.65 ng/g ww, n = 3) similar to experimental organisms from the low treatment group (23.0 ± 13.7 ng/g ww), suggesting that our exposure achieved environmentally realistic concentrations as recommended by Koelmans et al. (2017). Furthermore, to mirror the variety of CTRPs in the environment, the TTmix used in the experiment was made using a combination of 20 different tire types, which can vary in terms of additive concentrations and formulations. The concentrations of 6PPD in the mussel tissues showed a linear relationship with the exposure dose with mussels in the high TTmix treatment group showing concentrations of 6PPD that were approximately 8 times higher compared to the low treatment group, both during exposure and after depuration. Despite some variability observed in the data (high standard deviations), our data indicate a linear relationship between CTRP (i.e., TTmix) concentrations in the surrounding environment and 6PPD uptake by blue mussels. This further highlights the potential of 6PPD as a valuable biomarker for detecting the presence of CTRPs in the environment by monitoring its concentration in body tissues of mussels. However, it is worth noting that the study findings are based on a study measuring the uptake of 6PPD by mussels exposed to CTRPs in a closed aquarium. Such an experimental setup does not allow to conclude whether 6PPD first leached out from the CTRPs into the water and was then taken up by the mussels or whether the CTRPs were first taken up by the mussels and then leached out 6PPD after absorption, although both hypotheses are not mutually exclusive. Further investigation is needed to better understand the uptake of 6PPD in mussels in natural habitats where leachates may be less concentrated and CTRPs less abundant than in aquariums as in this study.

Throughout the exposure, the TTmix released chemicals into the seawater in the tanks, exposing the mussels to leachates containing concentrations of 6PPD, TPPD and DTPD greater than the other selected compounds (Figure 11). The proportion of these chemicals differed to some degree between the low and the high treatment tanks. Namely, the high treatment showed higher concentrations of 6PPD in relation to other compounds in the water samples compared to the low treatment tank (Figure 11). Nevertheless, the analyses were based on a single water sample from each tank at each sampling day, with only one extraction performed for each sample.

These factors introduce uncertainty and limitations when drawing conclusions regarding the differences in proportion observed in the water samples.

Even though the TTmix showed high concentrations of 6PPD-Q, CPPD and DPPD, these were not detected to the same extent in mussel tissues. The low concentrations observed in the mussels could either be caused by a low uptake and/or high metabolization, or a fast degradation in the tank before they can be taken up. This study however reported high concentrations of several other rubber related organic chemicals in the tissues of the exposed individuals. Since most of these chemicals decreased in concentrations after the depuration time, it is likely that they underwent metabolic transformation and/or were excreted. Nevertheless, when mussels were transferred to clean tanks, concentration of 6PPD, TPPD and DPTD decreased but remained relatively high after 7 days of depuration. While our results do not support that 6PPD accumulated during exposure, the concentrations of TPPD and DTPD increased linearly over time of exposure, showing accumulation when CTRPs were present in the tanks. Between day 7 and 14, 6PPD and TPPD had significantly decreased compared to the end of exposure, but concentrations of DTPD did not show significant decreases (Figure 15 & 16). Additionally, on day 7, DTPD was detected at the highest concentrations in both treatment groups compared to the other chemicals. This finding aligns with the results of a previous study where lumpfish were exposed to dietary crumb rubber (CR) for the same duration period as our study and several of the same compounds were analyzed (Hägg, 2022). In the latter study, DTPD was also detected at the highest concentrations in lumpfish blood on day 7 in relation to other compounds. In our study, after 7 days of depuration, DTPD remained the chemical with the highest concentrations in both treatment groups compared to other chemicals. However, in contrast to our study, Hägg (2022) had a longer depuration period of 14 days at the end of which they detected the highest concentrations of 6PPD and not DTPD as reported in the current study. Based on our findings, DTPD appears to be the most promising candidate marker for CTR in mussel tissue, given its capacity to accumulate during exposure and to persist at elevated levels following depuration.

6PPD was detected in the gill tissue of both shore- and edible crabs from both sampling sites (contaminated and reference sites) although more frequently in shore crabs compared to the larger species. At the reference site, both crab species were found to have detectable concentrations of 6PPD, comparable to the concentrations detected at the polluted site (Figure 17). Yet, a higher number of crabs collected from the polluted site had detectable concentrations

of 6PPD in their gill tissue compared to the reference sites. While 6PPD concentrations in the edible crabs were marginally significantly higher at the polluted site, no such difference was observed in the shore crabs. This could be explained by the sampling location of the reference shore crabs, which were collected from a boat harbour in Kristiansand that was located close to a parking lot (i.e. potential sources of CTR pollution), while the reference edible crabs were collected in open waters distant to obvious potential sources of CTR. Additionally, we cannot exclude that the observed inter-species differences could be due to differences in their physiology and metabolism (e.g., detoxifying and elimination processes).

The transformation product of 6PPD, 6PPD-quinone, has been suspected to accumulate in sediments and to biomagnify in the food chain (Hiki et al., 2021; Tian et al., 2021). However, 6PPD-Q was not detected in any of the crab gill, water, or mussel samples, despite its initial detection in the TTmix used in the exposure study (Figure 12). This could be due to several factors including the possibility that the chemical may not have been extracted with our methods, that it was already degraded or metabolized at the time of sampling, that it was not available for uptake in the mussels or crabs and/or that it may not have been present in the analyzed tissues. Regardless, since 6PPD-Q was not detected in any of our samples, it is not considered as a useful biomarker for CTRPs in biological tissues of mussels or crab gills.

$C_{23}H_{26}N_2$ was detected in gills of two crab individuals, and in both water and mussel samples from the exposure experiment (all tanks, including the controls) (Appendix H, Figure 27). $C_{23}H_{26}N_2$ concentrations were treatment dependent, with the lowest concentrations detected in the control, and the highest concentrations in the high treatment group. Considering that the control tanks were not exposed to TTmix, and given that $C_{23}H_{26}N_2$ is a CTRP related chemical, its presence could be attributed to potential contamination sources in the laboratory facilities. An alternative hypothesis could be that the mussels were contaminated with $C_{23}H_{26}N_2$ prior to the experiment, as they were collected in the field. The fact that the chemical concentrations were treatment dependent and that $C_{23}H_{26}N_2$ was also found in the TTmix suggest that the chemical also leached from the TTmix and was taken up in the mussels from the water or from ingested particles during the experiment.

4.2 Uptake of CTRPs by mussels and crabs

Mussels

As expected, small black particles were observed in the tissue of some exposed mussels (as shown in Figure 10). Previous studies have also shown that wild mussels ingest rubbery fragments and microplastics (Bråte et al., 2018; Bråte et al., 2020). It is unclear in this study whether particles remained in the mussels throughout the whole experiment or got excreted, as this aspect was not investigated. If particles remained in the mussels, they could have been translocated to other body compartments where they could have also leached chemicals. Furthermore, this study did not investigate how many particles remained in mussels over the study period. Previous studies have shown that small particles can remain in tissues for extended periods of time. For example, blue mussels exposed to low-density polyethylene (LDPE) particles of size 20-25 µm for 4 and 56 days were observed to have most of the particles in the intestinal lumen and in pseudofeces (Søbstad, 2020). Moreover, the latter study demonstrated that LDPE particles of this size were not significantly translocated from the digestive system to other tissues. Based on the latter findings, it seems likely that the CTRPs in our study, which had a size of 23.7 ± 16.7 µm, would be observed in the same compartments, if ingested. However, the TTmix used also included particles smaller than 20 µm, which could have been translocated to other tissues as observed in Browne et al. (2008). In their study, MPs of polystyrene (PS) with a size of ~ 3 -10 µm were found distributed in the circulatory system of blue mussels after ingestion, with a retention time of over 48 days. In addition, it is important to note that the specific characteristics and behavior of CTRPs may be different than those of LDPE and PS.

Our results showed that the relative composition of chemicals in the mussel tissues between day 7 and 14 did not vary much in either of the treatments, indicating an efficient transfer of chemicals from rubber to the tissue. This indicates that CTRPs may act as vectors for organic chemicals in mussels through ingestion and/or water leachates. Similarly, other studies on *Mytilus galloprovincialis* (Avio et al., 2015; Pittura et al., 2018) and *Mytilus edulis* have shown the transfer of polyaromatic hydrocarbon (PAH) into the tissue of mussels from exposure to sorbed MPs (Stollberg et al., 2021). However, an experimental study using *Mytilus galloprovincialis* exposed to both edible and inedible particles of expanded polystyrene (EPS) highlighted that leachate uptake is an important exposure route of EPS additive in mussels (Jang et al., 2021). Both pathways might have contributed to the uptake of chemicals by blue mussels

in our study. Nevertheless, the higher concentrations of CTRP-related chemicals in the mussels compared to the tank water indicate that the mussels were likely exposed through the ingestion of rubber particles to accumulate such high concentrations of chemicals. It is noteworthy that, in natural habitats, mussels have a constant water flow, and leachates will not be as concentrated as in a closed aquarium for seven days. In addition, the uptake and behavior of other additives cannot be directly compared to the rubber compounds in our study, as they have different physicochemical properties. To better address whether chemicals are absorbed through water or ingested particles, future studies should expose mussels to both CTRPs and leachates in parallel and compare the uptake of the same organic chemicals.

Crabs

Crabs have a powerful structured stomach and a relative short retention time of food (Štrus et al., 2019). Even though no observations of ingested CTRPs > 0.3 mm were reported in crabs in this study, rubber particles could potentially have been ingested but excreted upon capture in the crab traps. McGoran et al. (2020) have reported findings of rubbery fragments in the gastric mill of the Chinese mitten crab (*Eriocheir sinensis)* and Hägg (2022) demonstrated uptake of crumb rubber (CR) in lumpfish when exposed to CR mixed with food with as much as 76% of the exposed lumpfish found to have ingested CR. Other studies suggested that MPs masked with biofilm can have higher ingestion rates in marine organisms (Vroom et al*.,* 2017). In our study, as the shredded car tires have been at the seabed for several years at the polluted site, the rubber fragments could have been biofouled and thus be made more attractive for ingestion. Both shore crabs and edible crabs have a diverse diet that include mussels, echinoderms, worms and other crustaceans (Lawton, 1989; Mascaron & Seed, 2001; Van der Meeren et al*.,* 2022). Many of these prey items have been reported to ingest and contain different types of MPs, and it is plausible that they also ingest CTRPs (Browne et al., 2013; Bråte et al., 2020; McGoran et al., 2020). Additionally, the trophic transfer of MPs has been demonstrated in a study where shore crabs were fed blue mussels contaminated with 0.5µm polystyrene (PS) microspheres (Farrell & Nelson, 2013). This suggests that trophic transfer of CTRPs to higher-level crabs may occur through contaminated prey, as observed with PS in the latter study.

4.3 Uptake of CTRP-related additives by mussels and crabs

Mussels

Capolupo et al. (2021) demonstrated in a short-term study sub-lethal effects on early-stage mussels following exposure to CTR additive leachates. However, the mortality in the current exposure experiment was minimal, suggesting that it was unlikely to be associated with the TTmix exposure conditions. This suggestion is based on the observation of a higher number of control and low treatment mussels that died in the tanks compared to mussels exposed to high concentrations of TTmix. Only one mussel from the high treatment tank died during the exposure (at day 7), while six and four mussels died in the low treatment and control tanks, respectively. Additionally, *in-situ* sampled mussels showed concentrations of 6PPD similar to those observed in the experimental organisms from the low treatment group, indicating that mussels can tolerate such concentrations over the short exposure time. Adult mussels in the current study therefore did appear to have a high tolerance to CTRP, at least at the selected particle size and period of exposure. Interestingly, adult mussels can exhibit behavioral avoidance mechanisms when exposed to pollution. For example, Stollberg et al. (2021) observed that blue mussels exposed to polyvinyl chloride (PVC) MPs displayed lower valve opening during exposure compared to depuration, indicating avoidance response to the pollution. While the current study did not record the daily shell opening during or after exposure, the high exposed mussels showed the highest concentration of chemicals, indicating that they have taken up CTRPs and/or leachates from the surrounding waters and likely did not display any avoidance behavior.

Crabs

Despite some methodological adjustments, extraction from gill tissue showed concentrations of 6PPD. Even though 6PPD was found in gill tissue, our study does not allow to disentangle whether rubber chemicals originated from respiration across the gills and/or rubber fragments attached to the gills, which have been shown with microplastics on shore crabs (Watts et al., 2014). Moreover, during dissection and extraction, we could not avoid the gills to be in contact with body fluids and parts, including the hepatopancreas, which is a detoxifying organ known to accumulate toxins (Štrus et al., 2019). Therefore, the detected concentrations of 6PPD in the gills may have been impacted by chemicals from the hepatopancreas or body fluid from other compartments in the crab. Additionally, variations in the extent of contact with e.g. body fluids among individuals, may have contributed to observed variation in the detected concentrations of 6PPD.

4.3.1 Chemical concentration differences between study species

In our study, we observed differences in chemical concentrations between the study species. Mussel samples had much higher concentrations of several CTRP-related compounds compared to crab gills. Lump fish blood analyzed in the study by Hägg (2022) also exhibited much lower concentrations of organic chemicals compared to mussels. 6PPD in lump fish blood on day 7 of exposure was 0.330 ng/g, while it was 17.0 ± 10.6 ng/g ww in low treatment mussels and 138 ± 52.4 ng/g ww in high treatment mussels. However, there was not a significant difference in concentrations of 6PPD between lumpfish blood and crab gills (e.g., 6PPD in edible crab from the polluted site: 0.299 ± 0.298 ng/g w). Several factors could contribute to the differences in 6PPD concentrations among these study species. First, species-specific physiological mechanisms can affect the uptake and ingestion of CTRPs. Both crabs and lump fish are opportunistic feeders and respire through gills. However, ingestion of CR particles was reported in the exposed fish but not in the field-collected crabs in our study. Additionally, both crabs and fish can be exposed to CTRPs and leachates through respiration across their gills. In contrast, mussels filter water for respiration and feeding, being a less selective feeder and can potentially ingest smaller CTRPs compared to the other species. A second factor that could explain the differences in 6PPD concentrations among the species is the analysis of different tissues. In the current study, we analyzed homogenized whole soft body tissues from mussels, while only selected tissues were compared in crabs and lumpfish. It is possible that other compartments in the crabs and lumpfish may contain higher concentrations of the targeted chemicals than the analyzed tissues. The homogenizing of the entire soft body of mussels allowed for the inclusion of ingested CTRPs in the extraction processes (seen in Figure 10). This could potentially contribute to additional leaching of chemicals during extraction, affecting the concentrations detected in the analysis. An alternative explanation for this difference in 6PPD among the different tissues could be due to its expected tendency to bind with lipids based on a log Kow > 4 (Unice et al., 2015; Wagner et al., 2018). Accordingly, the higher concentrations of 6PPD in the whole mussel homogenate suggests that the whole mussel may contain more lipids compared to lumpfish blood and crab gills. However, the behavior and interaction of 6PPD in organisms as well as those of the other analyzed compounds are highly understudied, suggesting that other factors could also influence the observed the differences.

4.4 Bioindicators of local car tire rubber pollution

Detecting CTR in the environment and organisms has been challenging due to limited applicability of existing analytical methods such as RAMAN, FTIR or other techniques otherwise used for the detection of microplastics. Crabs seemed to be promising candidates to detect CTRP-related organic chemicals as they are a robust species, being able to live in heavily polluted areas such as in Frierfjorden. However, analyzing different crab tissues for CTR-related chemicals raised some challenges, such as absorbent properties in the gills. The highest detected concentration of 6PPD in crab gills was 0.830 ng/g ww, compared to 17.5 ± 6.65 ng/g ww in wild mussels from our study. To optimize the use of crab species in monitoring of CTRP chemicals, it may be necessary to develop methods for analyzing other tissues which may contain higher concentrations of CTRP-related chemicals, such as the hepatopancreas. Although the hepatopancreas could potentially be a valuable organ to analyze for CTR-related chemicals in crabs, it was not selected for this study due to previous issues with noise during GC/MS analysis experienced by Hägg (2022) in the same laboratory facilities. New methods should therefore be developed to analyze e.g. the hepatopancreas, as this organ can contain 100 nm and smaller particles from ingested food (McGaw & Curtis, 2013 and references therein), possibly including particles and chemicals of CTR origin.

Blue mussels are also a promising candidate, as they are already commonly used in biomonitoring of the surrounding pollution, being easily collected and semi-sessile filter feeders. However, mussels may not be as resilient as crabs, being more vulnerable to harsh conditions, as evidenced by their absence in Frierfjorden. Nevertheless, the mussels were found to have tissues that were more easily extractable for chemicals, compared to the crab gills. Even though blue mussels were not found at the highly polluted site, they are otherwise widely distributed and commonly used in biomonitoring studies, including an urban site in Tromsø. CTRP related chemicals such as 6PPD, TPPD and DTPD are used as vulkanox agents (antioxidants) in manufacturing of tires. These chemicals are mainly used in manufacturing of rubber materials and are characteristic for rubber. Observation of high concentrations of 6PPD and accumulation during exposure of TPPD and DTPD in blue mussels in this study, show that these chemicals could be promising candidate markers to detect the presence of CTR in the surrounding environments of blue mussels.

4.5 Future objectives

To gain a more comprehensive understanding on the effects of CTRP leachates and the particles themselves, it would be essential to conduct more in-depth studies. Investigating the potential negative and chronic effects of long-term exposure to these particles and leachates on blue mussels is important knowledge gaps that needs to be addressed. To do so, future studies could examine biomarkers of stress or toxicity at different life stages to detect any molecular and developmental changes or abnormalities. Additionally, it would be important to investigate the reproductive effects from exposure, and the occurrence of developmental abnormalities in offspring and effects across generations. Capolupo et al. (2021) have demonstrated sub-lethal effects on early-stage mussels following exposure to CTR additive leachates during a short-term study. However, a long-term study examining the effects of both particle and leachates exposure on mussels would provide a more comprehensive understanding of the potential impact that mussels may experience in their natural habitats.

5 Conclusion

In conclusion, this study showed that blue mussels can ingest CTRPs, and that several organic chemicals related to CTRPs are bioavailable to mussels and can remain in the organism for over 7 days. Wild mussels did also show 6PPD, indicating ongoing CTRP exposure in the environment. In wild crabs, no large particles CTR > 0.3 mm were detected although the CTR related chemical 6PPD was detected in gill tissues of both shore- and edible crabs from two sampling sites. Crabs sampled in the heavily polluted site in Frierfjorden and from the less polluted reference sites in Kristiansand showed both the highest occurrence and concentrations of 6PPD in gill tissue. DTPD in blue mussels appears as a promising candidate marker for CTRPs in surrounding environments, accumulating during exposure and slowly decreasing in concentrations after depuration.

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Appendix

Appendix A: Historical satellite pictures from Skjerkøya

Figure 18 - Historical satellite pictures over Skjerkøya, where Ragn-Sells car tire shredding facility is located. The picture shows storage of tires and shredded pieces on an orange dock. (Google, 2004; 2009; 2011).

Appendix B: Blue mussel sampling sites

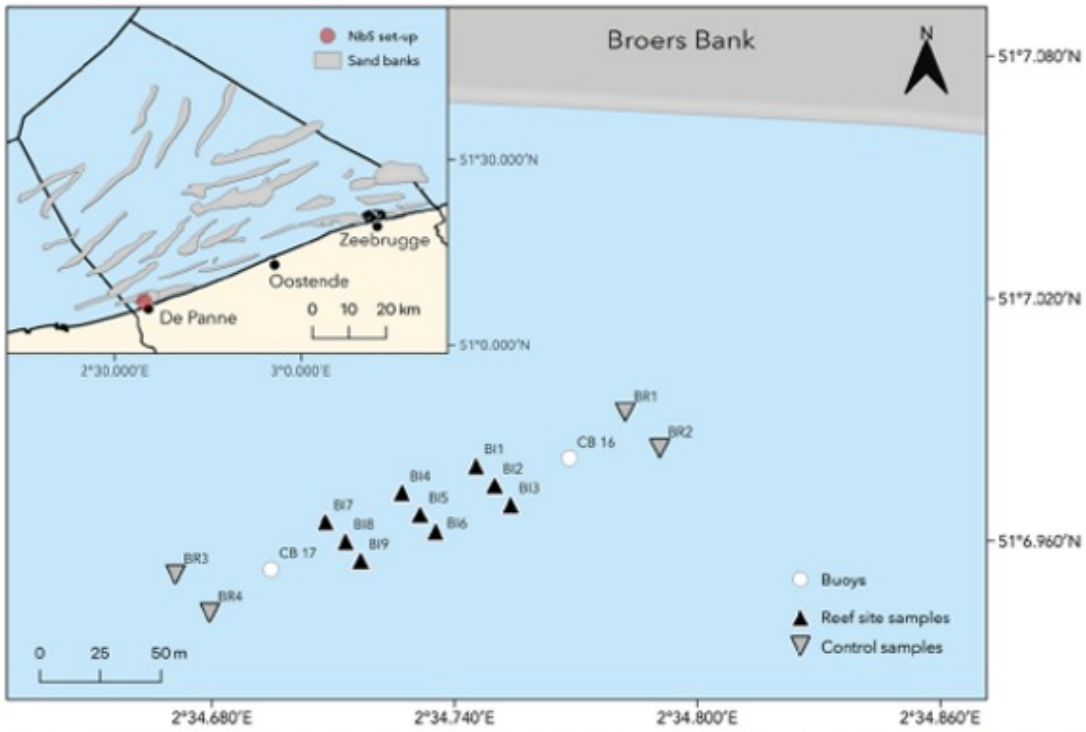


Figure 19 - Location of the Nature-based Solution (NBS) set-up and its sampling stations. Overview map depicts the Belgian Part of the North Sea with sand banks and the NBS set-up location (red dot) displayed.

Appendix C: Tire tread particle mixture (TTmix)

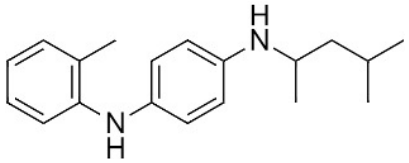
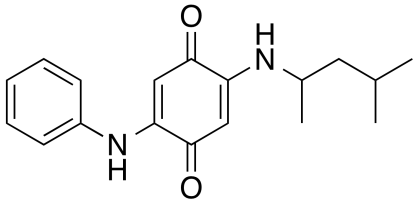
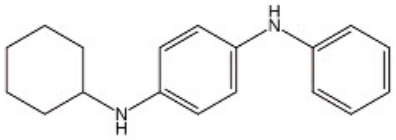
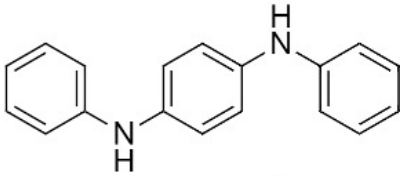
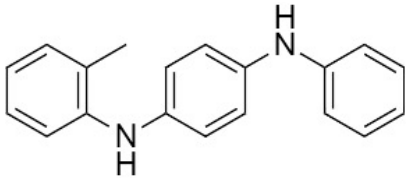
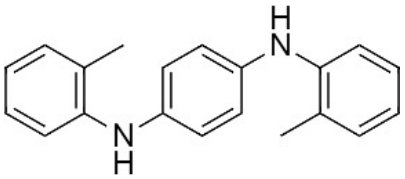
The 20 different types of tires used to make the TTmix exposed to mussels in the exposure experiment.

Table 5 – A list of 20 different types of tires used to create the tire tread particle mixture (TTmix). The table includes the sample ID, corresponding season suitability, and specific details on each tire.

Sample	Season	Details
TP1	all-season	Falken outside Euroall Season 225/50 R17 98 V
TP2	winter	WINTEC PN150 165/65 R15 91T M+S
TP3	winter	Goodyear Vector 5+ M+S 185/65 R15 88T
TP4	winter	Fulda Kristall Montero 2 M+S 195/60 R15 88T
TP5	winter	Continental ContiWinter Contact TS830 205/55 R16
TP6	summer	tire rubber summer (typical mixture)
TP7	winter	tire rubber winter (typical mixture)
TP8	not specified	Continental Germany
TP9	not specified	Triangle, China
TP10	not specified	Wanlitire, China
TP11	not specified	Cheng Shin, China
TP12	summer	Bridgestone DriveGuard 225/40R18 92Y DRGSFZ 67854 VRT7
TP13	winter	Pirelli Sottozero 3 225/40 R18 92Y M+S extra load studless tubeless
TP14	winter	Fulda Kristall Montero 3 205/65 R15 94T M+S
TP15	all-season	Continental VancoFourSeason 2 235/65 R16 C
TP16	summer	Dunlop SP Sport Maxx GT 235/65 R17
TP17	summer	Sava intensa uhp 225/50 R16 92W
TP18	summer	Continental ContiSportContact 5 235/45 R17 94W
TP19	summer	Hankook VentusPrime 3 205/55R16 91V
TP20	summer	Semperit Speed-Life 195/50 R15 82H alpine proven

Appendix D: Chemicals

Table 6 – Eight car tire rubber related organic chemicals (5 p-Phenylenediamines and two unidentified chemicals by structure) analyzed for in tissue samples from the study species. The chemicals are presented with abbreviation, formula, name and chemical structure (if known).

Abbreviation	Formula	Name	Chemical structure
6PPD	$C_{18}H_{24}N_2$	N-(1,3-dimethylbutyl)-N′-phenyl-p-phenylenediamine	
6PPD-Q	$C_{18}H_{24}N_2O_2$	N-(1,3-dimethylbutyl)-N′-phenyl-p-phenylenediamine-quinone	
CPPD	$C_{18}H_{22}N_2$	N-Cyclohexyl-N′-phenyl-p-phenylenediamine	
DPPD	$C_{18}H_{16}N_2$	N,N′-Diphenyl-p-phenylenediamine	
TPPD	$C_{19}H_{18}N_2$	N-o-Tolyl-N'-Phenyl- p-phenylenediamine	
DTPD	$C_{20}H_{20}N_2$	N,N′-Di-o-tolyl-p-phenylenediamine	
C21	$C_{21}H_{26}N_2$	Unknown	Unknown
C23	$C_{23}H_{26}N_2$	Unknown	Unknown

Appendix E: FTIR-analysis of the O-ring in percelly tubes

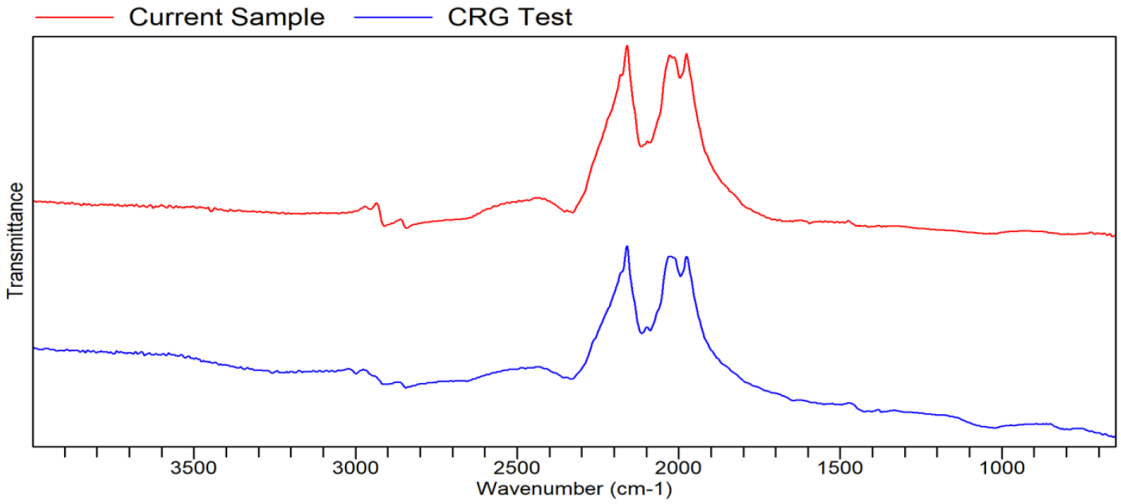


Figure 20 - FTIR result from the O-ring in the precelly tubes used in the first extraction of organic chemicals method on crab tissue. The blue graph shows the transmittance from the car tire rubber granulate (CRG) reference, while the red graph shows the transmittance from the O-ring.

Appendix F: Water parameters

Table 7 - Water parameters measured during the experiment at day 1, 3, 7, and 14. pH, salinity (ppt) and temperature(°C) was measured in each tank (control, low and high exposure).

		Water parameters		
Sampling day	Treatment	**Temperature (°C)**	**Salinity (ppt)**	**pH**
1	Control	12	33	7.69
	Low	12	33	7.68
	High	12	33	7.72
3	Control	12	33	-
	Low	12	33	-
	High	12	33	-
7	Control	12	33	7.88
	Low	12	33	7.91
	High	12	33	7.95
14	Control	12	33	7.95
	Low	12	33	7.99
	High	12	33	8.01

Appendix G: 6PPD (ng/L) analyzed in tank water from the blue mussel experiment

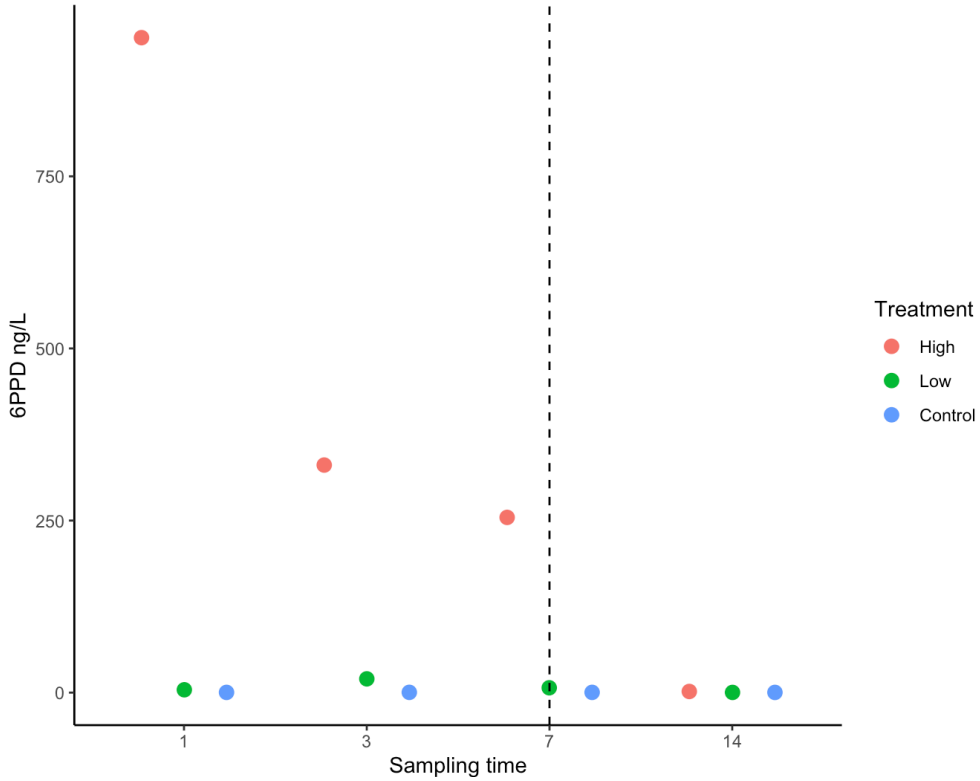


Figure 21 - Concentrations of 6PPD (ng/L) detected in water samples from control, low and high treatment tanks, on day 1, 3, 7, and 14 (Each point, sample n=1).

Appendix H: CTRP-related chemicals in blue mussels

The following individual boxplots displays the concentrations of CPPD, DPPD, TPPD, DTPD, $C_{21}H_{26}N_2$ and $C_{23}H_{26}N_2$ (ng/g ww) detected in blue mussels (*Mytilus edulis*) (n =60) from control, low and high treatment groups from exposure to car tire rubber particles (CTRPs).

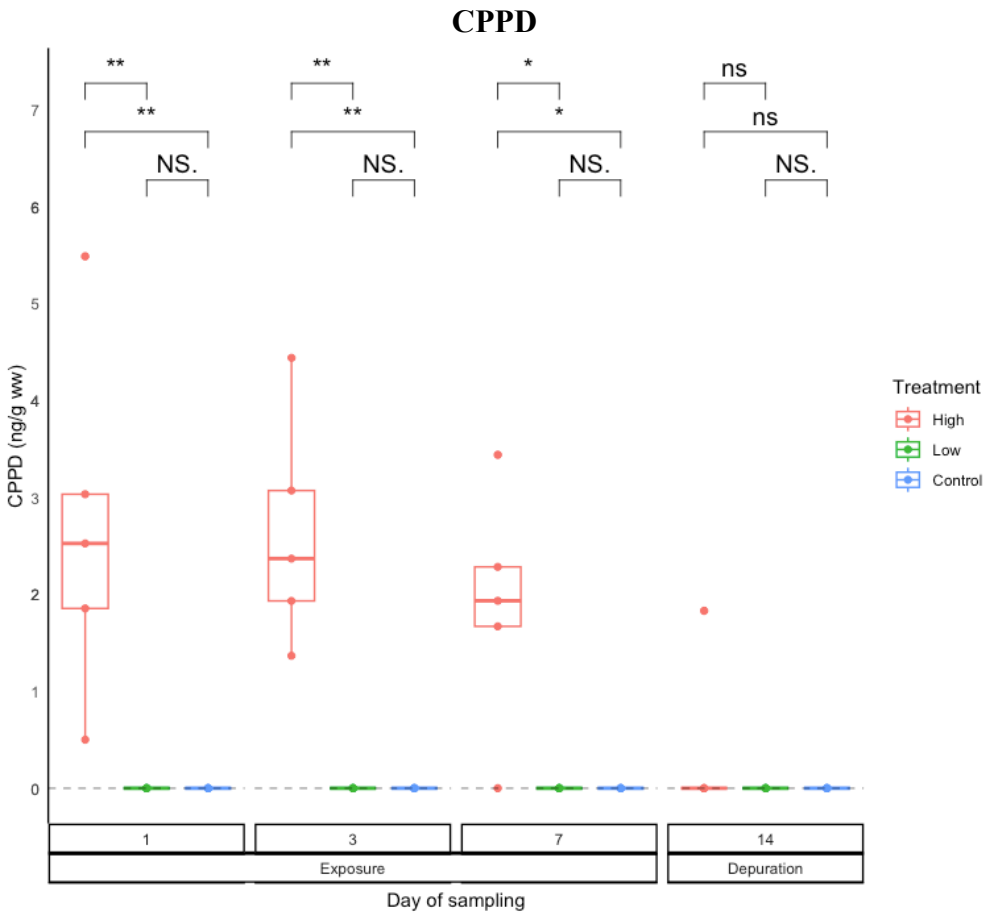


Figure 22 - Concentrations of CPPD (ng/g ww) detected in blue mussels (Mytilus edulis) (n =60) from control, low and high treatment groups from exposure to car tire rubber particles (CTRPs). The exposure lasted for 7 days, followed by a depuration time of 7 days, starting on day 7.. Asterisks (*) indicate statistically significant differences in CPPD (ng/g ww) according to a Wilcoxon rank test between the treatment groups from each sampling day (, p<0.05, **, p<0.01).*

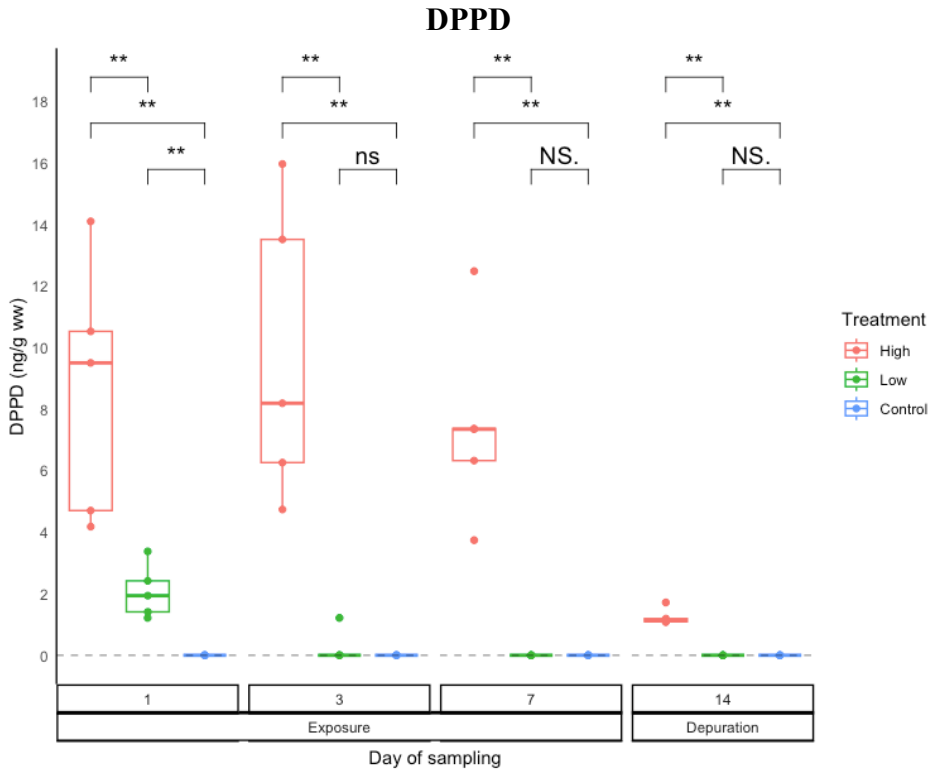


Figure 23 - Concentrations of DPPD (ng/g ww) detected in blue mussels (Mytilus edulis) (n =60) from control, low and high treatment groups from exposure to car tire rubber particles (CTRPs). The exposure lasted for 7 days, followed by a depuration time of 7 days, starting on day 7. Asterisks (*) indicate statistically significant differences in DPPD (ng/g ww) according to a Wilcoxon rank test between the treatment groups from each sampling day (, p<0.05, **, p<0.01).*

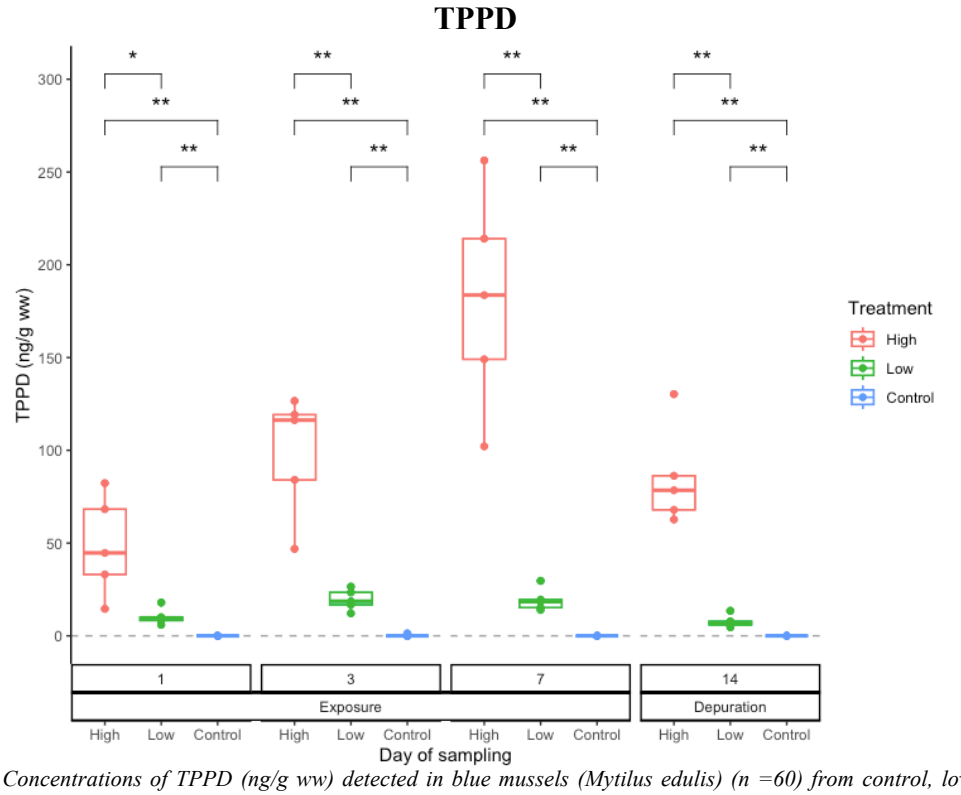


Figure 24 - Concentrations of TPPD (ng/g ww) detected in blue mussels (Mytilus edulis) (n =60) from control, low and high treatment groups from exposure to car tire rubber particles (CTRPs). The exposure lasted for 7 days, followed by a depuration time of 7 days, starting on day 7. Asterisks (*) indicate statistically significant differences in TPPD (ng/g ww) according to a Wilcoxon rank test between the treatment groups from each sampling day (, p<0.05, **, p<0.01).*

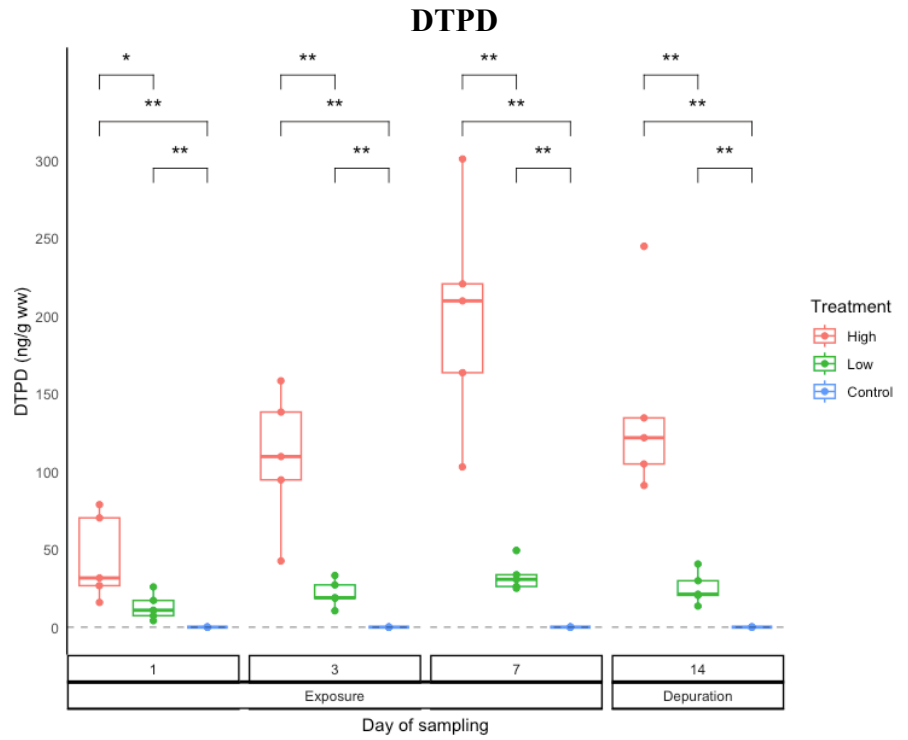


Figure 25 - Concentrations of DTPD (ng/g ww) detected in blue mussels (Mytilus edulis) (n =60) from control, low and high treatment groups from exposure to car tire rubber particles (CTRPs). The exposure lasted for 7 days, followed by a depuration time of 7 days, starting on day 7.. Asterisks (*) indicate statistically significant differences in DTPD (ng/g ww) according to a Wilcoxon rank test between the treatment groups from each sampling day (, p<0.05, **, p<0.01).*

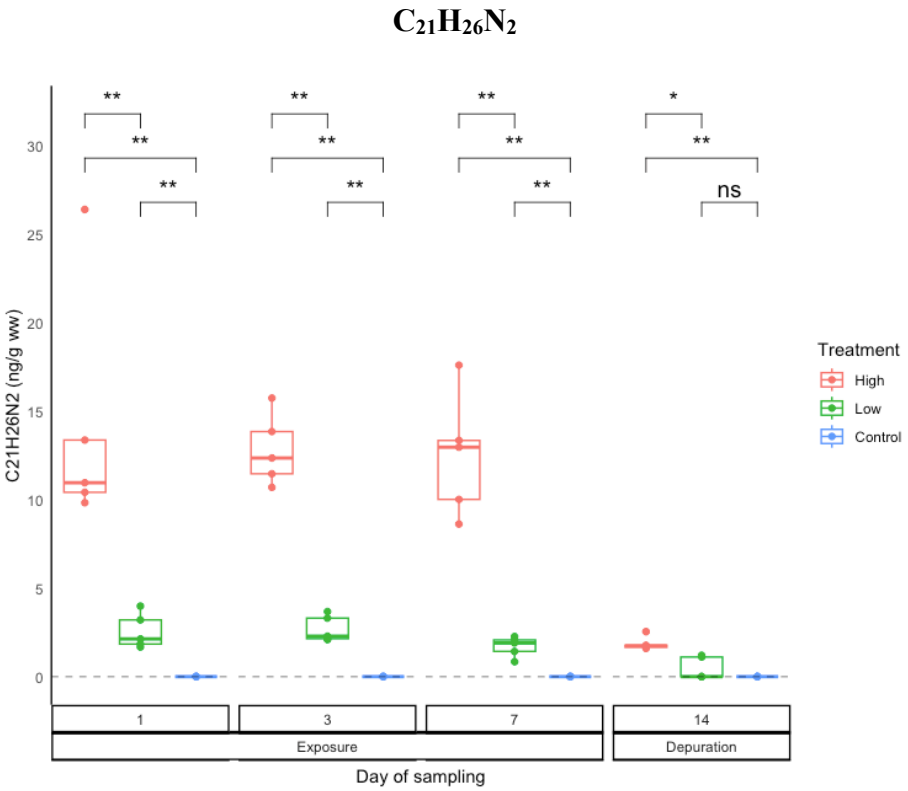


Figure 26 - Concentrations of C21H26N2 (ng/g ww) detected in blue mussels (Mytilus edulis) (n =60) from control, Concentrations of DTPD (ng/g ww) detected in blue mussels (Mytilus edulis) (n =60) from control, low and high treatment groups from exposure to car tire rubber particles (CTRPs). The exposure lasted for 7 days, followed by a depuration time of 7 days, starting on day 7. Asterisks (*) indicate statistically significant differences in C21H26N2 (ng/g ww) according to a Wilcoxon rank test between the treatment groups from each sampling day (, p<0.05, **, p<0.01).*

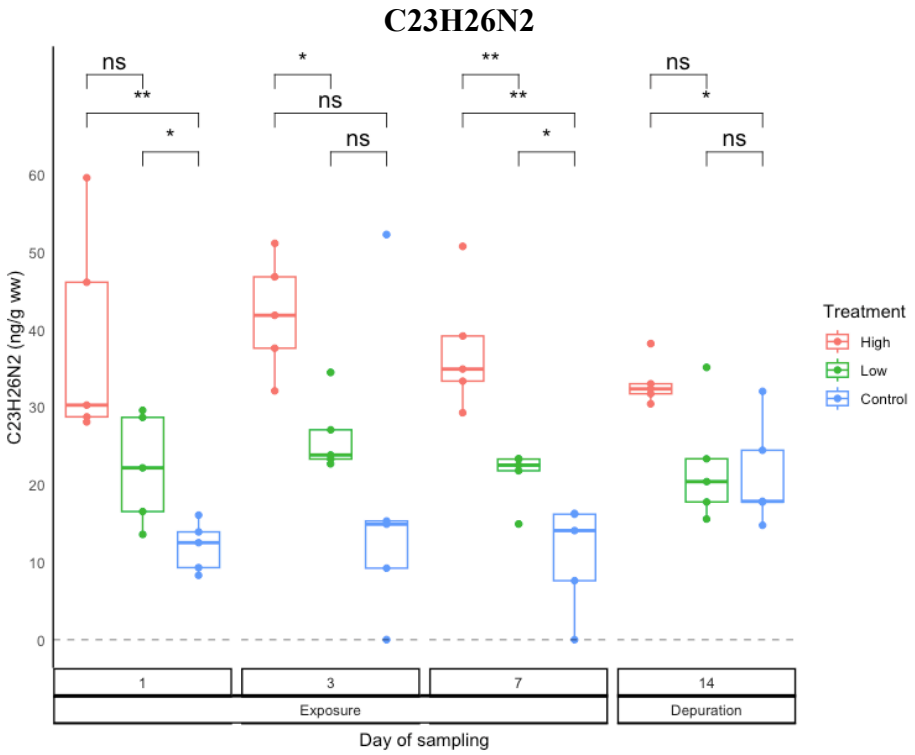


Figure 27 - Concentrations of C23H26N2 (ng/g ww) detected in blue mussels (Mytilus edulis) (n =60) from control, Concentrations of DTPD (ng/g ww) detected in blue mussels (Mytilus edulis) (n =60) from control, low and high treatment groups from exposure to car tire rubber particles (CTRPs). The exposure lasted for 7 days, followed by a depuration time of 7 days, starting on day 7. Asterisks (*) indicate statistically significant differences in C23H26N2 (ng/g ww) according to a Wilcoxon rank test between the treatment groups from each sampling day (, p<0.05, **, p<0.01).*

Appendix I: CTRP-related in blue mussels, low and high treatments

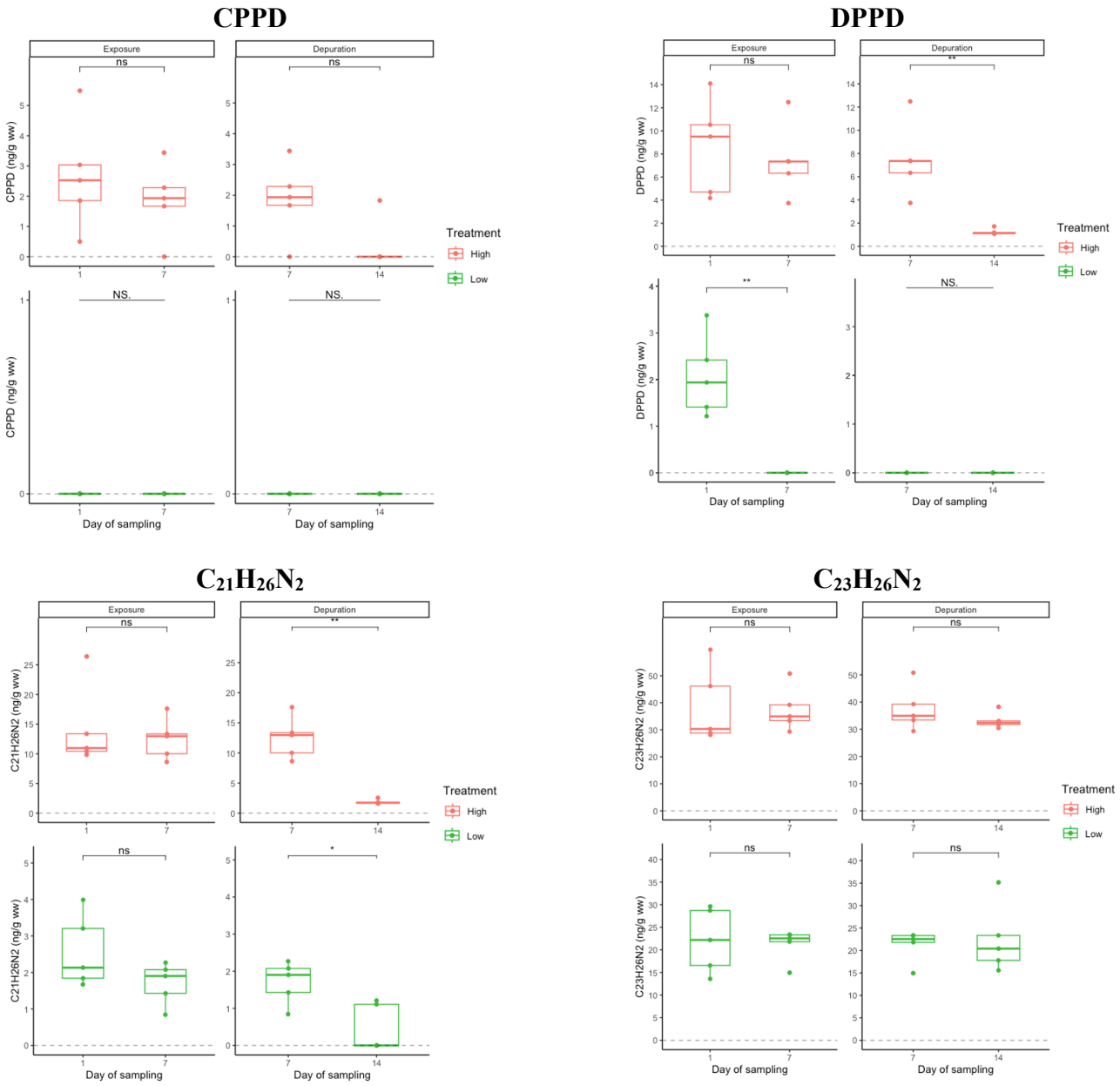


Figure 28 - Concentrations of CPPD, DPPD, $C_{21}H_{26}N_2$ and $C_{23}H_{26}N_2$ (ng/g ww) in blue mussels between the first (day 1) and the last day (day 7) of exposure, and between the first (day 7) and last day of depuration (day 14) for both the low- and high treatment. Each box corresponds to a sample size of n = 5. Asterisks (*) indicate statistically significant differences in 6PPD (ng/g ww) according to Wilcoxon tests between sampling day 1 and 7, and 7 and 14 (: p<0.05, **: p<0.01). The y-axis represent different ranges for the respective treatment groups. The vertical dashed line on day 7 marks the start of the depuration period.*

Appendix J: Morphological measurements of crabs

Table 8 - Morphological measurements (weight (kg or gram), length (cm) and carapace width (cm)) of edible- and shore crabs from the polluted site and the reference sites. Measurements are presented with mean and standard deviation (SD), median and range (minimum value - maximum value) of crabs analyzed for chemicals. The number of analyzed crabs from total catch is presented as n/n total.

	Edible crab						**Shore crab**					
Location	Polluted site			Reference site			Polluted site			Reference site		
n/n total	10/14			7/7			10/22			6/6		
Sex (M/F)	2 / 8			2 / 5			9 / 1			4 / 2		
	Mean ± SD	Median	Range	Mean ± SD	Median	Range	Mean ± SD	Median	Range	Mean ± SD	Median	Range
Weight (kg)	0.40 ± 0.08	0.38	0.27 – 0.50	0.30 ± 0.11	0.30	0.17 – 0.50						
Weight (g)							21.8 ± 11.2	18.9	9.3 - 39.9	57.0 ± 13.6	57.9	36.5 - 71.9
Length (cm)	9.5 ± 0.6	9.5	8.6 – 10.2	8.8 ± 0.9	8.5	7.9 – 10.2	34.4 ± 4.5	33.5	28.5 - 42.0	51.5 ± 3.1	51.0	47.0 - 55.0
Width (cm)	14.3 ± 0.7	14.2	13.1 – 15.5	13.8 ± 1.4	13.6	12.5 – 16.6	45.7 ± 8.3	43.8	35.5 - 58.5	64.3 ± 4.2	63.5	60.0 - 70.0

Appendix K: Model-analysis of 6PPD concentration drivers in crabs

Since model 2 has the lowest AICc score (Akaike Information Criteria, corrected to a small sample size), the lowest Delta AICc value, as well as the highest AICcWt of 0.84, indicating that it has a higher probability of being the best model compared to model 1. Model 2 is therefore regarded as the most parsimonious.

```
> model1 <- lme(log(seksppd)~species*width ,random = ~ 1|group_id ,data=cg, method = "ML")
> model2 <- lme(log(seksppd)~species*Location,random = ~ 1|group_id , data=cg,method = "ML")
>
> models <- list( model1, model2)
> model.names <- c('model1', 'model2')
>
> aictab(cand.set = models, modnames = model.names)

Model selection based on AICc:

       K   AICc Delta_AICc AICcWt Cum.Wt     LL
model2 6 127.64       0.00   0.84   0.84 -56.20
model1 6 130.97       3.33   0.16   1.00 -57.87

> model2 <- lme(log(seksppd)~species*Location,random = ~ 1|group_id , data=cg)
> model2
Linear mixed-effects model fit by REML
  Data: cg
  Log-restricted-likelihood: -55.43586
  Fixed: log(seksppd) ~ species * Location
                           (Intercept)                    speciesShore crab                 LocationPolluted site
                             -3.568944                             1.741632                             1.447290
speciesShore crab:LocationPolluted site
                            -1.382876

Random effects:
 Formula: ~1 | group_id
        (Intercept) Residual
StdDev:    1.315903 1.417367

Number of Observations: 33
Number of Groups: 4
```

Figure 29 - R-script showing model analysis of 6PPD concentration drivers in crabs. Model 1 including carapace width and model 2 including sampling location. The models were analyzed with Akaike Information Criteria (AICc)(Corrected to a small sample size).