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Ingestion of Tyre Crumb Rubber and Uptake of Associated Contaminants in Marine Invertebrates (Pandalus borealis) and Fish (Cyclopterus lumpus) – An Experimental Exposure Study

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Abstract

Crumb rubber (CR) produced from end of life vehicle tires is widely used on artificial sports fields and to create urban artificial surfaces. It is also used as a surrogate for tyre wear particles in experimental studies simulating the behaviour and effects of car tyre particles in the environment. It is known to contain a wide range of organic chemicals and metals, some of which are only just being identified and assessed. Weathering of such artificial surfaces releases both particles and the chemicals they contain into the environment, where they may be bioavailable and affect biota. *N*-(1,3-dimethylbutyl)-*N'*-phenyl-p-phenylenediamine (6PPD) has been identified as a chemical marker for CR and tyre wear particles. Both 6PPD and its transformation product, 6PPD-quinone, have been shown to be toxic to a number of different freshwater and marine organisms. The current study examines the ingestion of CR (1 - 2.8; < 1.2 mm) by a marine invertebrate and fish, the gut retention time of ingested CR, and the tissue burden of tire-derived chemicals. Lumpfish (*Cyclopterus lumpus*) and Northern shrimp (*Pandalus borealis*) were exposed to CR in laboratory experiments for 21 days; 7 days of exposure to CR followed by 14 days of depuration. The stomach contents have been analyzed for ingested CR and selected tissues (Lumpfish liver and blood; Northern shrimp hepatopancreas and muscle) have been characterized for tire-associated chemicals and metals. Analytical chemical techniques involved ICP-MS, pyrolysis GC-MS, GC-MS/MS and HRGC-HRMS (ThermoFisher, Orbitrap). CR were found in 76% of exposed Lumpfish stomachs (n=90), where the number of CR particles in the stomach increased during the exposure and peaked around day 8 followed by a decrease throughout the depuration. Ingested CR was still found in some Lumpfish stomachs at the end of the experiment (day 21). Ingestion by Northern shrimp was analyzed by pyrolysis-GC/MS but. Analysis by HRGC/HRMS (ThermoFisher, Orbitrap) detected 6PPD up to 700 pg/g in blood from exposed Lumpfish, 6PPD was found both at the end of the exposure and at the end of depuration. ICP-MS analysis of metals did not show increased concentrations of metals in any of the tissues for exposed individuals or over time for either Lumpfish or Northern shrimp. The current results suggest that ingested CR can stay in the digestive system of Lumpfish longer than 14 days and that the particles may be leaching 6PPD during this period, suggesting bioavailability of both CR particles and associated chemicals to ecologically and commercially important marine organisms.

CR	Crumb Rubber Granulate
MP	Microplastics
NP	Nanoplastics
SBR	Styrene Butadiene rubber
n	Number
GI-tract	Gastrointestinal tract
ISTD	Internal standard
RSTD	Recovery standard
ELT	End of life tyres
TWP	Tyre wear particles
TRWP	Tyre and road wear particles
ww	Wet weight
dw	Dry weight
n.d.	Not detected

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1. Introduction

1.1 Tyre rubber particles

Tyre rubber particles are abundant in the environment. There are different types of tyre particles. Tyre wear particles (TWP) are produced from the abrasion of tyre tread (Figure 1.1 A), resulting in tyre particles. Similarly, tyre and road wear particles (TRWP) are produced from tyre abrasion as well and are defined as particles encrusted by other smaller particles or minerals from break- or road-wear, entailing in a mixture of TWP and other road-related materials (Sommer *et al.*, 2018a; Goßmann, Halbach and Scholz-Böttcher, 2021). Crumb rubber (CR) are intentionally produced from retired tyres, so called end of life tyres (ELT) and used in different aspects as material recovery or for civil engineering (Erman, Mark and Roland, 2013; WBCSD and Tire Industry Project, 2019). Tyre particles can be considered both as primary and secondary MP. As primary MP are intentionally produced (GESAMP, 2015), such as CR, whereas secondary MP is unintentionally produced from larger items (GESAMP, 2015), such as TWP and TWP.

1.2 Tyre rubber composition

Tyre tread consists of rubber polymers, such as natural rubber, butadiene rubber (BR) and styrene-butadiene rubber (SBR). Car tyres predominately consists of SBR or BR. Chemicals are added in the production of tyres (Figure 1.1 B; Rogge *et al.*, 1993; Erman, Mark and Roland, 2013; Verschoor, 2016) to achieve durable properties. Sulfur is often used in the curing of tyre rubber, where the rubber polymer is cross-linked for increased retractile capacity and decrease permanent deformity. Zinc oxide (ZnO) is often added as an accelerator in the curing process to reduce the process time. Carbon black and silica is commonly used as fillers. These fillers act as reinforcement, to prolong the tyre rubber lifespan. Carbon black increases abrasion resistance, while silica reduced heat buildup and both carbon black and silica increases tear resistance. Rubber polymers are sensitive to oxygen, ozone and thermal degradation. To avoid degradation antioxidants and antiozonants are added. Para-phenylenediamines (PPDs) are widely used as antiozonants (Erman, Mark and Roland, 2013) where diamines are commonly used as antiozonants in tyre production to preserve the tyre and avoid degradation by oxidation (Rogge *et al.*, 1993; Erman, Mark and Roland, 2013).

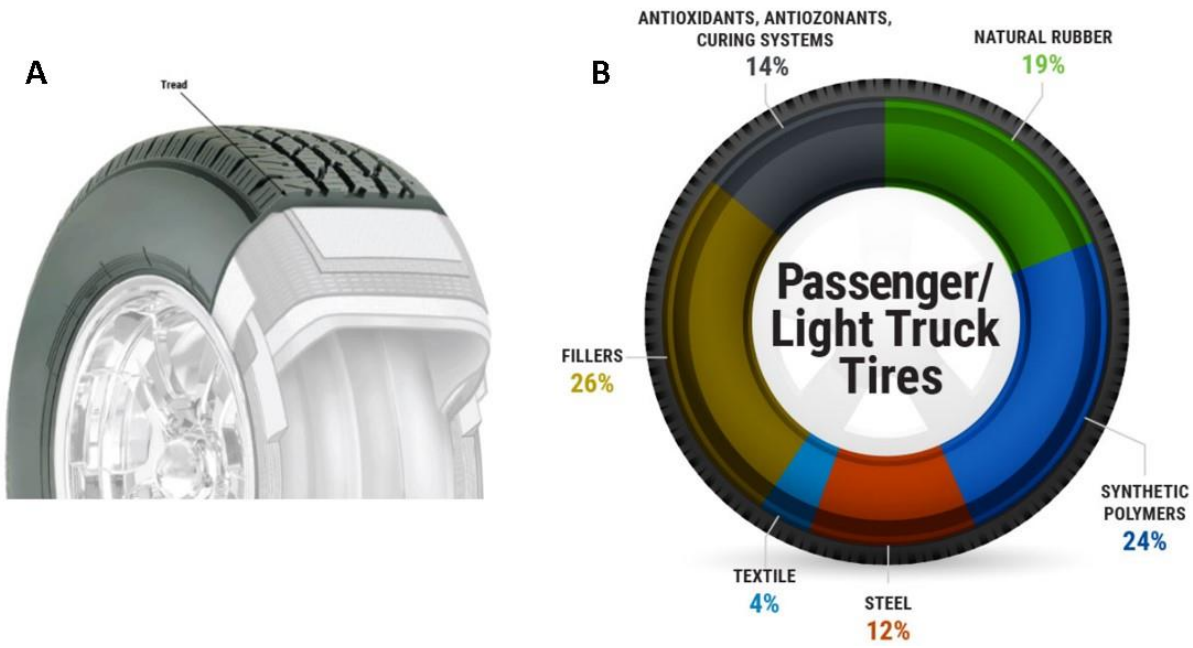


Figure 1.1: Tyre tread (A) and the composition of passenger and light truck tyres (B). Modified from the US Tire Manufacturer Association (UStires.org/innovation).

1.3 Crumb rubber granulate

CR are produced from ELTs and re-used in urban facilities (Erman, Mark and Roland, 2013; WBCSD and Tire Industry Project, 2019). The annual recovery of ELT in Europe and 13 countries (China, India, United states, Japan, Indonesia, Brazil, Thailand, South Korea, Mexico, Russia, South Africa, Argentina and Nigeria) has been estimated to be approximately 26.1 million tonnes. The annual generation of ELT in Europe and these 13 countries has been estimated to be approximately 29.1 million tonnes. In Europe the recovery of ELT was estimated to 92 %, of which 1.9 million tons (54 %) were material recovery, including the production of CR (43 %; WBCSD and Tyre Industry Project, 2019). CR is used in various urban artificial surfaces and in artificial turfs CR is used as fillers. CR is lost during utilization and therefore the turf fields need maintenance with re-filling. Pathways of which CR is lost is by shoes and clothes and through snow removal, drainage systems and water runoff. The estimated annual loss of infill from artificial turfs in Sweden is 2300 – 3900 tonnes, of which 2070 – 3510 consists of styrene butadiene rubber (SBR). There are knowledge gaps in the amount of lost CR that reaches the storm water systems and the marine environment (Magnusson *et al.*, 2016). Usage of artificial turfs entail losses of CR and thus a source of CR

and associated chemicals to the environment. Rainwater from outdoor artificial football turfs have shown chemical leaching of CR. Leaching of such urban artificial surfaces created out of CR may amplify the dispersal of associated chemicals and particles to the environment and reach the marine environment. Research show a relationship between the chemical characterisation of CR and aquatic pollution (Celeiro, Armada, Ratola, *et al.*, 2021).

1.4 Sources, environmental distribution and transport of tyre rubber particles

Tyre rubber particles originate almost exclusively from land-based sources of tyre rubber usage (Rogge *et al.*, 1993; Erman, Mark and Roland, 2013; Boucher and Friot, 2017; Knight *et al.*, 2020; Goßmann, Halbach and Scholz-Böttcher, 2021) and is the second largest source for marine primary MP. Tyre particles are estimated to constitute 28 % marine MP (Figure 1.2; Rogge *et al.*, 1993; Erman, Mark and Roland, 2013; Knight *et al.*, 2020; Goßmann, Halbach and Scholz-Böttcher, 2021). The global number of vehicles has increased with 78 % between 2000 and 2015 and the annual rubber sales for tyre production were 13.9 million tonnes in 2010, of which 46 % were synthetic rubber (Boucher and Friot, 2017). TWP are abraded from tyres, where breaking and acceleration of vehicles increases the tyre abrasion and thus the release of TWP (Rogge *et al.*, 1993; Erman, Mark and Roland, 2013; Knight *et al.*, 2020; Goßmann, Halbach and Scholz-Böttcher, 2021). The predominant source for TWP and TRWP seems to be car tyres rather than truck tyres (Goßmann, Halbach and Scholz-Böttcher, 2021) and the amount of tyre tread lost through traffic depends on various factors of: tyre, vehicle and road surface characteristics and operation of vehicle (Verschoor, 2016).

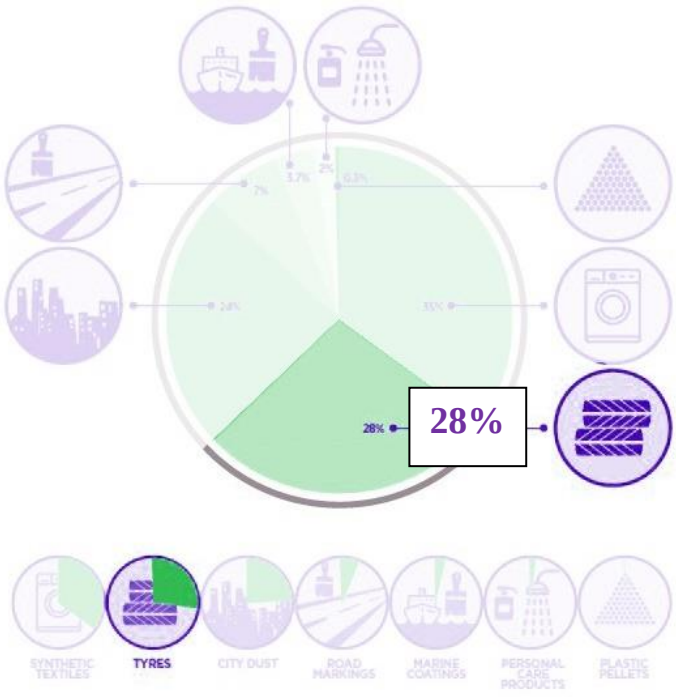


Figure 1.2: Global release of primary MP to the oceans. Tyre particles constitute 28 % of the global primary MP release, being the second largest source for primary MP in the marine environment (Modified from IUCN. 2017).

According to the United Nations Environment Programme (UNEP) mapping over plastic losses, tyre abrasion is the greatest source for MP to the environment (UNEP, 2018). Thus, tyre abrasion represents a major source for rubber-derived MP to the environment (Figure 1.3). Knight et al., (2020) highlight the knowledge gaps concerning the fate and transport of TWP and TRWP and the need for more research on these processes. However, several pathways may be possible, such as aerosols, road and storm water run-off, and wastewater effluent (Knight *et al.*, 2020). Accordingly, a major pathway for TWP may be through aerosols – particle suspension in air. Aerosol samples comprise 89 % of particles derived from traffic-related sources, where 33% were constituted tyre wear (Sommer *et al.*, 2018a). By volume, 93 % were derived from traffic-related sources, where 54 % constituted tyre wear. Extended traffic infrastructure points to extensive emission of TWP which fuel environmental concerns (Sommer *et al.*, 2018a). TWP are also found in road dust (Rogge *et al.*, 1993; Goßmann, Halbach and Scholz-Böttcher, 2021). Road dust samples TWP concentrations between 1.7 and 11.3 g/kg, where TWP were positively correlated with heavily trafficked routes (Goßmann, Halbach and Scholz-Böttcher, 2021). Furthermore, road dust had a similar chemical characterization as aerosol samples, indicating a dynamic

relationship of resuspension and deposition between road dust and aerosols (Rogge *et al.*, 1993). PPD-antioxidants used in tyre production have been found in urban dust samples (Huang *et al.*, 2021), as well as snow (Seiwert *et al.*, 2022), indicating abundance of tyre particles and derived chemicals. Furthermore, this points to the potential for much more derivatives that are yet unknown or just being discovered.

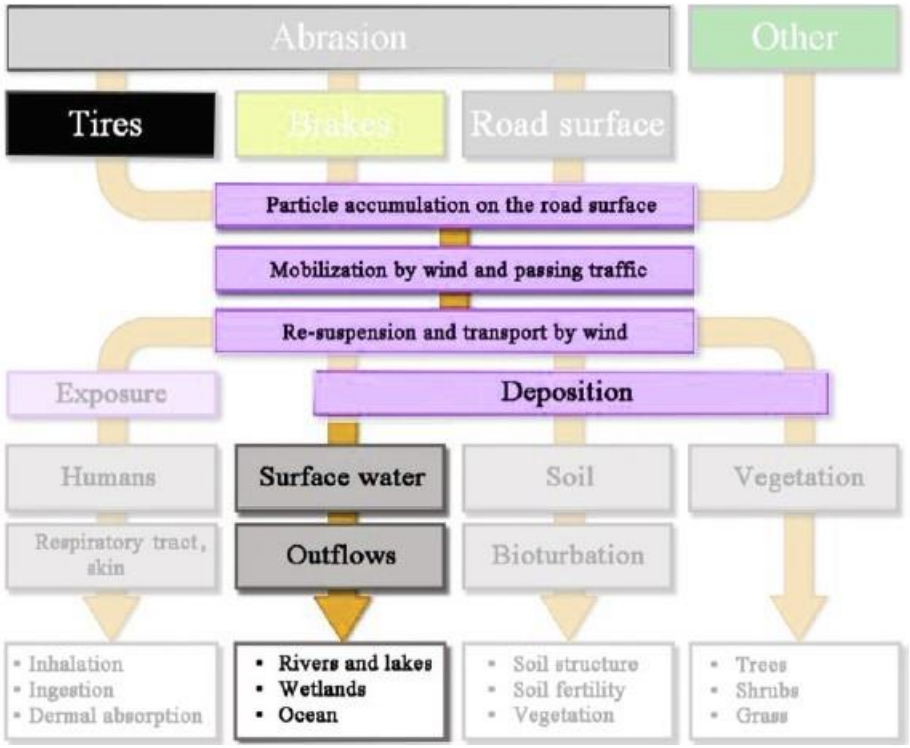


Figure 1.3: Traffic-related emissions of non-exhaust particulate matters. Highlighted are the source of tyre abrasion and the pathways to the aquatic environment where potential impact may occur (Modified from Sommer *et al.* 2018).

Modelling of the release and transportation of MP, including TWP, showed that population densities and connection to sewage treatment plants is a vital factor (Siegfried *et al.*, 2017). Increased number of people connected to sewage treatment facilities increases the MP load that enter the sewage treatment facility. However, these MP may also be discharged untreated depending on the sewage treatment facility efficiency in filtering MP. TWP were the predominant source of MP in European rivers, accounting for 42 % of total MP load exported. Notably, this model only considered MP and TWP emissions from point sources, not including diffuse sources (Siegfried *et al.*, 2017).

TWP have been found in sediments of coastal rivers and TWP concentrations seemed to depend on proximity to highways or the level of urbanization (Leads and Weinstein, 2019; Knight *et al.*, 2020; Goßmann, Halbach and Scholz-Böttcher, 2021). Intertidal and subtidal river sediments and the sea surface microlayer (the boundary layer interface between the ocean and the atmosphere) have been shown to contain TWP. In both intertidal and subtidal sediments, TWP abundance were the second highest MP whereas in sea surface microlayers TWP abundance were the highest. TWP were predominately found in size classes < 500 μm. These findings indicate the abundance and distribution of TWP in and throughout rivers. River characterisations such as volume and flow of water may influence the abundance and transportation of TWP, where higher volume may dilute TWP concentration and higher water flow may increase transportation and dispersal of TWP. Rivers may play a vital role in the downstream transportation in TWP (Leads and Weinstein, 2019).

1.5 Marine tyre rubber pollution

Tyre rubber particles is one of the greatest sources for marine MP (Boucher and Friot, 2017). Although not much is known about the pathways to the marine environment, the main routes for marine tyre particles have been suggested as through aerosols and surface runoff (Kole *et al.*, 2017). Knowledge gaps remains in the transport of tyre rubber by aerosols and the airborne TWP fraction in particulate matter < 10 μm (PM_{10}) may be generally low (< 1 %). However, considering that > 70 % of the earth's surface is covered by ocean, the deposition of aerosols may be an extensive source for the marine load of tyre particles (Kole *et al.*, 2017). Organic content of marine aerosols from the Arctic contained chemicals used as plasticizers (Fu *et al.*, 2013). Even though, these chemicals are not exclusively found in tyre rubber, the results indicate the potential for long-range transportation of MP and tyre rubber particles to remote areas as the Arctic Ocean (Kole *et al.*, 2017).

Modeling of the annual global TWP emissions were estimated to 2907 kilotonnes (kt; Figure 1.4), where the deposition of TWP particulate matter < 2.5 μm ($PM_{2.5}$) were 28.4 kt/year and PM_{10} were 283.4 kt/year (Figure 1.5). The aerosol transportation of TWP $PM_{2.5}$ and PM_{10} ranged between a few ng/m^3 to 20 ng/m^3 and up to 50 ng/m^3 respectively. The mean lifetime for TWP-aerosols of $PM_{2.5}$ and for PM_{10} were 28 and 8.3 days respectively. Accordingly, smaller TWP like $PM_{2.5}$ were dispersed more widely compared to larger PM_{10} that deposit close to the sources. long-range transport of smaller TWP can deposited them in the oceans

and on ice- and snow-covered surfaces of polar and mountain regions. The influence of TWP from both European and North American sources predicts that the snow and ice between northern Greenland and Europe has the highest TWP concentrations. Northern Eurasia is also predicted to have high snow concentrations of TWP (Figure 1.6), influenced by sources from the south. These estimations show a high transportation of fine TWP as $PM_{2.5}$ to Greenland and the Arctic as well as the potential for long-range transportation to remote and vulnerable regions. However, there are still uncertainties as there are knowledge gaps in the size distribution of TWP, influencing the potential for atmospheric transportation of TWP. Moreover, the potential for re-suspension may influence the transportation of TWP where re-mobilization of deposited particles can enhance the transportation and further transport particles (Evangeliou *et al.*, 2020). Similarly, TWP have been found in Arctic marine sediments from the Canadian Arctic. The highest concentrations found in the Beaufort sea and the north Baffin island region/Baffin bay, indicating the potential for long-range transportation of TWP and Arctic sediments acting as sinks (Adams *et al.*, 2021). In contrast, information for TWP or rubber contamination is sparse for the North Atlantic and European sector of the Arctic.

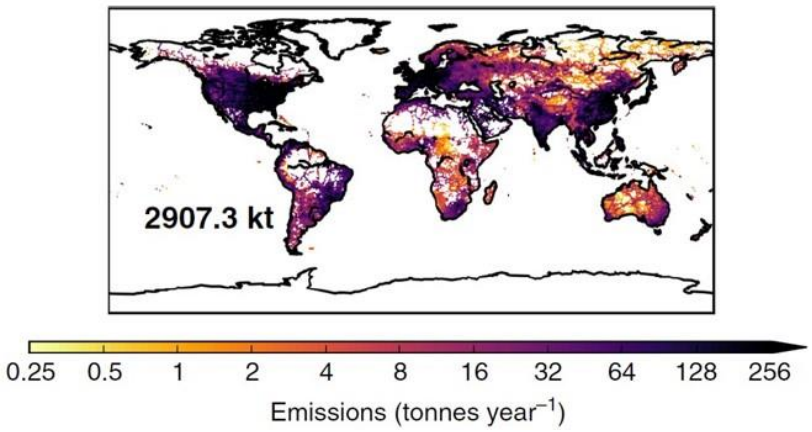


Figure 1.4: Annual global TWP emissions from road vehicles in tonnes/year showing a global annual emission of 2907.3 kt from 2014 (Adams *et al*., 2021).

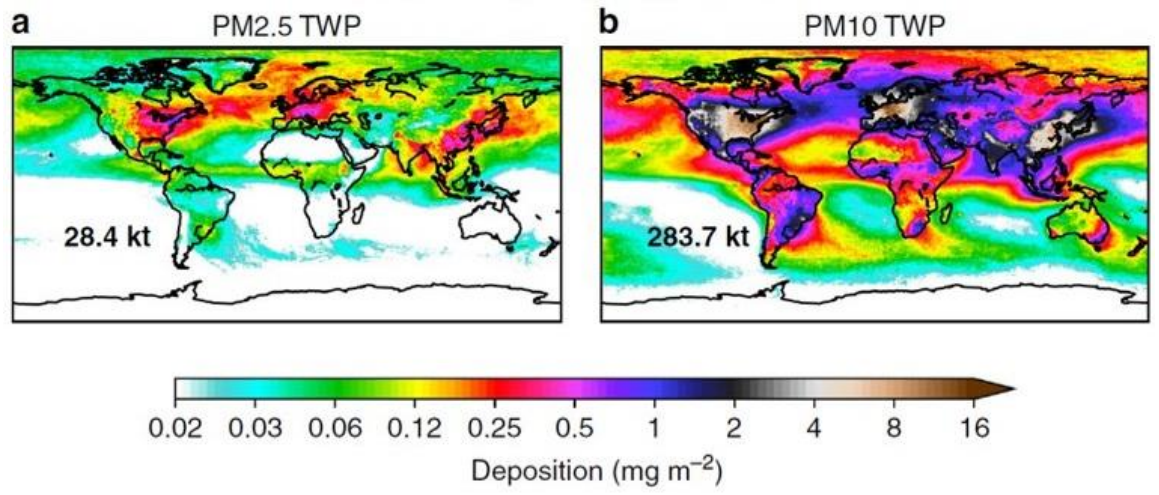


Figure 1.5: Annual total global (wet and dry) deposition in mg/m^2 of TWP $PM_{2.5}$ and PM_{10} where 28.4 kt/year of $PM_{2.5}$ were deposited (a) and 283.4 kt/year of PM_{10} were deposited (b) from 2014. Modified from Adams *et al*., (2021).

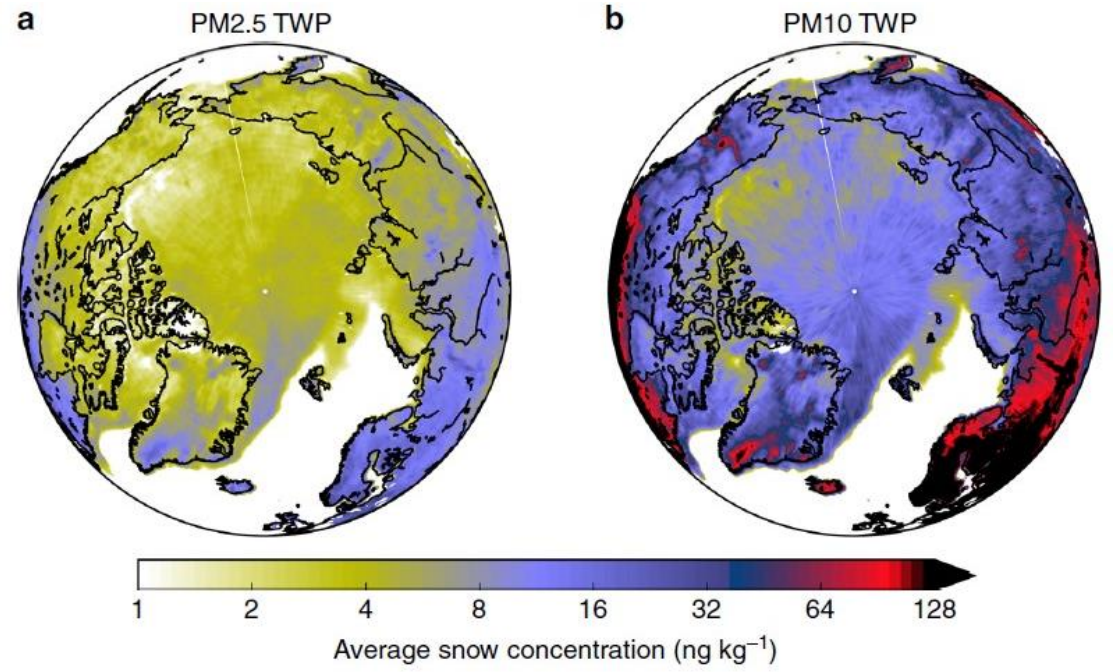


Figure 1.6: Annual average concentration of road MP in Arctic snow and ice in ng/kg. Modified from Adams *et al*. (2021).

1.6 Chemicals and toxicity

Tyre rubber is a technical engineered material consisting of BR or SBR enhanced with mixtures of both metals and organic chemicals (Erman, Mark and Roland, 2013). The predominant chemicals found in tyre rubber particles are Benzothiazole, zinc (Zn) and the polycyclic aromatic hydrocarbon (PAH) pyrene. When tyre particles are dispersed to aquatic environments, the associated chemicals can leach and produce a toxic leachate. Leachates of

tyre particles do not necessarily consist of the same proportions of chemicals as found in the tyre material itself (Halsband *et al.*, 2020; Halle *et al.*, 2021). This could be due to different water solubilities of the chemicals that may influence the potential for leaching (Halsband *et al.*, 2020). Benzothiazoles and zinc are some of the most abundant chemicals and metal in both tyre particles and leachate of sea water (Halsband *et al.*, 2020) and freshwater (Halle *et al.*, 2021). GC/MS-analysis of tyre rubber particle has identified over 100 organic compounds for several classes of compounds, such as n-alkanes, PAHs and Benzothiazoles (Rogge *et al.*, 1993). Further chemicals found in tyre rubber and CR in high abundance are N-(1,3-dimethylbutyl)-N'-phenyl-p-phenylenediamine (6PPD; National Toxicology Program, 2019; Schneider *et al.*, 2020) and DPG 1,3-diphenylguanidine (DPG; Schneider *et al.*, 2020).

Chemical characterization of CR leachate include cyclic amines such as DPG; cyclohexyl‐3‐phenylurea (CPU); N,N‐dicyclohexylurea (DHU) and N,N‐diphenylurea DPU) as the most abundant, but also benzothiazoles (Chibwe *et al.*, 2021). Zinc is by far the most abundant metal found in both particulate tyre rubber (Klöckner *et al.*, 2019; Halsband *et al.*, 2020; Halle *et al.*, 2021) and leachate (Halsband *et al.*, 2020). The high leachability of zinc may be attributed to the high concentration of zinc in tyre materials (Li *et al.*, 2010). PAHs have also been found in CR used in artificial football fields and playgrounds, pyrene being the most abundant (Celeiro, Armada, Dagnac, *et al.*, 2021; Celeiro, Armada, Ratola, *et al.*, 2021), but leaching into seawater is limited (Halsband et al. 2020). Artificial football fields seem to contain higher concentrations of PAHs compared to playgrounds (Celeiro, Armada, Dagnac, *et al.*, 2021) and indoor turf surfaces contained higher overall concentrations of PAHs and other chemicals compared to outdoor turfs. This is likely due to weathering of outdoor surfaces. Accordingly, leachability of zinc seems to be higher in weathered crumb rubber materials (Li *et al.*, 2010) and rainwater from outdoor artificial football pitchers showed leaching of associated chemicals, the most abundant being pyrene. Thus, CR is a source for associated contaminants to the environment (Celeiro, Armada, Ratola, *et al.*, 2021).

Roadway runoff is similar to tyre rubber particle leachate (McIntyre *et al.*, 2021) and also a source for tyre related contaminants (Spies, Andresen and Rice Jr, 1987; McIntyre *et al.*, 2021). A study showed that roadway runoff containing benzothiazoles can pollute sediments and organisms in adjacent areas. Liver extracts from flounder in an bay in close proximity to a busy road contained benzothiazole and 2-methylmercaptobenzthiazole, indicating the

potential for bioaccumulation of these compounds and potential introduction into the food web (Spies, Andresen and Rice Jr, 1987). Leachate have been shown to be toxic to both freshwater (Kellough, 1991; Day *et al.*, 1993; Nelson, Mueller and Hemphill, 1994; Skinner, Peyster and Schiff, 1999; Wik and Dave, 2009a; McIntyre *et al.*, 2014; Young *et al.*, 2018; Khan, Halle and Palmqvist, 2019; Capolupo *et al.*, 2020; Chibwe *et al.*, 2021; Halle *et al.*, 2021) and marine organisms (Skinner, Peyster and Schiff, 1999; Turner and Rice, 2010; Young *et al.*, 2018; Capolupo *et al.*, 2020; Halsband *et al.*, 2020) and have shown adverse effects on growth and development (McIntyre *et al.*, 2014; Young *et al.*, 2018; Capolupo *et al.*, 2020; Chibwe *et al.*, 2021) and survival (Kellough, 1991; Day *et al.*, 1993; Nelson, Mueller and Hemphill, 1994; Skinner, Peyster and Schiff, 1999; Halsband *et al.*, 2020).

Toxicity tests of 6PPD and the transformation product, 6PPD-quinone have shown that freshwater organisms are predominately sensitive to 6PPD with high mortality in freshwater crustaceans and induction of adverse developmental effects and deformities in freshwater fish. In contrast, the same study found no observed effects from exposure to 6PPD-quinone (Hiki *et al.*, 2021). Contrary to this, marine fish seems to be predominantly sensitive to 6PPD-quinone, where low concentrations were lethally toxic to Coho salmon (*Oncorhynchus kisutch*; LC_{50} 0.82 µg/L), compared to 6PPD (LC_{50} 250 µg/L; Tian *et al.*, 2020). This response seems species-specific, as chum salmon (*Oncorhynchus keta*) exposed to 6PPD-quinone showed no sensitivity or toxicity at concentrations up to 320 mg/L (McIntyre *et al.*, 2021).

Deposited TWP that are not washed off by precipitation may be a stealthy source of continuous tire-derived contaminants for the environment (McIntyre *et al.*, 2021), where chemicals from roadway runoff can stay in reservoirs and be mobilized during and after rain events (Peter *et al.*, 2020; Johannessen *et al.*, 2021). Furthermore, besides 6PPD and 6PPD-quinone, a large fraction of TWP chemicals, along with their derivatives are unknown, with unknown behaviors and effects (Johannessen *et al.*, 2021; Johannessen, Helm and Metcalfe, 2021; Müller *et al.*, 2022; Seiwert *et al.*, 2022). Derivatives have been determined experimentally (Seiwert *et al.*, 2022) and in the environment (Huang *et al.*, 2021; Seiwert *et al.*, 2022). Besides the chemical constituents of the tyre rubber material itself, these additive chemicals can give rise to a multitude of derivatives, some of which are known and some are unknown. Some of these chemicals and derivatives are just being identified and assessed (Seiwert *et al.*, 2022).

1.7 Interactions between marine biota and tyre rubber particles

Tyre rubber particles is one of the greatest sources for marine MP (Boucher and Friot, 2017). Benthic ecosystems are heavily affected by MP due to accumulation (Woodall *et al.*, 2014) of sinking particles (Booth *et al.*, 2017a). Sediment samples from oceans indicate high concentrations of MP (Woodall *et al.*, 2014; Booth *et al.*, 2017a) where 91.6 % of the global marine MP is found in the sediments (Booth *et al.*, 2017a) and can act as both sinks and sources for particles and contaminants (Grung *et al.*, 2021). Despite this, there are not many publications available on the ingestion and effect of tyre rubber particles. However, tyre particle exposure have shown that marine fish (LaPlaca and van den Hurk, 2020; Siddiqui *et al.*, 2022) and invertebrates (Siddiqui *et al.*, 2022) ingest small rubber particles (< 355) and that exposure induces adverse effects (Siddiqui *et al.*, 2022).

Marine biota interacts with plastic litter in various ways and the size of the plastic items influence the impact (Figure 1.7). Smaller items like MP, including tyre particles, may be directly ingested or indirectly ingested, the latter referring to the ingestion of prey that contains MP. Ingested MP may block the digestive tract while smaller MP (GESAMP, 2019) or NP may translocate to other tissues (GESAMP, 2019; Clark *et al.*, 2022). Small MP in the environment also have the capacity to adsorb and leach chemicals, acting as vectors of contaminants that may be taken up by organisms (GESAMP, 2015; EFSA, 2016; Booth and Sørensen, 2020).

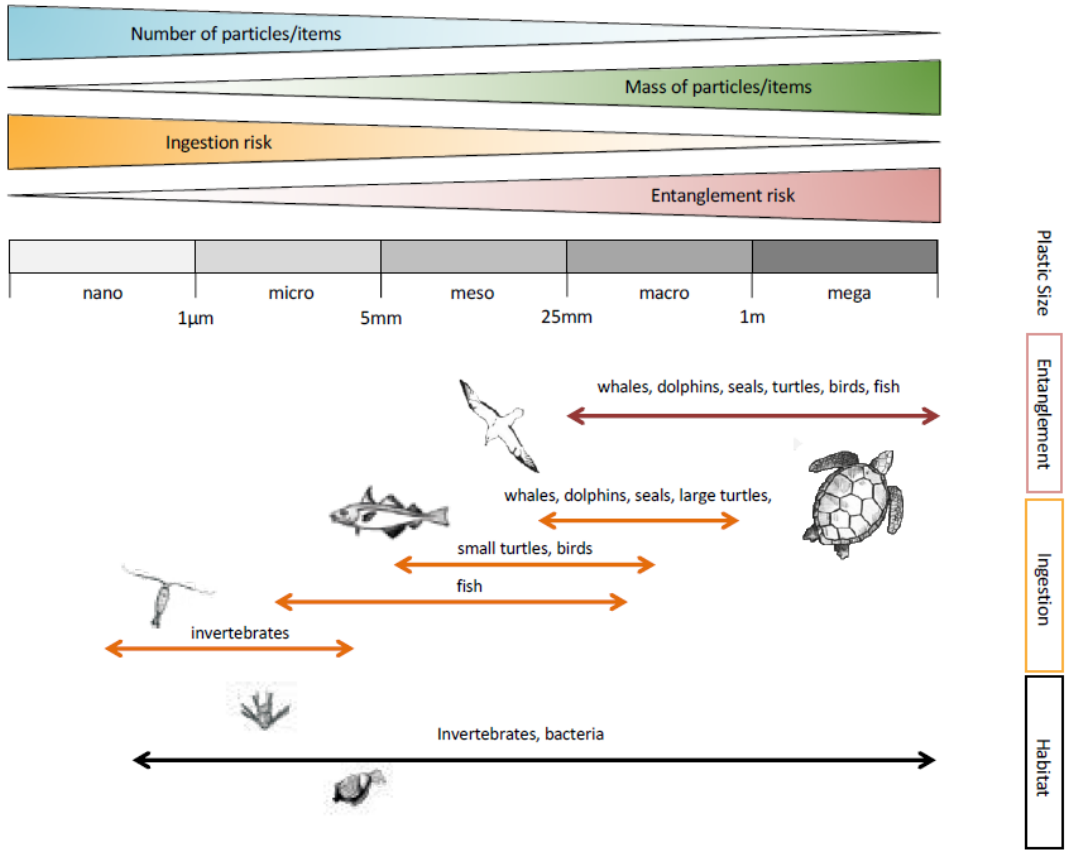


Figure 1.7: Interaction between different animal groups and size classes of plastic and the different associated effects and risks. GESAMP (2019).

1.8 Study species

In the current study, the marine fish (*Cyclopterus lumpus*) and the marine invertebrate (*Pandalus borealis*) were used as model organisms representing different habitats (pelagic and benthic) and feeding strategies (opportunistic and selective).

1.8.1 Lumpfish

The lumpfish (*Cyclopterus lumpus*) is a semi-pelagic fish (Cox and Anderson, 1922; Davenport, 1985; Kennedy *et al.*, 2016) found in the north Atlantic Ocean (Cox and Anderson, 1922) and the Barents sea (Eriksen, Durif and Prozorkevich, 2014). Lumpfish are found in both coastal ecosystems with rocky bottoms and a lot of substrates and pelagic ecosystems with floating substrates where they can attach (Cox and Anderson, 1922; Kennedy *et al.*, 2016). Lumpfish prefer low temperatures (~ 4 - 7 °C) and high salinities (Cox and Anderson, 1922, Eriksen, Durif and Prozorkevich, 2014). Because lumpfish perform diel

vertical migration they are found in varied depths up to 300 m and are both found at the sea floor and the surface layer. It is likely that this behavior is at least to some extent linked to finding prey (Kennedy *et al.*, 2016). Lumpfish prey on smaller organisms such as molluscs, annelids and crustaceans (Cox and Anderson, 1922, Davenport, 1985). In the Norwegian Sea lumpfish have a widespread distribution that increases northward with high abundance close to the polar front and predominately spend their lifetime in the open sea over great depths. It may be that regions close to the polar front represent important feeding grounds. Observations suggest that the Norwegian Sea represents an important nursery for Lumpfish with lower predation and abundant recourses of zooplankton, suggesting migration of Lumpfish fry from the coast to the open sea (Holst, 1993). Thus, Lumpfish seems to be quite a mobile fish, migrating both horizontally and vertically between different marine habitats (Cox and Anderson, 1922; Holst, 1993; Kennedy *et al.*, 2016).

1.8.2 Northern shrimp

Northern shrimp (*Pandalus borealis*) are found in circumboreal regions of the Barents Sea, Greenland and north America. Northern shrimp prefer low temperatures between 0 – 5 °C and are temperature sensitive. They inhabit soft bottoms of mud or sand and are usually found at depths of 50 – 500 m and form schools. Migration of Northern shrimp constitutes of diel vertical migration, ascending in the evening and descending in the mornings, and spawning where females carry eggs inshore throughout autumn – spring. During this period of carrying eggs the diel vertical migration stops and the female stay at the seafloor. The diel vertical migration is likely linked to the feeding as northern shrimps feed both at the sea floor and in the water column. The feeding strategy is opportunistic consisting of both scavenging and predating feeding behavior (Shumway *et al.*, 1985), using chemoreceptors to recognize dissolved emitted substances from the prey (Ceccaldi, 1989). The diet is wide and includes polychaetes, copepods, diatoms, small crustaceans, detritus and organic matter. It is likely that the feeding strategy varies with season, reproductive cycle and geographical area. Northern shrimp is an important species in the marine deep-water ecosystem acting both as predator and prey, thus being a vital source of food for numerous fish species. However, there are records of northern shrimp serving as prey for octopus and harbor seal as well (Shumway *et al.*, 1985).

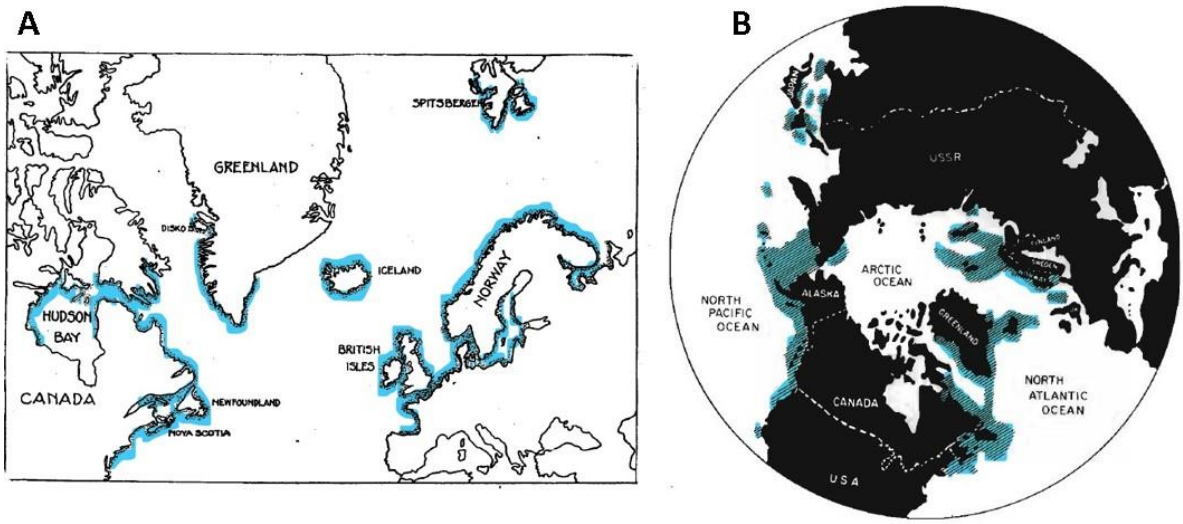


Figure 1.8: Distribution of lumpfish (*Cyclopterus lumpus*) (A) and northern shrimp (*Pandalus borealis*) (B) modified from Cox and Abderson (1922) and Shumway (1985) respectively.

1.9 Knowledge gaps

The apparent widespread abundance of tyre rubber particles in the environment demands increased knowledge of the toxicity of tyre particles to organisms (Siegfried *et al.*, 2017; Sommer *et al.*, 2018b; Knight *et al.*, 2020). A lot of knowledge gaps in tyre rubber particles remain, the effect on aquatic ecosystems being a large area. Although, these questions have been addressed to some degree, the available data is dispersed and much is still not known (Kole *et al.*, 2017), especially in fragile and remote regions like the Arctic where the effects are mostly unknown (Evangeliou *et al.*, 2020). Furthermore, the multitude of tyre-derived chemicals are yet to be fully characterized, not to mention the transformation products produced during degradation of the rubber. Not much is known about what transformation products are produced along different pathways in different environments and even less is known about their effects in the environment (Seiwert *et al.*, 2022). Chemical characterization, reliable harmonized libraries, fate and transport and effect of chemicals constituents of tyre rubber as well as transformation products in various stages and environments are needed.

Furthermore, the European Chemical Agency (ECHA) identified a list of substances of concern in CR used in synthetic turf pitchers. They evaluated the environmental risk and need for further risk evaluation of selected substances of greatest potential environmental concern.

ECHA conclude that there are both human health and environmental risk of certain substances used in infill materials produced by CR. The substances of concern and further risk assessment found were: cadmium, cobalt, copper, lead, zinc, 4-tert-octylphenol, 4,4´-isopropylidene diphenol (BPA), bis(2-ethylhexyl)phthalate (DEHP), benzyl butyl phthalate (BBP) and benzothiazole-2-thiol (ECHA, 2021). Thus, further toxicity studies are needed to evaluate if lower thresholds for such chemicals are needed (Celeiro, Armada, Dagnac, *et al.*, 2021).

The fact remains that not much is known about the behavior and effect of the ubiquitous tyre rubber particles as well as the derived chemicals along with the transformation products ,and few aquatic species have been evaluated for the toxicological effect of such particles, leachates and the complex chemical mixture related to tyre rubber (McIntyre *et al.*, 2021). Therefore, the need for increased and broad knowledge on environmental and toxicological effect of tyre rubber particles and the associated chemicals is needed.

1.10 Objective

The objective of the present study was to provide a better understanding of the ingestion of CR and the leaching and uptake of tyre-related metals and organic chemicals by marine fish and invertebrate exposed to CR. Therefore, the aim was to answer the questions: I) Do fish and invertebrates ingest CR? II) Do CR leach tyre-derived chemicals and are these taken up into different tissues? and III) Do CR and associated contaminants accumulate in tissues and can fish and invertebrates depurate CR and chemicals?

To address these question, two representative marine organisms of different feeding strategy and habitat, Lumpfish (*Cyclopterus lumpus*) and Northern shrimp (*Pandalus borealis*), were chosen for experimental exposure experiments.

2. Materials and Methods

Two laboratory exposure experiments were conducted for lumpfish and northern shrimp. A semi-long-term exposure of three weeks compiled of a one-week exposure following a two-week depuration without exposure. Lumpfish and northern shrimp were exposed to tyre CR and associated contaminants through the food. The study species were provided by FISK (Forsknings- og Innovasjonsstasjon Kraknes). Lumpfish were bread and reared at FISK for 11 months, thus the lumpfish in our experiment were juveniles. The northern shrimps were caught from Laksefjorden in northern Norway. The environmental setting was imitating the summer conditions of northern fjords with a temperature ~5 - 10 °C and an O_2 saturation of > 90 %. The light conditions were different for lumpfish and shrimps. Lumpfish were kept in dark-light conditions, whereas shrimps were kept in dark conditions.

2.1 Experimental design and sampling

2.1.1 Lumpfish set up

Lumpfish (n = 180) were acclimated to the experimental conditions (9.2 °C, 99.9 % O_2 and light-dark cycles) 24 hours prior to experimental start. Lumpfish were randomly and equally distributed (n = 30 per tank) across six tanks (~ 240 l) in two treatment groups: control (tank 1, 3 and 5) and exposed (tank 2, 4 and 6; Figure 2.1) for 21 days. Lumpfish were exposed to CR for seven days through the prepared food that was fed daily through an automatic feeder that dispensed food regularly for approximately 10 – 15 hours. Followed by a depuration period where lumpfish were fed clean food for 15 days. Sampling of lumpfish was conducted for 10 (days 1, 2, 4, 7, 8, 9, 11, 14, 18 and 21) of total 21 days (Figures 2.1 and 2.2). For every sample day, three random lumpfish from each tank was sacrificed. The fish was transferred to a bucket with sea water mixed with anesthetic (Finquel, 1600 mg/l) where they were sacrificed by sedation. The sacrificed fish weight was measured and blood was drawn. The fish were frozen at – 20 °C until dissection.

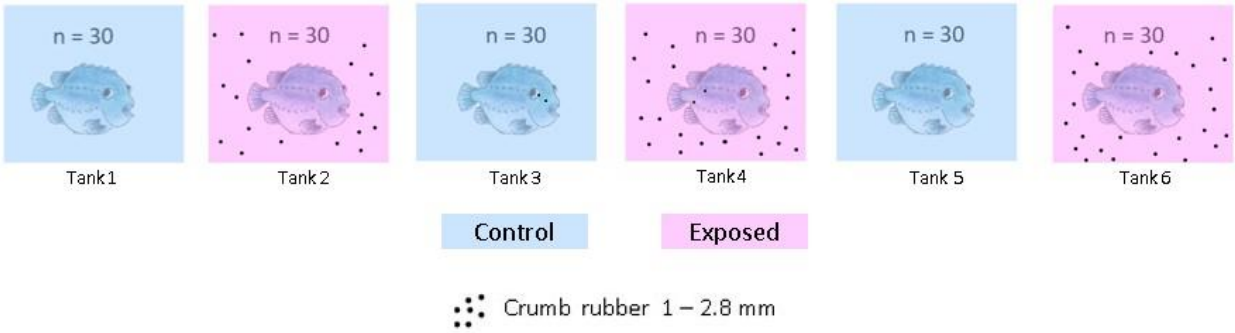


Figure 2.1: Lumpfish experimental set up at experimental start (day 0). Blue represents the control treatment and pink represents the exposed treatments.

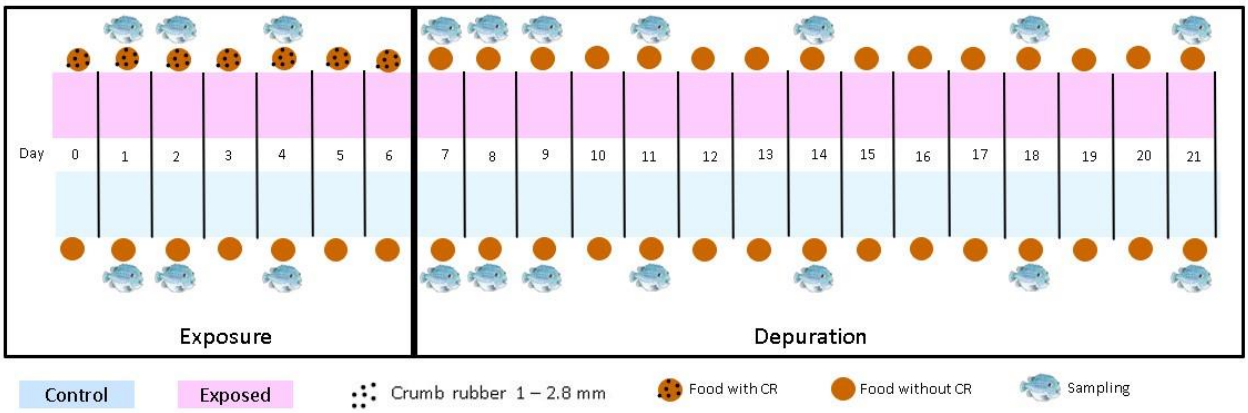


Figure 2.2: Timeline of lumpfish experiment. Exposure experiment of 21 days, starting with 7 days of exposure to CR (1-2.8 mm) through the food following 14 days of depuration without CR exposure.

2.1.2 Shrimp set up

Shrimp (n = 54) were acclimated to the experimental conditions (~ 6 °C, O_2 > 90 % and dark conditions) 24 hours prior to experimental start. Shrimps were randomly and equally distributed across 18 tanks (45 l), i.e. 3 shrimps each, resulting in 6 control treatments (n = 18 shrimps), 6 low CR exposed treatments (n = 18 shrimps), and 6 high CR exposed treatments (n = 18 shrimps). They were incubated in a flow-through system (50 – 60 l/h) for 21 days (Figures 2.3 and S2.1). During the distribution of shrimps five shrimps were lost and replaced with five shrimps (2 shrimps placed in tank 15 and 3 shrimps placed in tank 14) from another catch that had been acclimated for a shorter time than the other 49 individuals and not dark-adapted. To keep the shrimps in dark conditions the laboratory area was separated from the main room with tarps installed from ceiling to floor. Shrimps were offered the prepared food

balls three times in the first week, half spiked with CR, that were fed to the shrimps, followed by a depuration period with food (Vitalis Clean 7 mm) without CR for another two weeks. Shrimps (n=27; from tanks 1, 2, 3, 4, 13, 21, 22, 23 and 24) were sampled after the exposure (day 7) and the remaining shrimps (n=27; from tanks 6, 7, 14, 15, 17, 18, 19 and 20) were sampled at the last day of the depuration (day 21; Figure 2.4). The weight of the shrimps was measured prior to freezing (- 20 °C) and stored until dissection.

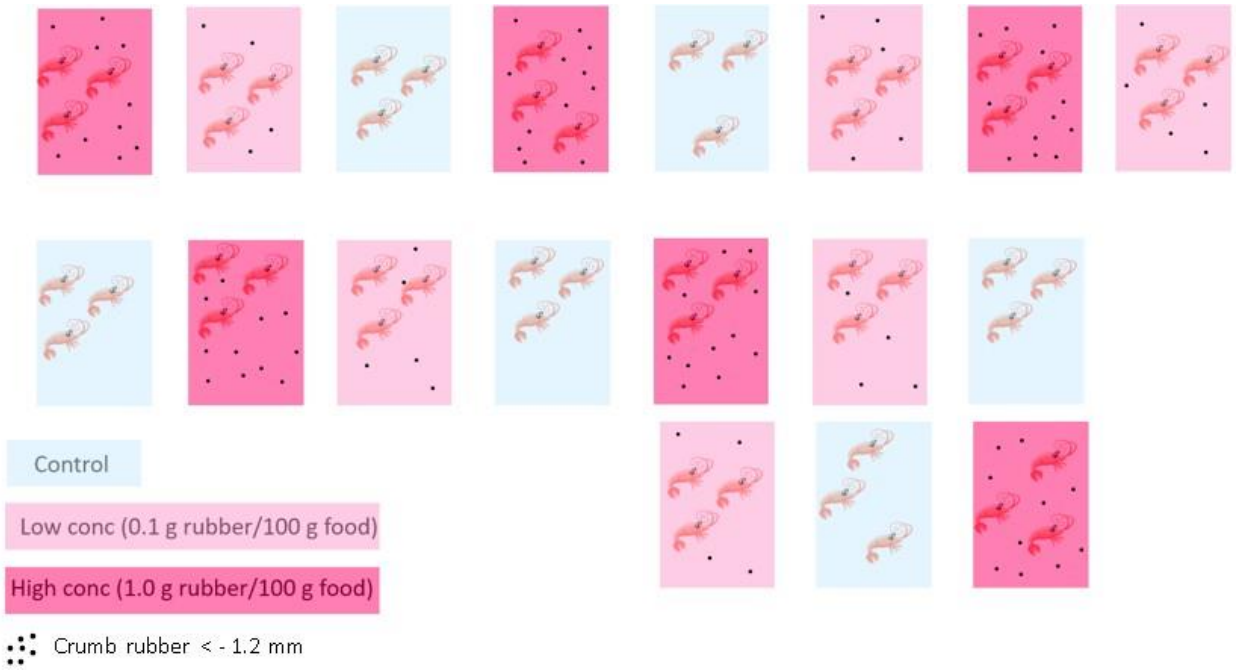


Figure 2.3: Shrimp experimental set up at start. Blue represent control treatments, light pink represent low CR exposed treatments and dark pink represent high CR exposed treatments.

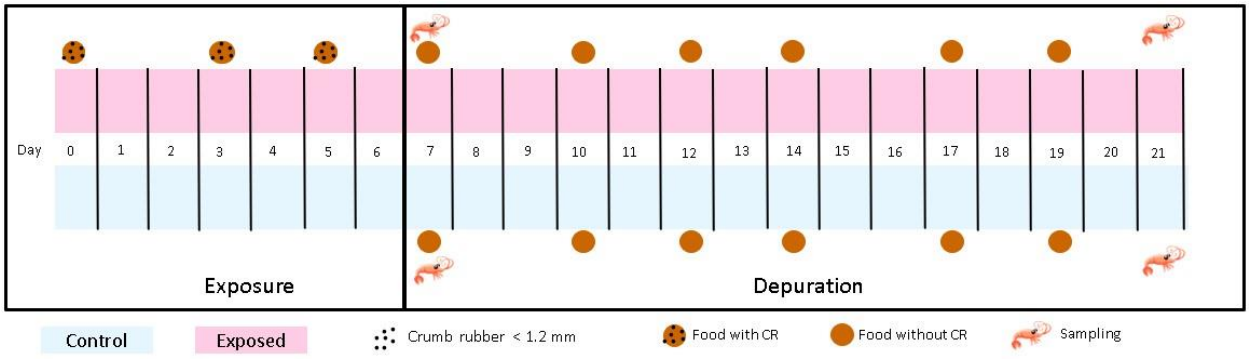


Figure 2.4: Timeline of the shrimp exposure experiment where the pink represents the exposed treatments and blue the control treatments. Sampling

2.2 Exposure preparation

Food used in the exposure of both Lumpfish and Shrimp was prepared in advance and stored at 4 °C in a refrigerator overnight until experimental start.

2.2.1 Lumpfish exposure preparation

Lumpfish dietary requirements (~ 2.4 g/day) were calculated based on the biomass of fish per tank and day, starting with 30 fish/tank (Table S2.1). Food (stamfiskfoder) was prepared by mixing 1.5 l food and 1 l CR (Medium Styrene-Butadiene rubber granulate;1 – 2.8 mm; Ragn-Sells AB; Table S2.2) before transferred to containers labeled by feeding day and tank. The food and CR mix were stored in a fridge until feedings.

2.2.2 Shrimp exposure preparation

Shrimp dietary requirements per week were calculated based on prior food consumption of shrimps at FISK (Table S2.4). One shrimp required 5 g of food per week, requiring a total of 270 g of food per week for all 54 shrimps. Food was prepared for each treatment group, mixing food (Vitalis Clean 7 mm) and CR (fine crumb rubber granulates; < - 1.2 mm; Ragn-sells AB). Food for low concentration treatments was prepared by mixing 100 g of food and 0.1 g of CR. Food for high concentration treatments was prepared by measuring and mixing 100 g of food and 1 g of CR. Food for control treatments measured 100 g of food (Table S2.5). Sea water (~ 5 – 10 mL) was mixed with the respective food mixes for a stickier texture. Balls (n = 54; 1.6 – 1.8 g respective; Table S2.2) were rolled from respective food mixes, acquiring 162 food balls, one per shrimp for three feedings during the exposure week. The food balls were transferred by three to containers marked with treatment group (control, low concentration and high concentration) and feeding day. Food balls were stored in a fridge until feedings (Figure 2.5).

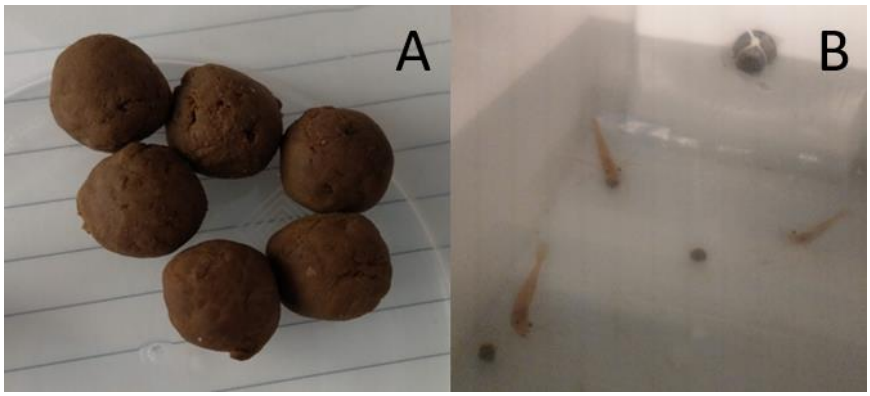


Figure 2.5: Food balls (~ 1.6 – 1.8 g) for control groups (A) and a tank with shrimps being fed with one food ball each (B).

2.3 Dissection and Examination of GI-tract

Both shrimps and lumpfish were thawed and dissected using scalpel, scissor and forceps on a cutting board covered in burnt aluminum foil. Dissection was carried out on control groups first, followed by the exposed groups where, for the shrimps, low CR exposed groups before the high CR exposed groups. The equipment was rinsed in ethanol (96 %) between the dissections and nitrile gloves (TouchNTuff) where used. The stomachs and intestines were dissected and will forward be referred to as the gastrointestinal tract (GI-tract).

2.3.1 Dissection of lumpfish

The length of the lumpfish was measured, nose to tail fluke. Lumpfish was then cut abdominally from the anus, over the abdominal and over the suction disc and continued laterally on the left side towards, and over, the gills. From the gills the left side was cut dorsally toward the exterior end until reaching back to the anus and the left side exposed the internal organs. The livers were carefully removed and transferred to glass vials (4 – 7 mL). During the dissections some of the livers were put in one glass vial of either 4 or 7 mL while some livers were aliquoted in two glass vials (4 or 7 mL). The GI-tract were then carefully cut out to not puncture the tissues and transferred to a marked zip lock bag. All samples were frozen until further analysis.

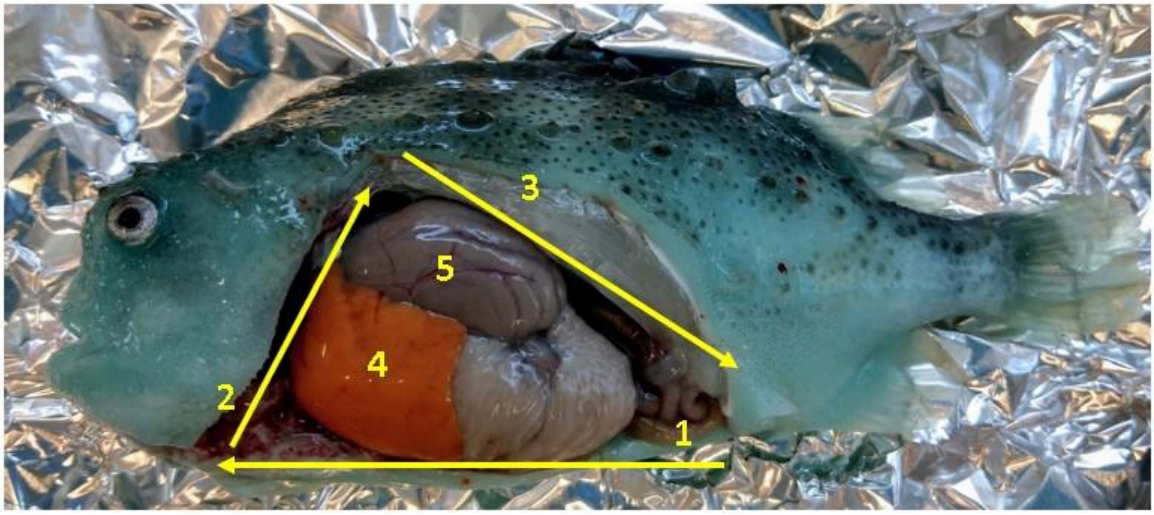


Figure 2.6: Dissection of lumpfish. Yellow arrows and numbers indicate the order of opening up and collecting of tissues. First opening the fish (arrows 1-3), followed by collecting the liver (4) and finally collecting the GI-tract (5). Fish were dissected on burned aluminum foil to avoid contamination.

2.3.2. Dissection of P. borealis

The length of the carapace was measured (along arrow 2 in Figure 2.7). Shrimps were cut below the end of the carapace, separating the abdomen from the cephalon. The exoskeleton of the head was cut dorsally towards the rostrum followed by the anterior end of the exoskeleton that was cut laterally towards the rostrum on both sides, releasing the exoskeleton. The thin membrane covering the organs was cut open to expose the organs. The hepatopancreas was carefully removed and transferred to a glass vial (7 mL) followed by the stomach that was transferred and wrapped in burned aluminum foil. The exoskeleton was dorsally cut to the exterior end and then with scalpel cut laterally down to the pleopoda to release from the tail muscle. After relieving the tail muscle of the exoskeleton, a piece of the left muscle was carefully cut to avoid cutting the intestine, which was also collected and wrapped with the stomach in foil. A part of the muscle (0.3 g) was removed and transferred to a plastic tube (2 mL; Precelly's), the remaining muscle was wrapped in burned aluminum foil. All the samples were frozen until further preparation and analysis (Figure 2.7).

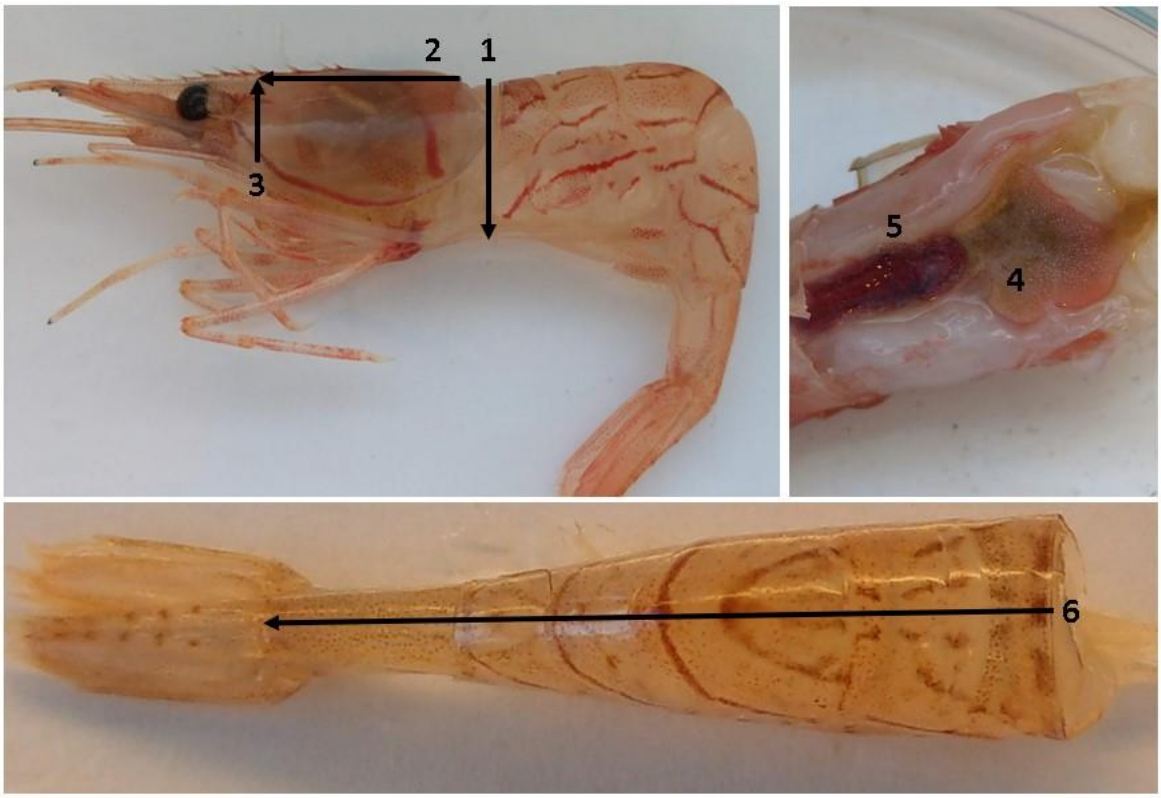


Figure 2.7: Dissection of shrimp. Black arrows and numbers indicate the order of dissection. First separating the cephalon from the abdomen (arrow 1), followed by opening the cephalon (arrows 2-3) and followed by collecting the hepatopancreas (4) and the stomach (5). Finally, exposing the tail muscle for collection of muscle and intestine. Shrimps were dissected on burned aluminum foil to avoid contamination.

Stomach and intestine of Lumpfish were examined manually on glass petri dishes with forceps and scalpel. The stomach and intestine were separated to be examined stomach separately. The tissues were opened by scalpel to release the content and CR particles were counted. Both particles in intestine and stomach were counted and noted and the total number of ingested particles throughout the GI-tract. All of the exposed GI-tracts were examined and all of the control GI-tract that was analyzed for chemicals were examined. For the remaining controls two random GI-tracks were examined to control for ingested CR.

2.3.3 Digestion and filtration of shrimp GI-tract

Digestion of shrimp stomach and intestine was performed according to Enders *et al.*, (2017). The tissues were weighted and transferred to glass vials (mL). Solutions were calculated according to amount of tissue digested, 5 g of digestion solution per 1 g of tissue. KOH pellets were dissolved in H_2O (1120 g/l). Water (42 mL) were transferred to a beaker following N_aClO (9 mL) and KOH (9 mL). The digestion solution was transferred (1g/ g

tissue sample) to the samples. The samples were sonicated in an ultrasonic bath for 15 min followed by overnight shake.

Digested samples are filtered on glass microfiber filters (Whatman, pore size 1.5 µm, diameter 27 mm) with underpressure. Tubes were rinsed with water and filtered. Filters were dried overnight on aluminum foil in a clean cabinet. Dried filters were folded, wrapped in aluminum foil and stored in a glass bottle covered in aluminum foil until final preparations and pyrolysis.

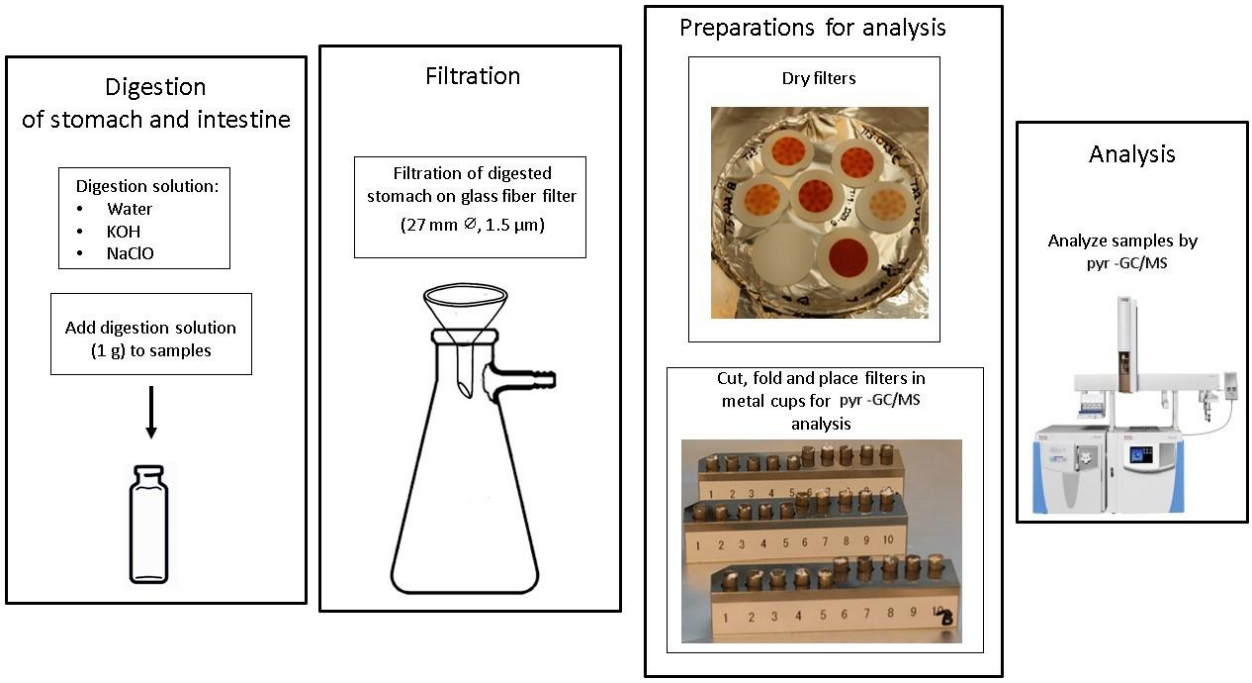


Figure 2.8: Shrimp GI-tract were digested and filtered on glass fiber filters (27 mm in diameter and 1.5 µm pore size) with underpressure. Filters were dried overnight and folded into metal cups for analysis by pyr-GC/MS. Samples were analyzed for the tyre rubber trace SBR-vinylcyclohexene for signs of ingestion of CR.

2.4 Extraction of organic chemicals

The chemical analysis was done for the samples after the exposure (day 7) and after the depuration (day 21). These were chosen to compare and evaluate leaching, uptake into tissues and depuration over time. In addition, CR was extracted and analyzed for characterization of organic chemicals.

2.4.1 Extraction of CR

Fine crumb rubber (20 mg) was measured and transferred to glass vials (7 ml). Solvents (2 ml) were added to the samples, one solvent per sample. Solvents used were Hexane, Ethylacetate, DMSO, Isopropanol, Toluene, and Acetonitrile. The samples were sonicated at 40 °C for 30 minutes and left to settle for 30 minutes before sonication at 40 °C for 30 minutes again. A subsample (100 µl) was transferred to a GC/MS glass vial (300 µl) with insert and screw cork. The remaining samples were sonicated at 40 °C for 30 minutes, left to settle for 30 minutes and sonicated at 40 °C for 30 minutes. The samples were shaken for 72 hours before a subsample (100 µl) was transferred to a GC/MS glass vial (300 µl) with insert and screw cork. Samples extracted with Isopropanol and Acetonitrile was evaporated by nitrogen and re-dissolved in Ethylacetate (100 µl) before analyzed.

Crumb rubber used in the exposure were extracted for characterization of organic chemicals present. Because the medium crumb rubber used in the lumpfish exposure and the fine crumb rubber used in the shrimp exposure were from the same material (Styrene butadiene rubber) and manufacturer (Ragn-Sell AB) and only differ by size, only the fine crumb rubber was extracted. Sonication was used to allow the crumb rubber particles to disperse while the shaking enhanced particle surface contact with the solvents. The shaking and heating induced the leaching of the crumb rubber. Samples extracted with Isopropanol, Acetonitrile and DSMO could not be analyzed as these solvents are not suitable for GC-analysis. Samples extracted with Isopropanol and Acetonitrile were therefore evaporated and re-dissolved for analysis. As there were no clear difference between the chemical profile between the solvents (Hexane, Ethylacetate, Isopropanol, Toluene and Acetonitrile) DSMO were not processed furthered and analyzed.

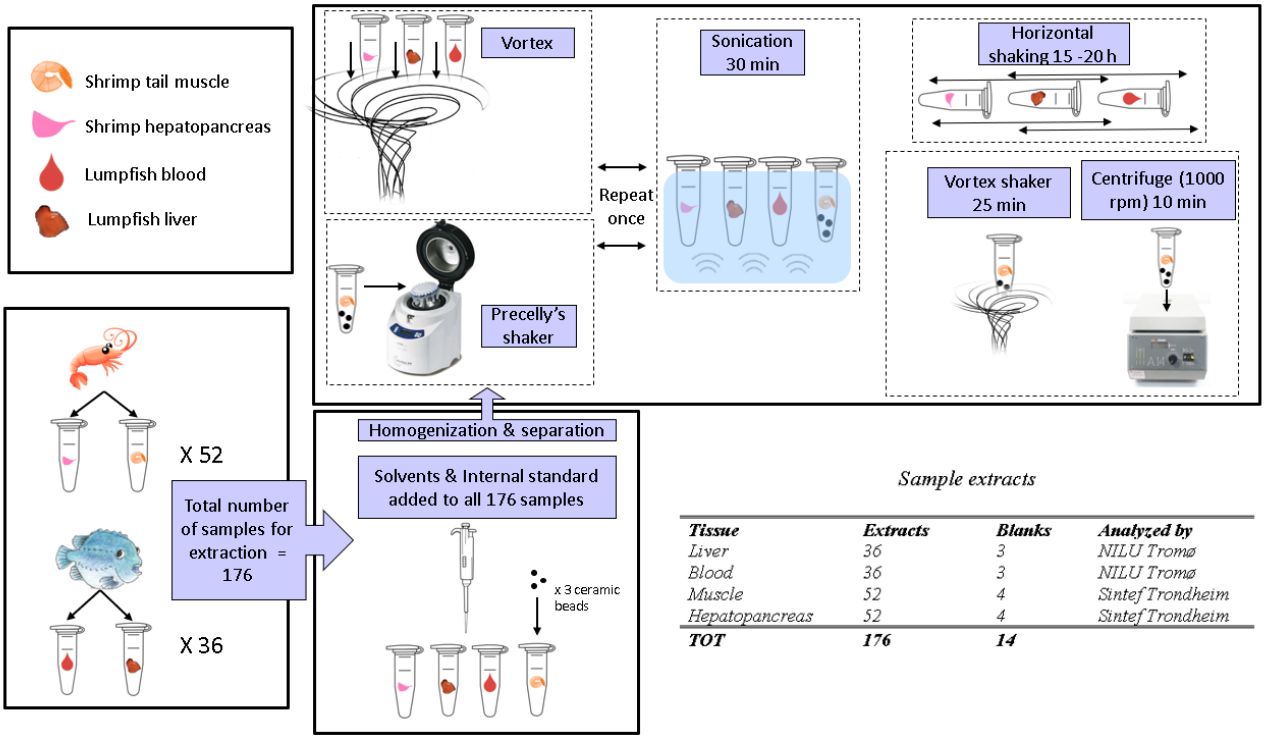
2.4.2. Extraction of organic chemicals

For the analysis of organic chemicals an extraction process was performed (Figure 2.8). All samples were prepared in a clean cabinet to avoid and decrease contamination and lab blanks of Milli-Q water was included for every 10 – 15 samples to control for contamination. A tyre rubber internal standard (6PPD-Quinone-D_5; 0.4 ng/µl) was used to detect and control for loss of the analyte (6PPD-Quinone). Hexane, a non-polar solvent, and Acetonitrile, a polar solvent, were used to extract the organic chemicals from the samples. Lumpfish blood (0.5 g) and liver (0.5 g) were measured and transferred to burned glass tubes (15 mL). Shrimp tail

muscle (0.3 g) was transferred to plastic tubes (2 mL) and hepatopancreas (0.5 g) was transferred to glass vials (7 mL) prior to the extraction process.

Solvents and standards were added to the samples. Samples were separated and homogenized by precelly's shaker and vortexed followed by 30 minutes of sonication. The process was repeated once. The samples were horizontally shaken overnight for ~ 15 – 20 hours before they were vortexed (liver, blood and hepatopancreas) or centrifuged (muscle). For detailed extraction protocol see supplementary information. All samples were frozen until further preparations. The caps were checked and screwed tight before and after every step of the extraction process as these easily unscrewed. All tubes were carefully marked with sample ID and date (Figure 2.8).

Acetonitrile and Hexane was used as solvents to ensure extraction of both polar and non-polar organic chemicals. The standard, 6PPD-Quinone-D_5, was used as an internal standard to control for measuring and loss of the analyte, as well as for the quantification of the analyte. Likewise, the standard, 6PPD-quinone-$^{13}C_6$, was used as a recovery standard to control for loss of internal standard and the efficiency of the extraction method. Ceramic beads were added to the tail muscle samples to enable homogenization by bead beating. This was necessary as muscle tissue are firmer compared to the other tissues analyzed in this study. Plastic tubes were necessary to use for the tail muscle samples to avoid breakage in the process of homogenization by bead beating. The overnight horizontal shaking of the samples was used to increase the mixing of solutions and tissue. For more details see supplementary section S3.



Sample extracts

Tissue	*Extracts*	*Blanks*	*Analyzed by*
Liver	*36*	*3*	*NILU Tromø*
Blood	*36*	*3*	*NILU Tromø*
Muscle	*52*	*4*	*Sintef Trondheim*
Hepatopancreas	*52*	*4*	*Sintef Trondheim*
TOT	***176***	***14***	

Figure 1.8: Extraction method of the organic chemicals in the tissues (lumpfish liver and blood and P. shrimp hepatopancreas and tail muscle). Solvents used was Acetonitrile and Hexane (95 %). Internal standard used was 6PPD-Quinone-D5, (0.4 ng/µl).

2.4.3 Preparations and pooling of samples for metal analysis

Lumpfish blood and liver, and shrimp tail muscle were analyzed for metals. Samples were pooled by tank and sample day (Figure 7; Table 3). Same sample days were analyzed for metals as for the organic chemicals (day 7 and 21). Samples were pooled by tank and day, thus three individuals per tank and treatment comprised one sample, acquiring 12 blood and 12 liver samples and 18 tail muscle samples for analysis. One pooled sample required 1 g of tissue, entailing approximately 0.33 g of each sample. Acid cleaned plastic tubes (15 mL) were used for the samples.

Liver samples (~ 0.33) were prepared on glass petri dishes with a ceramic knife and glass pipette tips to avoid and minimize contact with metals and contamination of the samples. Metal forceps were used to transfer liver pieces. Livers were cut, transferred to plastic tubes, measured and mixed with a glass pipette. The samples were frozen until further preparation and analysis. Blood samples (~ 0.33 g) were transferred to plastic tubes by glass pipettes and measured. The samples were frozen until further preparations and analysis. Tail muscle

samples (~ 0.33 g) were prepared on the burned aluminum foil they had been kept in. Muscles were cut, transferred to plastic tubes, measured and mixed with a glass pipette. The samples were frozen until analysis. Samples were sent to and analyzed for metals at NILU, Kjeller according to Halsband *et al.*, (2020). For more details see supplementary section S3.

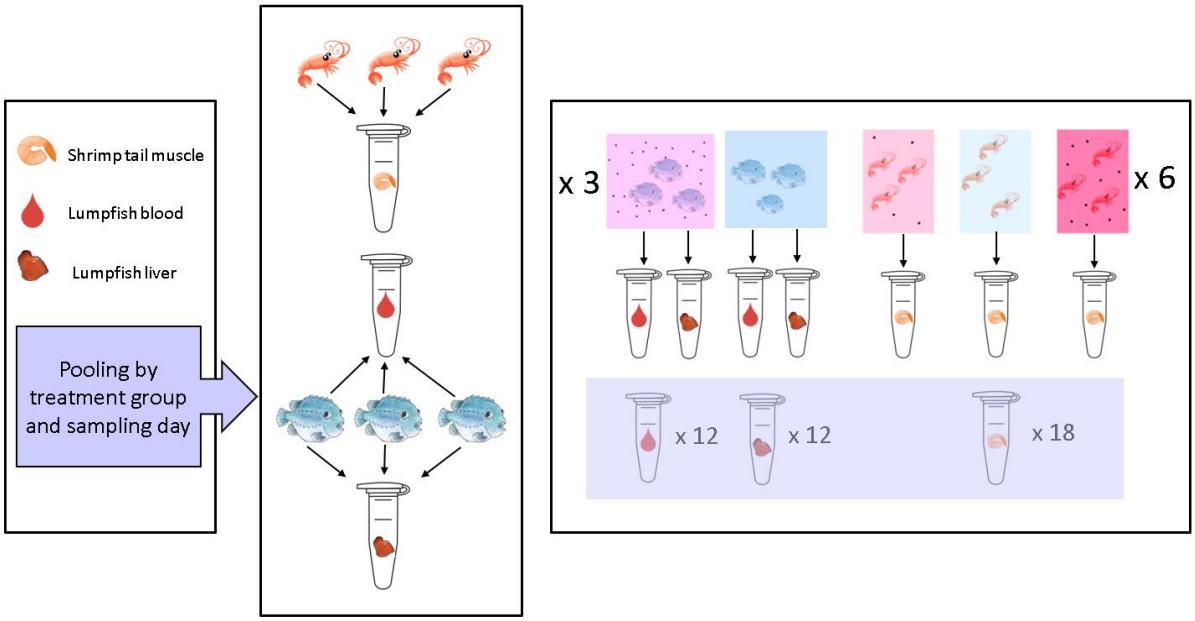


Figure 2.9: Pooled metal samples by tank and day. After the exposure period (day 7 for lumpfish and day 8 for shrimp) and after the depuration period (day 21 for lumpfish and day 22 for shrimp). Pink tanks represent exposure and blue tanks represents controls.

Table 2.1: Metal samples by tissues from two days, after the exposure (day 7 for lumpfish and day 8 for shrimp) and after depuration (day 21 for both lumpfish and shrimp).

Tissue	Liver	Blood	Tail muscle	Total
Number of pooled samples	12	12	18	42

2.5 Sample analysis

Fine CR extracts and extracts of blood samples from lumpfish were analyzed by gaschromatography high resolution mass spectroscopy (GC/HRMS-orbitrap; ThermoFisher)

by NILU, Tromsö. Extracts of blood and liver samples from lumpfish were analyzed by liquid chromatography (LC/HRMS) by UFZ Lepzig, Germany. Extracts of shrimp hepatopancreas and tail muscle were removed of biological molecules by solid phase extraction (SPE) by SINTEF ocean and analyzed by GC-MS/MS triple quadrupole (Agilent) by SINTEF ocean, Trondheim. Shrimp GI-tract were analyzed by pyrolysis-GC/MS by NILU, Tromsö. Blood and liver samples from lumpfish and tail muscle samples from shrimp were analyzed by inductively coupled plasma mass spectroscopy (ICP-MS) by NILU Kjeller, Oslo.

Chromatograms show peaks, the relative response, the peak area, of the analyte. The response correlates to the concentration and can be interpreted as the relative concentration as it is the ratio between the signal (the peak area) and the quantity of the analyte (Figure 2.10).

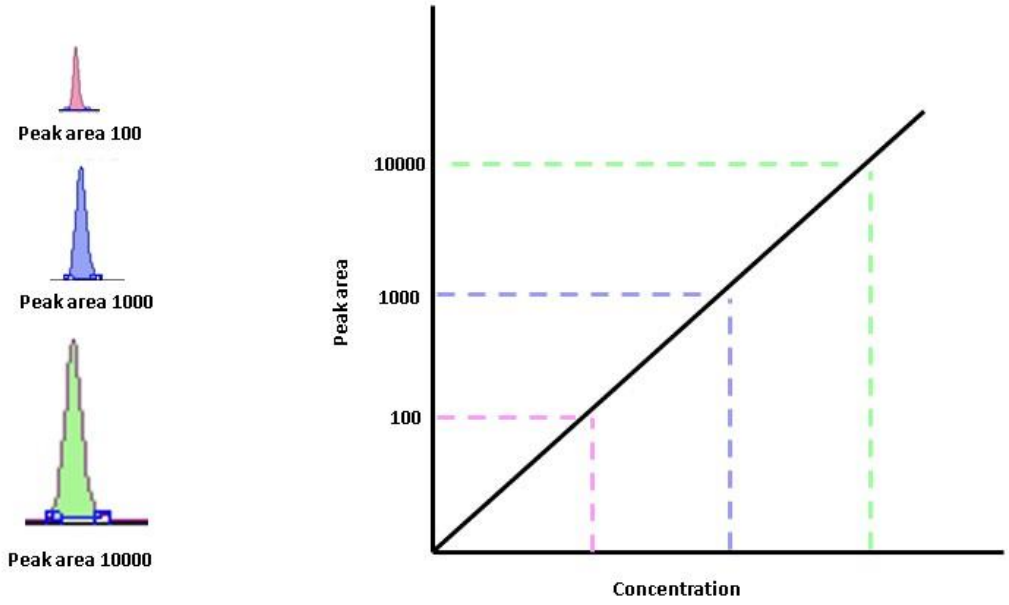


Figure 3.8: Schematic over response of peaks in a chromatogram. The peak area, also called the response, of a chemical, correlates to the concentration. The greater the peak, the greater the signal and the higher the concentration.

2.6 Statistical analysis

Shapiro-Wilks test for normality was run for the data over distribution of CR ingestion for lumpfish, SBR-vinylcyclohexene concentration for shrimp, metal concentrations and organic chemical concentrations for bot lumpfish and shrimp. Mann-Whitney U tests were run for the lumpfish metal and organic chemical data to test for statistical differences in concentrations

between treatment groups. Kruskal-Wallis were run for the shrimp pyrolysis and metal data to test for differences in various metal concentrations between treatments. All boxplots in the results show median (horizontal line), the 1st quartile (bottom half of the box), the 3rd quratile (the upper half of the box) and the whiskers (vertival lines, extends to highest and lowest values within 1.5 * IQR (inter-quartile range). Data points beyond the end of the whiskers are, according to the plot, considered outliers.

2.7 Ethical considerations

The use of fish and shrimps in the current study was approved by Mattilsynet and killed humanely in a researched and approved manner where animals were respected by reduced stress and suffering to possible extent.

3. Results

3.1 Ingestion and gut retention time in lumpfish

Many of the exposed lumpfish in tanks 2, 4, and 6 ingested CR under laboratory conditions during the exposure period of 6 days. Feeding on CR was observed both in situ (observations of fish behaviour in the tanks) and based on dissections of the gastrointestinal tract (GI-tract; Figures 3.1 and 3.2). Based on counts of CR particles in the GI-tract, 68 individuals out of 90 (76%) had CR in their GI-tract at the time of sampling. As expected, no CR was found in the GI-tract of the control groups in tanks 1, 3, and 5 with 30 fish each. The average number of ingested CR per fish (median) and interquartile range (IQR) found in exposed lumpfish on the 10 sampling days ranged between 0 (1) on days 14 and 18 and 28 (7) on day 8 (Figure 3.3 and Table S3.1). The total number of CR particles found per fish varied between individuals, ranging between 0 and 84 for the minimum and maximum numbers of CR particles found. The number of CR particles found increased over the exposure period from day 1 to 6, and until the beginning of the depuration period (day 7). On day 8, the median reached a peak, followed by a decrease throughout the remaining depuration period (days 9 - 21). The number of exposed lumpfish without CR in the GI-tract increased after day 8 and was highest on days 14 and 18 (5 fish, respectively). After 14 days of depuration, at the end of the experiment (day

21), there were still CR particles found in 6 out of 9 (67 %) remaining lumpfish (Figures 3.3, 3.4 and Table S3.1).

There was no significant difference in the amount of CR found between the exposed groups, tanks 2, 4 and 6 (Kruskal-Wallis test, $p > 0.05$). In all three tanks, there were individuals with either high, low, or no CR particles present in the GI-tract. Nevertheless, tank 2 had a higher number of fish with few or no CR particles present in the GI-tract (days 4, 9, 11 and 14 with median 0, Figure 3.5) compared to tank 6 (average 0 CR particles on days 14, 18 and 21) and tank 4 that never had fish without CR in the GI-tract. Nonetheless, two individuals from tank 2 had high CR particle count in the GI-tract both early in the exposure (day 2) and late in the depuration (day 18). In tank 6, one individual contained 60 CR particles on day 2, and three others contained 57 - 60 particles on day 7. In tank 4, one individual contained 56 CR particles on day 7, one 57 on day 8, and two individuals > 80 CR particles each on day 9 (Figure 3.3 and Table S3.2). At day 21 there were 2 fish with 8 and 3 CR particles in the GI-tract from tank 2, 3 fish with 3, 10 and 33 CR particles in the GI-tract from tank 4 and 1 fish with 1 CR particle in the GI-tract from tank 6 (Figure 3.3).

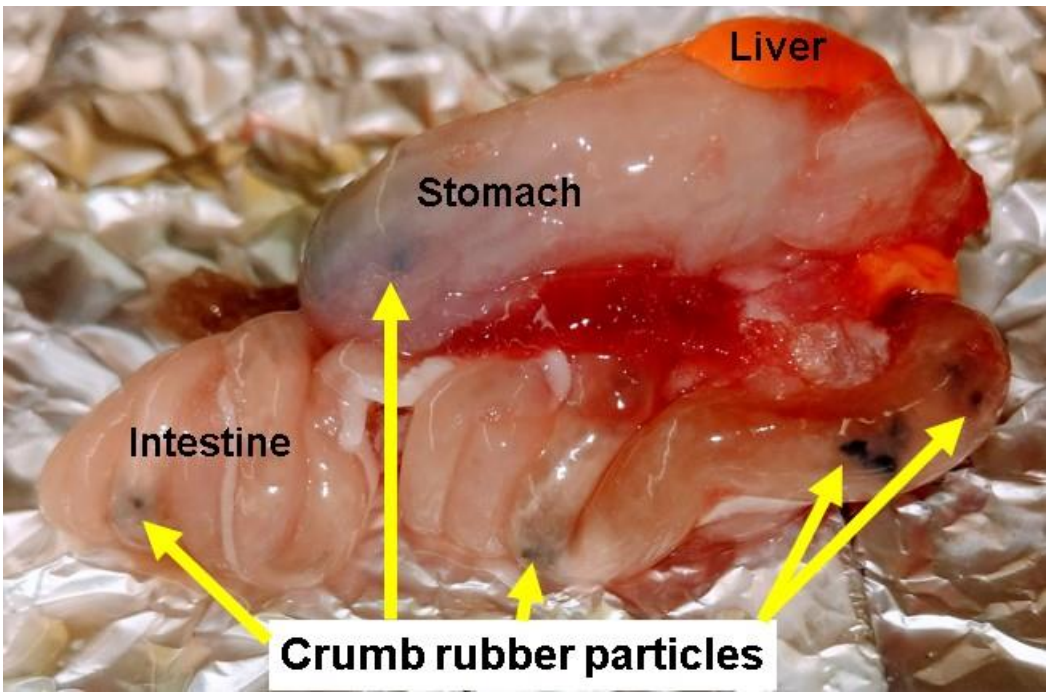


Figure 3.1: Dissected GI tract of an exposed lumpfish containing CR particles. Yellow arrows mark crumb rubber particles in the GI-tract. Intestine in the bottom, stomach above and parts of the liver on top (the liver is behind the stomach.

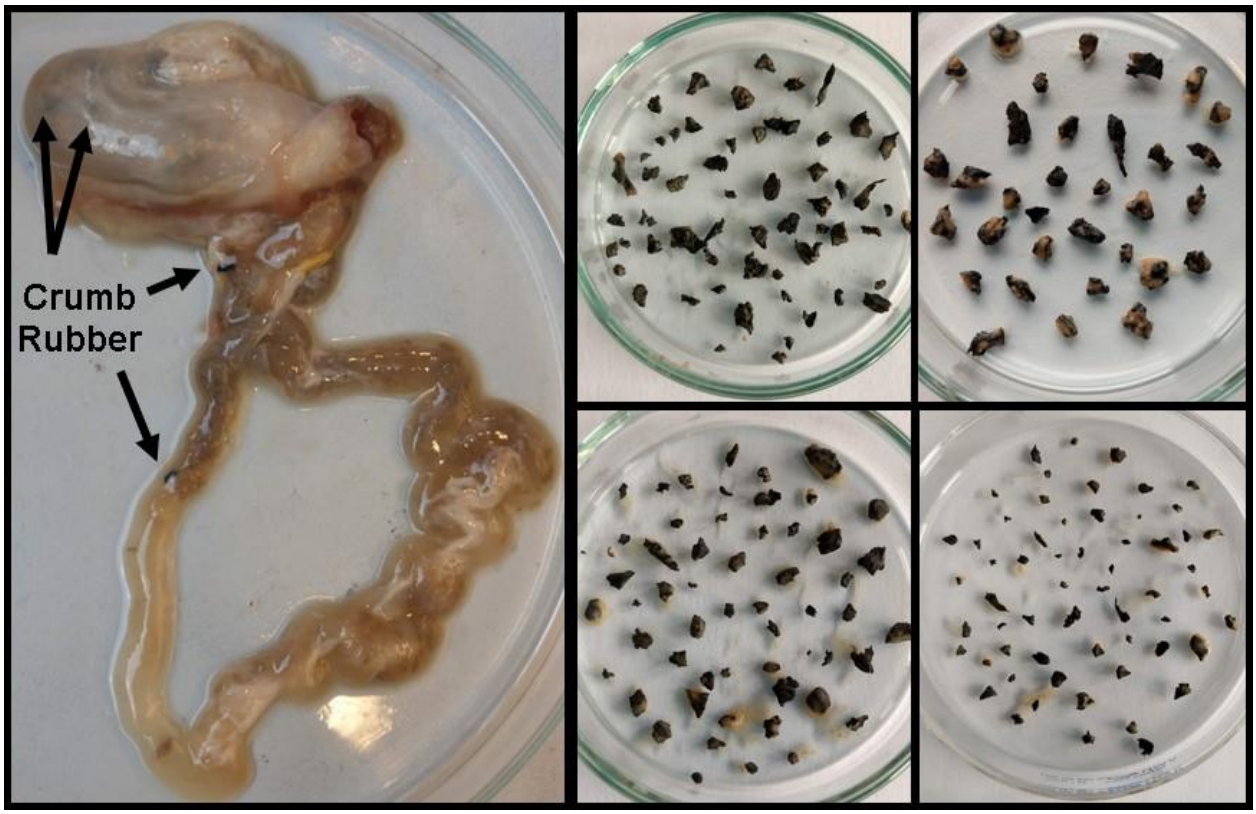


Figure 3.2: Lumpfish GI-tract with crumb rubber particles (Left). Retrieved crumb rubber particles from 4 exposed lumpfish (Lower right corner tank 6 day 7, lower left corner tank 6 day 7, upper left corner Tank 4 day 21, upper right corner tank 6 day 7) collected and counted in petri dishes.

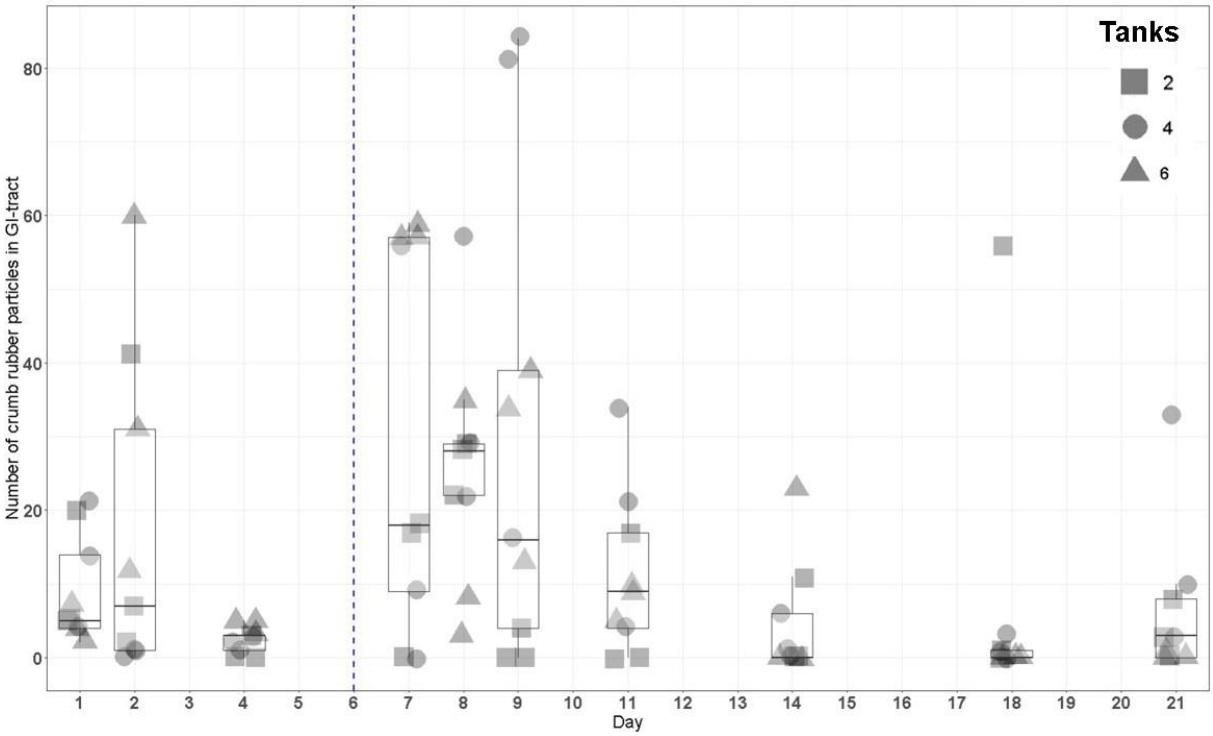


Figure 3.3: Number of crumb rubber found in exposed lumpfish (n=90) related to time of sampling (day) during exposure (day 1-6) and depuration (day 7-21). Tanks 2, 4 and 6 were exposed treatments. Control (n = 90) lumpfish not shown. Dashed line marks the end of the exposure.

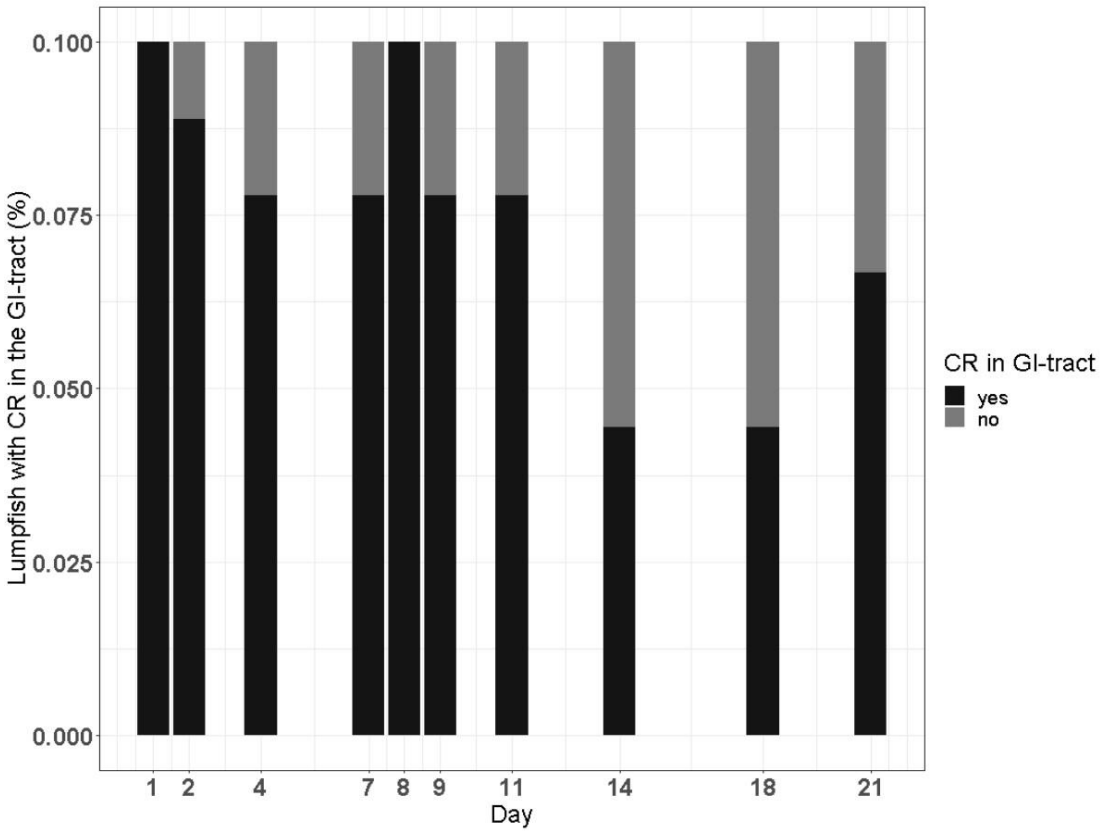


Figure3.4: Barplot over the proportions (%) of fish that contained (black) and fish that did not contain (grey) CR particles in the GI-tract over days of sampling.

3.2 Chemical characterization of crumb rubber

Characterisation of organic chemicals in fine CR showed a mixture of various chemicals (Figure 3.5) that formed a list of compounds that was further narrowed to a suspect list of ~ 50 characteristic tyre-related chemicals (Table 3.20). Only peaks detected in all five solvents, but not in blanks were chosen. Six chemicals were found in significantly higher concentrations in exposed lumpfish than in controls (Figure 3.6). These were amines, five of which diamines: $C_{20}H_{20}N_2$, $C_{18}H_{24}N_2$, $C_{21}H_{26}N_2$, $C_{23}H_{26}N_2$, $C_{19}H_{18}N_2$, $C_{12}H_{15}N$, and were focused on further in Section 3.3.

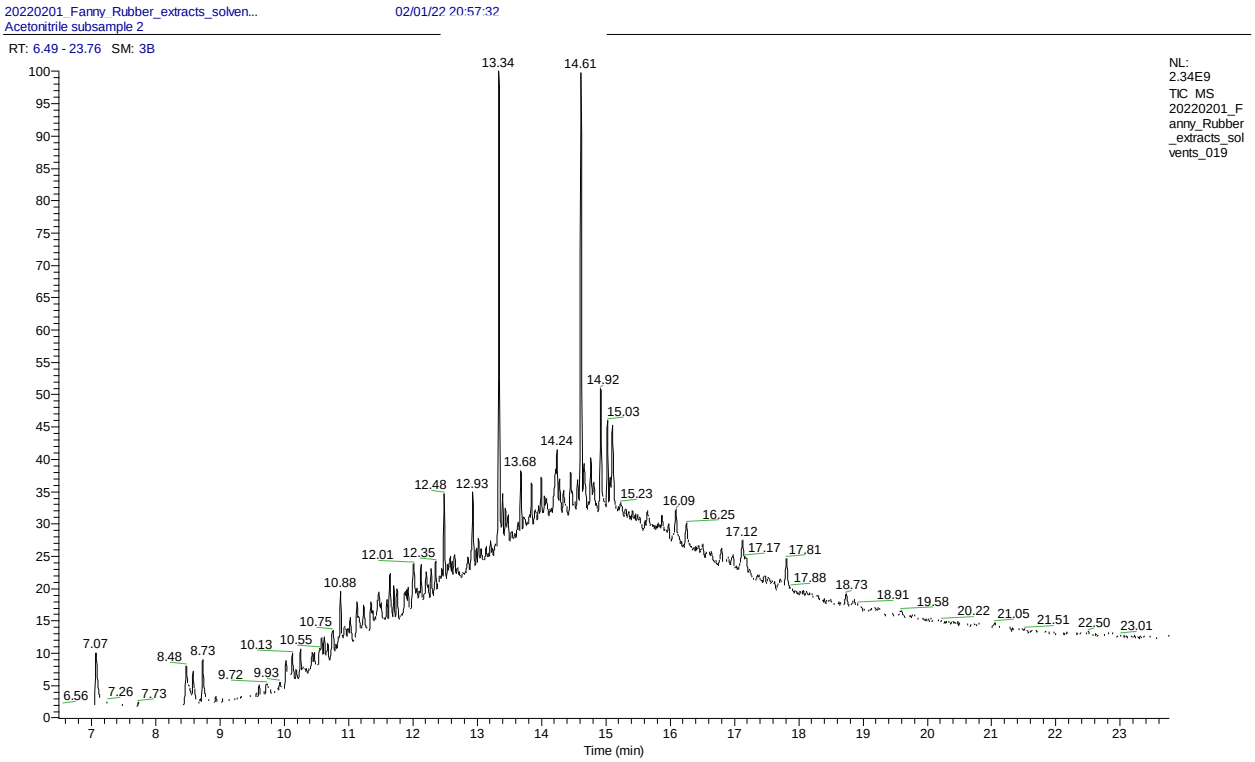


Figure 3.5: Full chromatogram of fine crumb rubber extract in Acetonitrile. The first major peak is 6PPD.

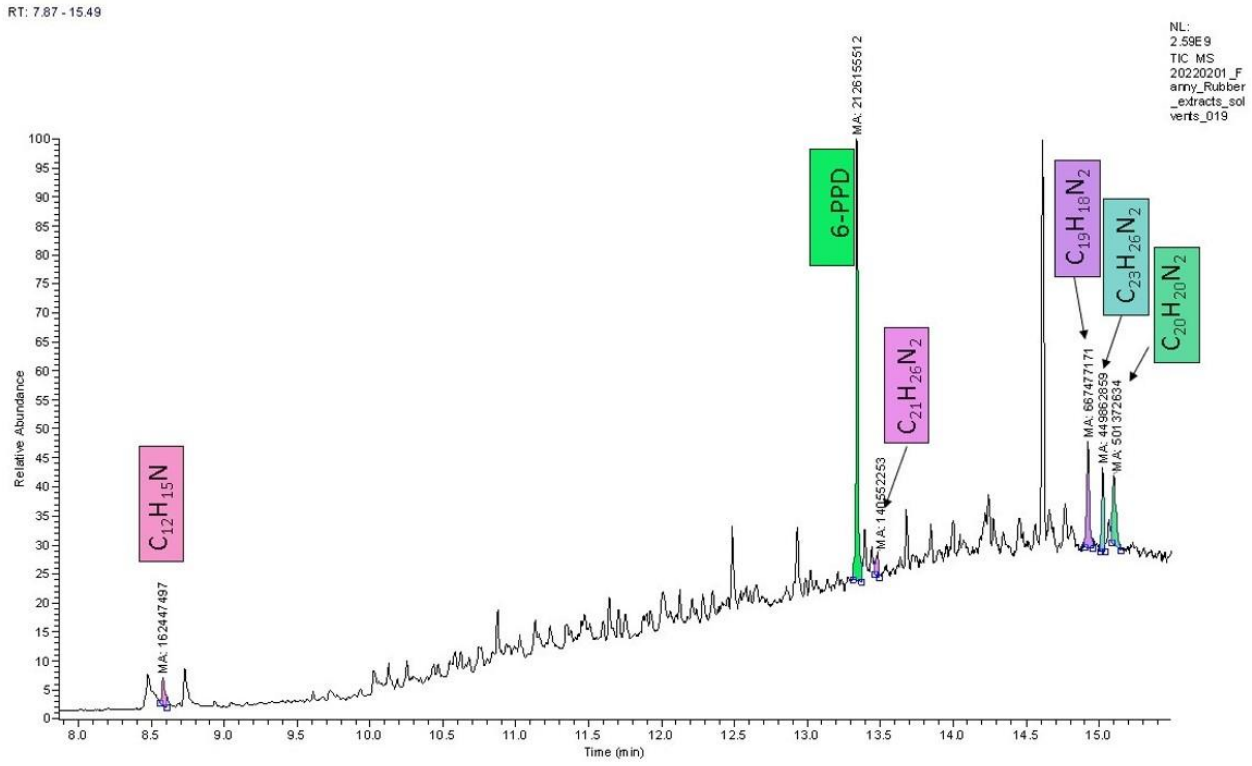


Figure 3.6: Chromatogram of fine crumb rubber extract in Acetonitrile showing the six compounds detected in significantly amount in blood from exposed lumpfish. 6-PPD($C_{18}H_{24}N_2$); $C_{21}H_{26}N_2$; $C_{19}H_{18}N_2$; $C_{23}H_{26}N_2$; $C_{20}H_{20}N_2$ and $C_{12}H_{15}N$.

3.3 Uptake of Organic chemicals by Lumpfish

To evaluate the capability of organic chemicals present in the CR to leach from the CR and be taken up into tissues of lumpfish, we analyzed both blood and liver samples of exposed and unexposed lumpfish. A number of organic compounds were detected in higher concentrations the blood and liver of exposed fish be $C_{20}H_{20}N_2$, $C_{18}H_{24}N_2$, $C_{21}H_{26}N_2$, $C_{23}H_{26}N_2$, $C_{19}H_{18}N_2$, $C_{12}H_{15}N$, Methyldicyclohexylamine, Methyldicyclohexylamine, Diphenylguanidine, Tributylamine, $C_{16}H_{16}N_3O$ and $C_{13}H_{20}N_3$.

3.3.1 Organic chemicals in blood

Six amines, 5 of which were diamines, were detected in significantly higher relative concentrations in blood samples of exposed lumpfish (n=18; p-values < 0.05) compared to control lumpfish (n=18; Figure 3.6 and Table 3.3). No other compounds characteristic for tyre rubber were detected with similar or higher relative concentrations. The molecular formulas of the detected amines were determined to be $C_{20}H_{20}N_2$, $C_{18}H_{24}N_2$, $C_{21}H_{26}N_2$, $C_{23}H_{26}N_2$, $C_{19}H_{18}N_2$ and $C_{12}H_{15}N$. Due to the use of a chemical standard, the chemical identity of $C_{18}H_{24}N_2$ could be proven to be *N*-(1,3-dimethylbutyl)-*N'*-phenyl-p-phenylenediamine or 6PPD (CAS: 793-24-8; Figures 3.7 and 3.8).

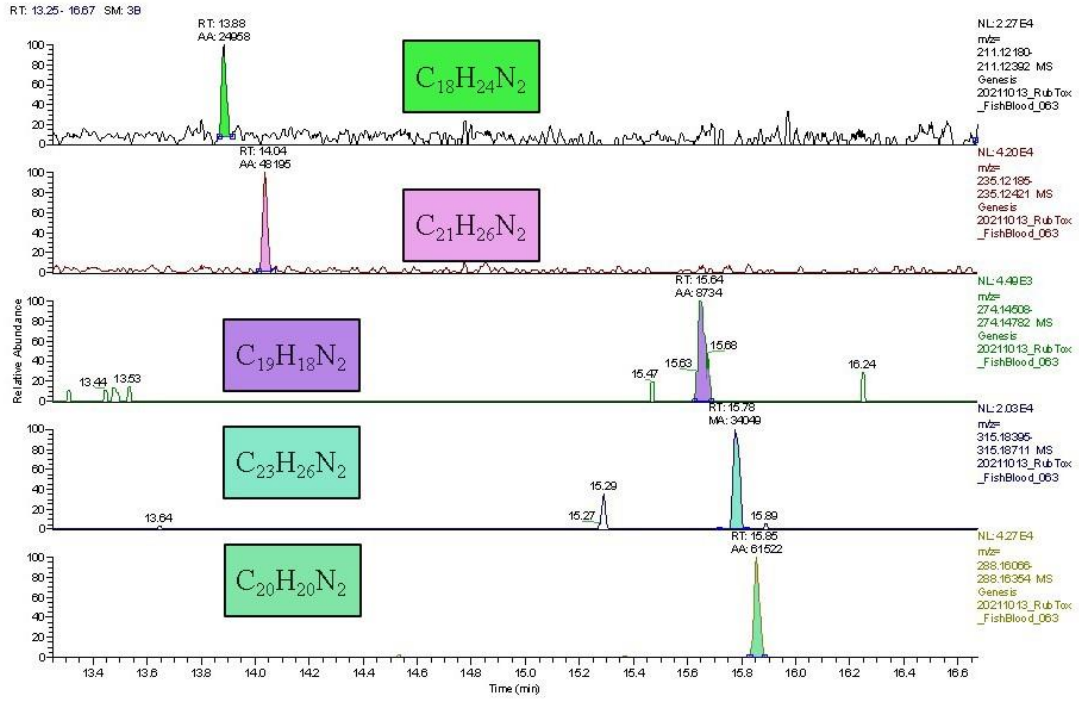


Figure 3.7: Chromatogram of detected chemicals in blood of exposed lumpfish (n=90). Top to bottom: 6-PPD($C_{18}H_{24}N_2$); $C_{21}H_{26}N_2$; $C_{19}H_{18}N_2$; $C_{23}H_{26}N_2$; $C_{20}H_{20}N_2$.

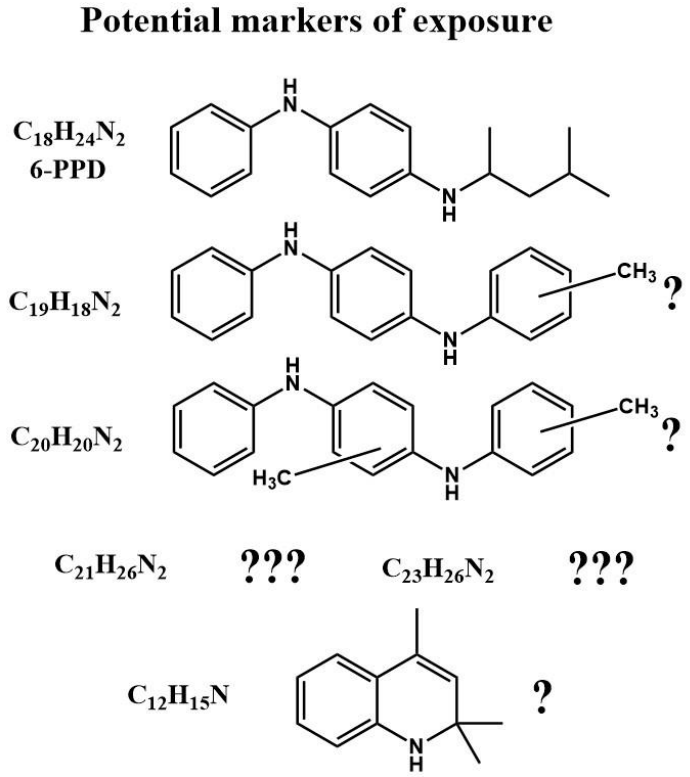


Figure 3.8: Molecular formula and structure for the detected amines. Known molecular formula and structure presented for $C_{18}H_{24}N_2$ (6PPD) and suggested molecular formula and structure presented for, $C_{20}H_{20}N_2$, $C_{19}H_{18}N_2$ and $C_{12}H_{15}N$, and suggested molecular formula for $C_{21}H_{26}N_2$ and $C_{23}H_{26}N_2$.

The chemical standard also enabled a quantification of the concentrations of 6PPD. Based on the concentration of 6PPD the relative concentrations for $C_{21}H_{26}N_2$, $C_{20}H_{20}N_2$, $C_{23}H_{26}N_2$, and $C_{19}H_{18}N_2$ were calculated in the assumption that the peak area (response) is the same or very similar for the diamines detected. It was not possible to quantify $C_{12}H_{15}N$ because the molecular mass of the compound was too small for detection. Therefore, it was not possible to quantify $C_{12}H_{15}N$ and the measurements are given as relative peak are.

All six of the detected amines were found in significantly elevated relative concentrations in exposed lumpfish compared to unexposed (Mann-Whitney U test, $p < 0.05$, Figure 3.9 and Table S3.3). The average concentrations of the 5 quantified amines were found in decreasing order as follows: $C_{20}H_{20}N_2$ > 6PPD > $C_{21}H_{26}N_2$ > $C_{23}H_{26}N_2$ > $C_{19}H_{18}N_2$. The relative concentration were higher in exposed lumpfish on day 7 ($C_{20}H_{20}N_2$, 384 pg/g; 6PPD, 330 pg/g; $C_{21}H_{26}N_2$, 139 pg/g; $C_{23}H_{26}N_2$, 111 pg/g; $C_{19}H_{18}N_2$, 43.3 pg/g; and $C_{12}H_{15}N$

399789(peak area)), compared to controls at day 7 ($C_{20}H_{20}N_2$, n.d.; 6PPD, 20 pg/g; $C_{21}H_{26}N_2$, n.d.; $C_{23}H_{26}N_2$, n.d; $C_{19}H_{18}N_2$, n.d; $C_{12}H_{15}N$, (92978); Tables S3.4.1 - S3.4.6). $C_{20}H_{20}N_2$ and $C_{12}H_{15}N$ were still found in low relative concentrations in exposed lumpfish on day 21 (average n.d and 92978 (peak area), respectively). 6PPD was still found in low average concentrations in exposed fish on day 21 (40 pg/g) and two fish still contained high concentrations on day 21 (280 pg/g and 680 pg/g). There were no relative concentrations of $C_{20}H_{20}N_2$, $C_{21}H_{26}N_2$, $C_{23}H_{26}N_2$ and $C_{19}H_{18}N_2$ in the control groups, whereas 6PPD was detected in negligible concentration < LOQ (Figure 3.9 and Tables S3.3.1 - 3.3.5).

The metabolization product of 6PPD, 6PPD-quinone, was not detected in the blood samples of either treatment group (control and exposed). 6PPD concentrations increased rapidly after the beginning of the exposure and were detected in higher concentration on day 7 (average 330 pg/g) than day 21 (average 40 pg/g). The individual concentrations varied, ranging from n.d. to 120 pg/g on day 0, 20 to 700 pg/g on day 7 and 20 to 680 on day 21. 6PPD increased throughout the exposure and had an average peak on day 7, following a decrease in the beginning of the depuration and then flattened out towards the end of the depuration phase (Figure 3.10 and Table S3.4.2).

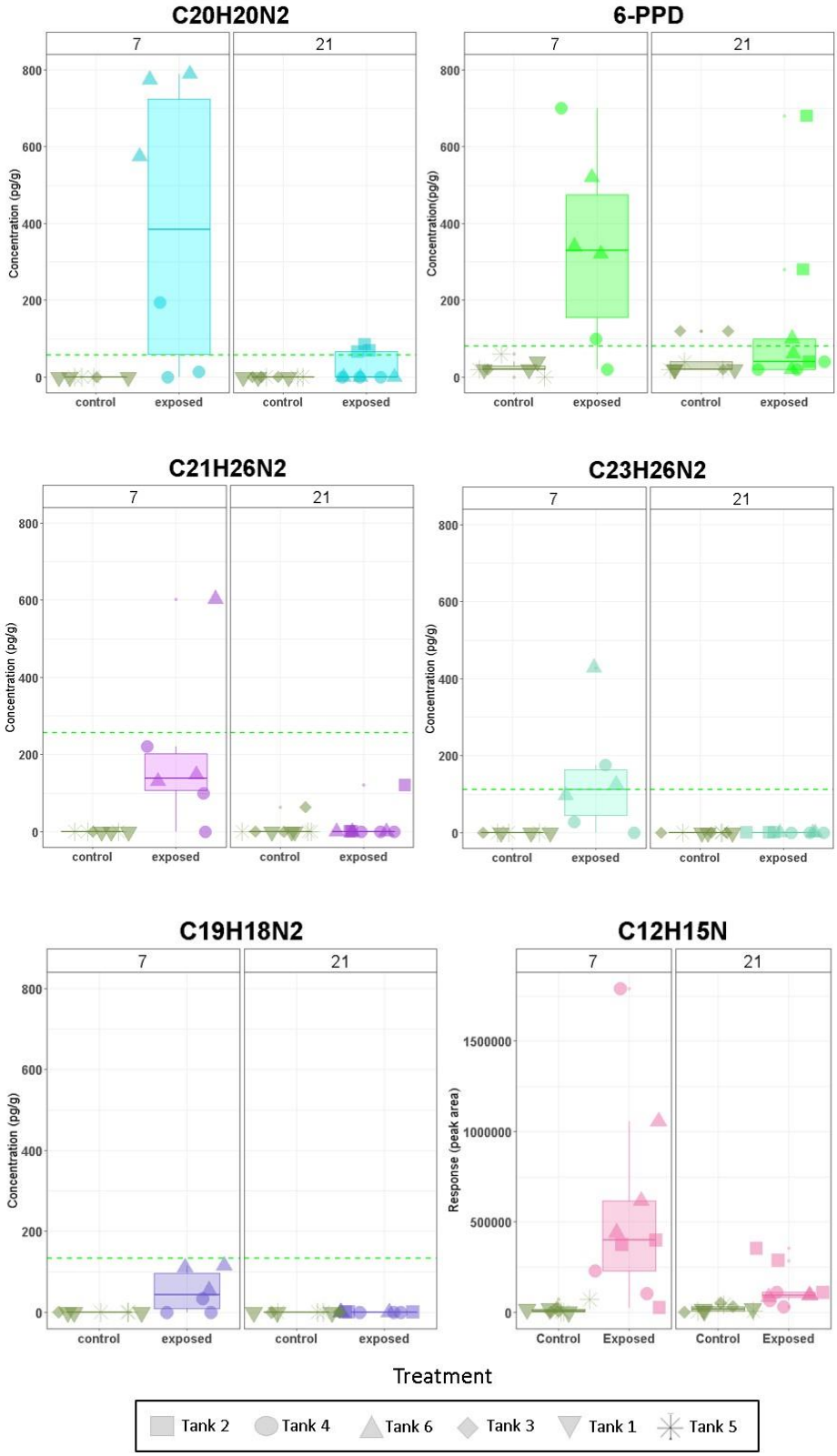


Figure 3.9: Concentration (pg/g) of detected diamines ($C_{21}H_{26}N_2$; $C_{20}H_{20}N_2$; $C_{23}H_{26}N_2$; $C_{18}H_{24}N_2$ (6PPD); and $C_{19}H_{18}N_2$) and the relative response (Peak area) of detected amine ($C_{12}H_{15}N$) in analyzed blood from lumpfish (n=36)) over timepoints (day 7 and 21) and treatments (controls and exposed). As $C_{19}H_{18}N_2$ was not possible to quantify the compound is reported in relative response instead of the concentration., $C_{19}H_{18}N_2$. Level of quantification (LOQ; $C_{20}H_{20}N_2$, 58; 6PPD, 80; $C_{21}H_{26}N_2$, 397; $C_{23}H_{26}N_2$, 112; and $C_{19}H_{18}N_2$, 134) indicated by the green dashed line.

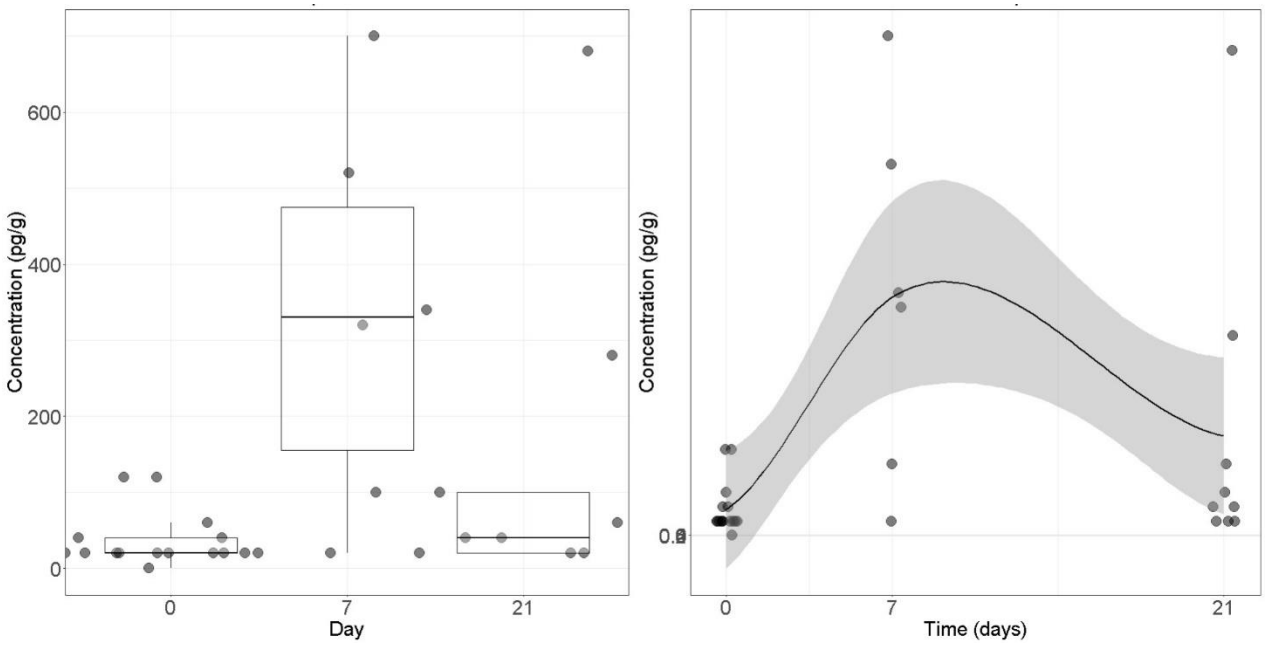


Figure 3.10: Concentration of 6PPD in Lumpfish (n=36) blood over time (days). Concentrations ranged between 0-700 pg/g. The trend shows increasing concentrations throughout the exposure and peaks at day 7, followed by a decrease through the depuration.

Methyldicyclohexylamine (M-DCH; CAS: 7560-83-0), Methyldicyclohexylamine (DCH; CAS: 7560-83-0), Diphenylguanidine (DPG; CAS: 102-06-7), Tributylamine (TBT; CAS: 102-82-9), and $C_{16}H_{16}N_3O$ and $C_{13}H_{20}N_3$, were detected by LC/MS-orbitrap in significantly higher relative concentrations in exposed lumpfish (n=18; Mann-Whitney U test, p-values < 0.05) than control lumpfish (n=18: Figure 3.11 and Table S3.5). Because of differences in standard use in the extraction of the samples between laboratories, the concentrations for these compounds are not comparable to the amines described above. The average relative concentration of the chemicals found in decreasing order was as follows: DCH > DPG > M-DCH and TBT > $C_{16}H_{16}N_3O$ > $C_{13}H_{20}N_3$. The relative concentrations were higher in exposed lumpfish on day 7 (M-DCH, 308 (pg/g); DCH, 29068 (pg/g); DPG, 12436 (pg/g); $C_{16}H_{16}N_3O$, 3950 (peak area); TBT, 2166 (peak area); $C_{13}H_{20}N_3$; 286 (peak area)), than controls on day 7 (M-DCH, 36 (pg/g); DCH, n.d.; DPG, n.d.; $C_{16}H_{16}N_3O$, 1246 (peak area); TBT, 992 (peak area); $C_{13}H_{20}N_3$; n.d.). All six chemicals were still found in low relative concentrations and responses in exposed lumpfish on day 21; and $C_{13}H_{20}N_3$ had higher average relative response on day 21 than day 7 (790 and 286, respectively). M-DCH was detected in negligible relative concentrations > LOQ and $C_{16}H_{16}N_3O$ and TBT in low response in controls while DCH, DPG and $C_{13}H_{20}N_3$ were not detected in controls (Figure 3.11 and Tables S3.6.1 – S3.6.6).

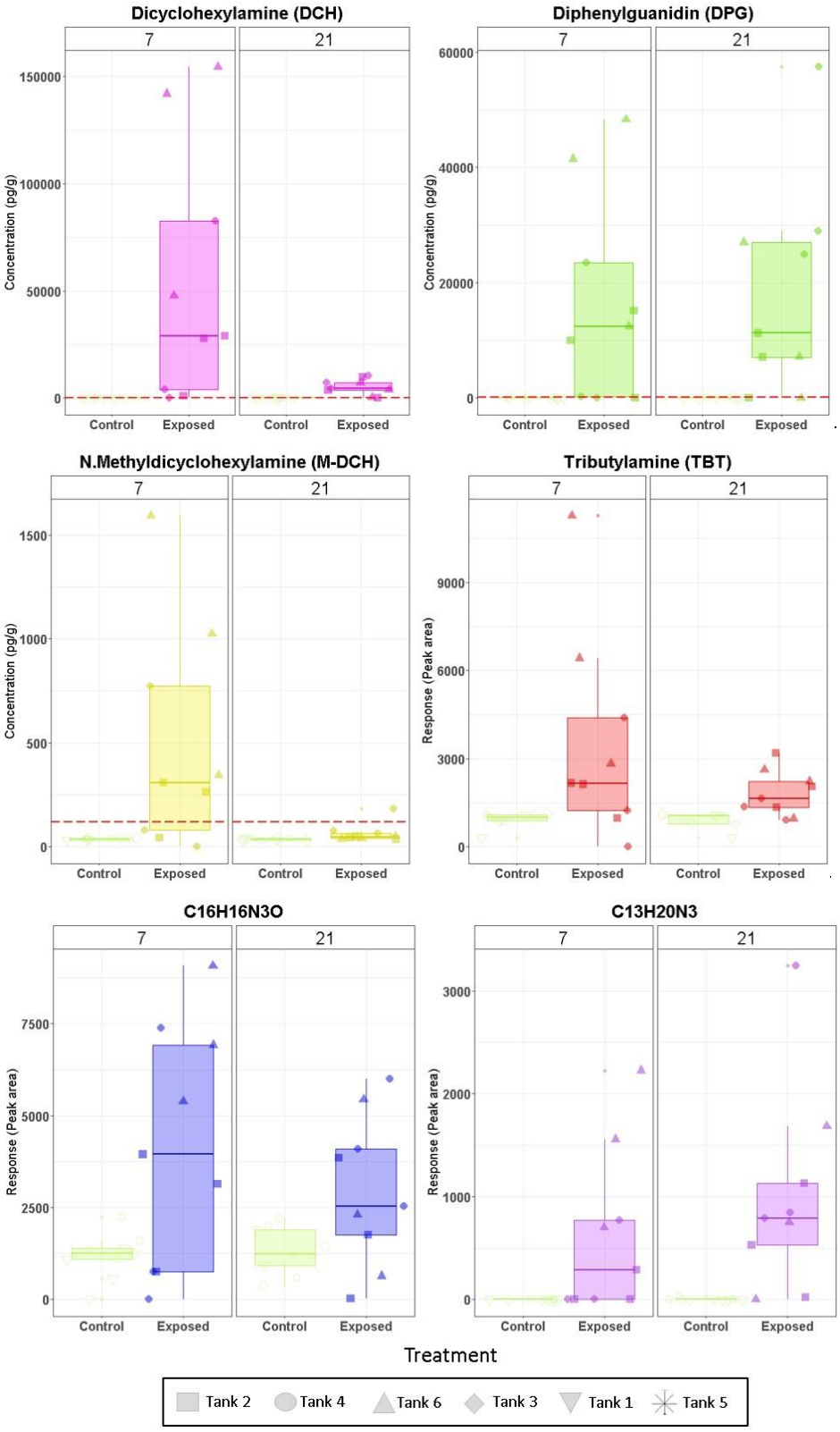


Figure 3.11: Concentration (pg/g) of detected chemicals Methyldicyclohexylamine (M-DCH), Methyldicyclohexylamine (DCH), Diphenylguanidine (DPG), Tributylamine (TBT), and $C_{16}H_{16}N_3O$ and $C_{13}H_{20}N_3$ in analyzed blood from lumpfish (n=36)) over timepoints (day 7 and 21) and treatments (controls and exposed).

3.3.2 Organic chemicals in liver

The same six compounds, N-Methyldicyclohexylamine (M-DCH; CAS: 7560-83-0), Methyldicyclohexylamine (DCH; CAS: 7560-83-0), Diphenylguanidine (DPG; CAS: 102-06-7), Tributylamine (TBT; CAS: 102-82-9), $C_{16}H_{16}N_3O$ and $C_{13}H_{20}N_3$ were also detected in liver tissues. All six had significantly higher relative concentrations and responses in samples of exposed lumpfish (n=18) than in the unexposed group (n=18: Mann-Whitney U test, p-values < 0.001; Figure 3.12 and Table S3.7) in decreasing order as follows: DPG > DCH > M-DCH. The average relative response of the chemicals found in decreasing order as follows: $C_{16}H_{16}N_3O$ > TBT > $C_{13}H_{20}N_3$. The relative concentration and response were higher in exposed lumpfish on day 7 (M-DCH, 140 (pg/g); DCH, 1685 (pg/g); DPG, 2145 (pg/g); $C_{16}H_{16}N_3O$, 1249 (peak area); TBT, 289 (peak area); $C_{13}H_{20}N_3$; 150 (peak area)) than in controls on day 7 (M-DCH, 6 (pg/g); DCH, 15 (pg/g); DPG, n.d.; $C_{16}H_{16}N_3O$, n.d.; TBT, 33 (peak area); $C_{13}H_{20}N_3$; n.d.). M-DCH, DCH, DPG, TBT and $C_{16}H_{16}N_3O$ had higher average relative concentration on day 7 than in day 21, whereas $C_{13}H_{20}N_3$ had higher average relative concentration on day 21 than in day 7 M-DCH and DCH were detected in low relative concentrations < LOQ and TBT in low response in control samples (Figure 3.11 and Tables S3.8.1 – S3.8.6).

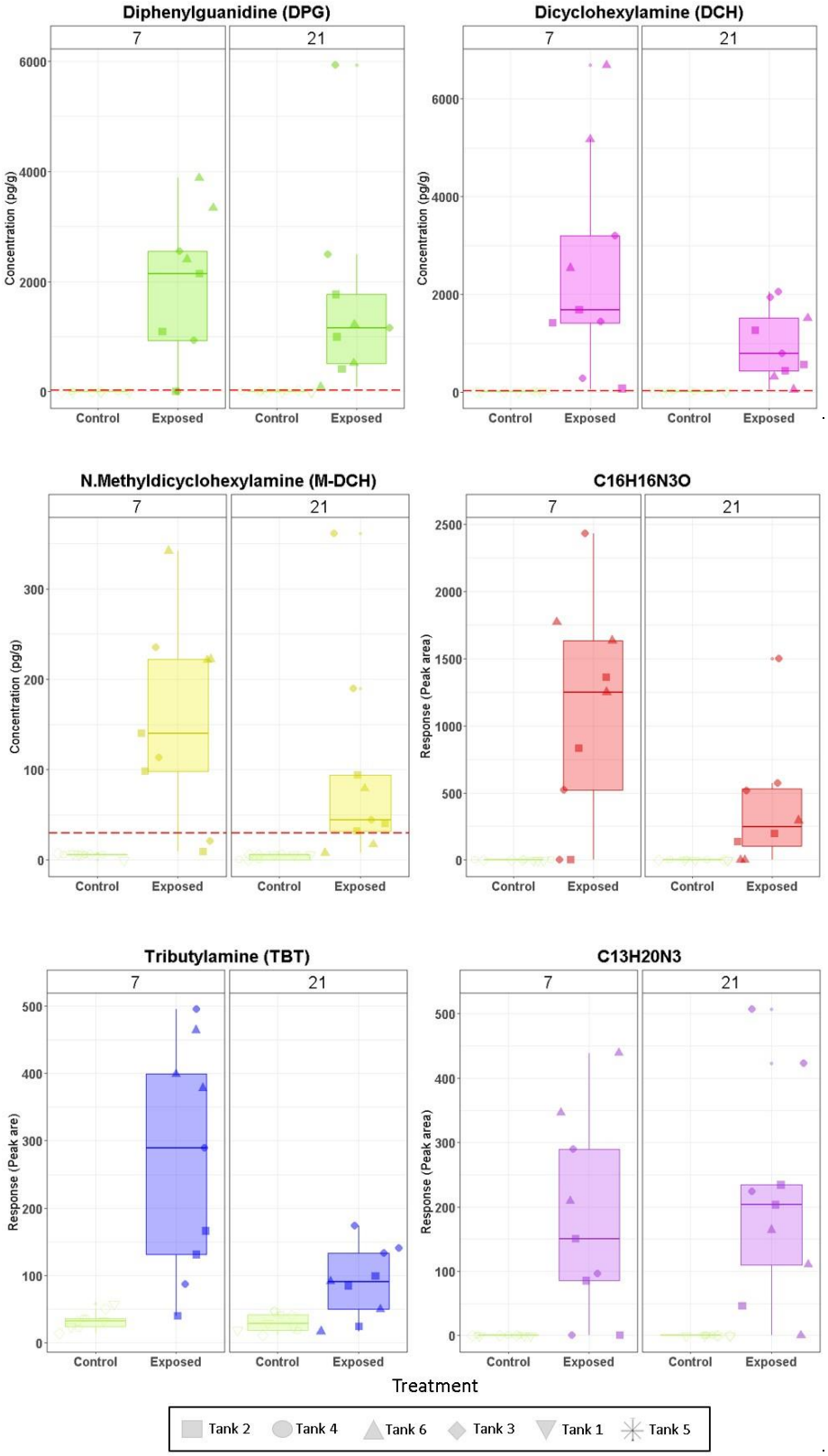


Figure 3.12: Organic chemicals detected in lumpfish blood by LC/MS-Orbitrap. Six chemicals were detected in significantly higher concentrations in analyzed exposed samples (n=18) compared to analyzed controls (n=18). Detected compounds were M-DCH, DCH, DPG, TBT, $C_{16}H_{16}N_3O$ and $C_{13}H_{20}N_3$. Red dashed line marks the LOQ for the quantified compounds.

3.4 Correlation between number of crumb rubber particles and amount of chemicals

There were correlations of varied strength between the number of CR particles found in lumpfish GI-tract and the relative concentrations and responses for most of the detected chemicals ($C_{21}H_{26}N_2$, $C_{20}H_{20}N_2$, $C_{23}H_{26}N_2$, 6PPD, $C_{19}H_{18}N_2$, $C_{12}H_{15}N$, M-DCH, DCH, DPG, TBT $C_{16}H_{16}N_3O$ and $C_{13}H_{20}N_3$).

In blood samples, $C_{20}H_{20}N_2$, $C_{19}H_{18}N_2$, M-DCH, DCH and DPG showed a strong correlation ($R^2 \geq 0.7$ and p-value < 0.05), whereas $C_{21}H_{26}N_2$, $C_{23}H_{26}N_2$, 6PPD, $C_{16}H_{16}N_3O$ TBT and $C_{12}H_{15}N$ showed a moderate correlation ($0.4 \leq R^2 \leq 0.7$ and p-value < 0.05). $C_{13}H_{20}N_3$ did not show a correlation between relative response and number of CR (Figures 3.13 and 3.14 and Tables S3.9 and S3.10).

In liver samples, DCH, $C_{16}H_{16}N_3O$ and TBT showed a strong correlation ($R^2 \geq 0.7$ and p-value < 0.05) between relative concentration or response and number of CR particles present, whereas M-DCH, DPG and $C_{13}H_{20}N_3$ showed a moderate correlation ($0.4 \leq R^2 \leq 0.7$ and p-value < 0.05) between relative concentration or response and number of CR particles (Figure 3.15 and Table S3.11).

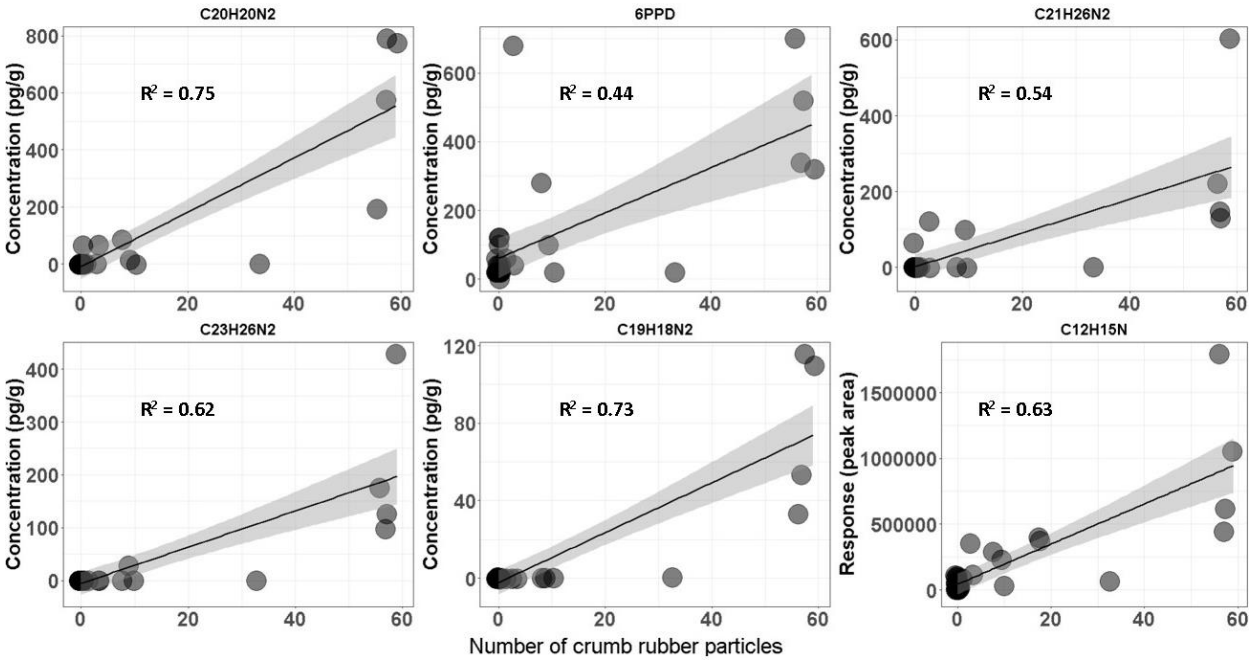


Figure 3.13: Correlation between number of crumb rubber and relative concentration and level of detected chemicals by GC/MS in in blood samples of exposed lumpfish (n=18).

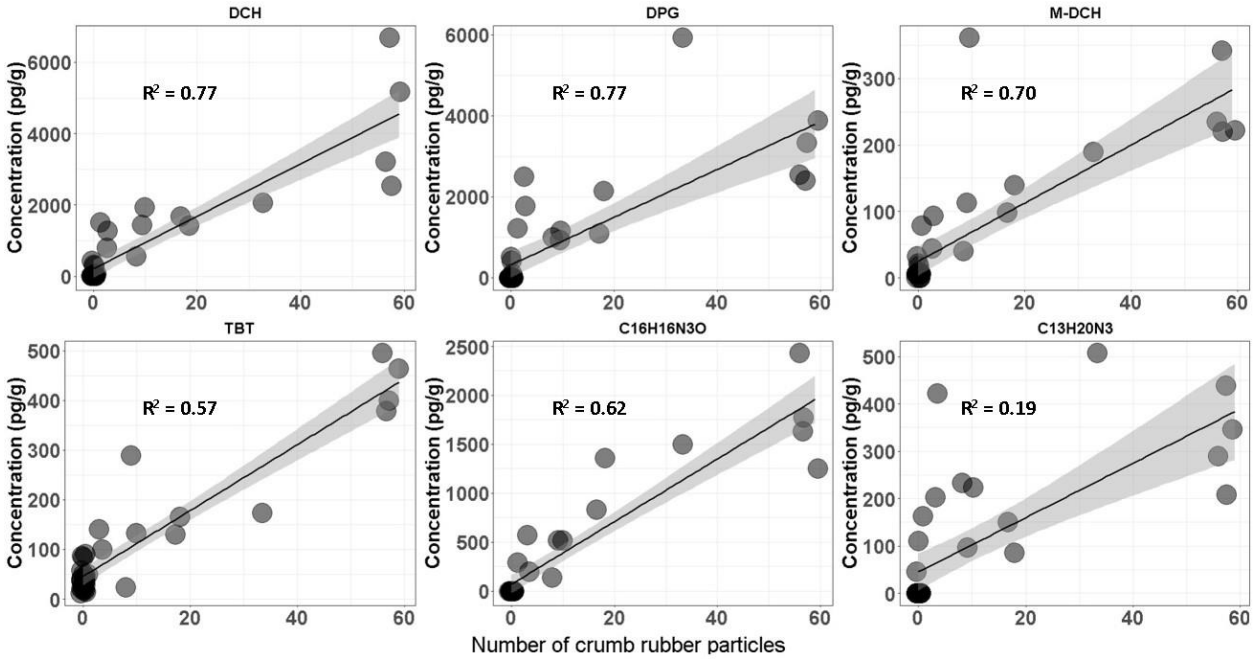


Figure 3.14: Correlation between number of crumb rubber and relative concentration and level of detected chemicals by LC/MS in in blood samples of exposed lumpfish (n=18).

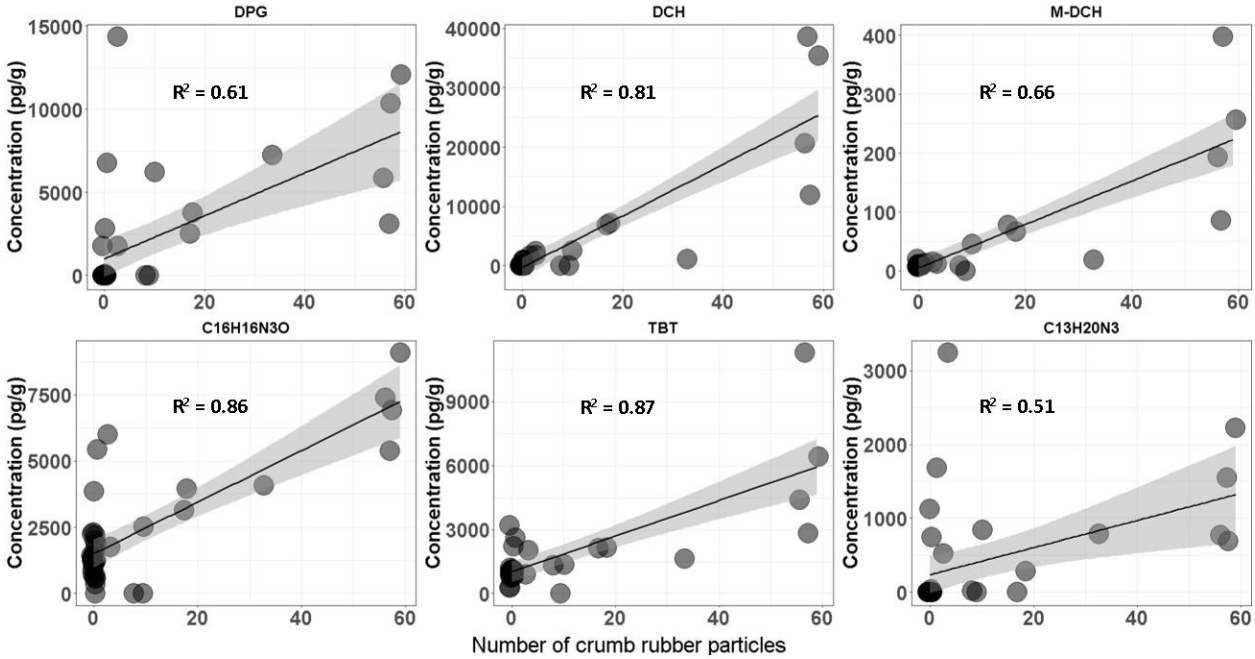
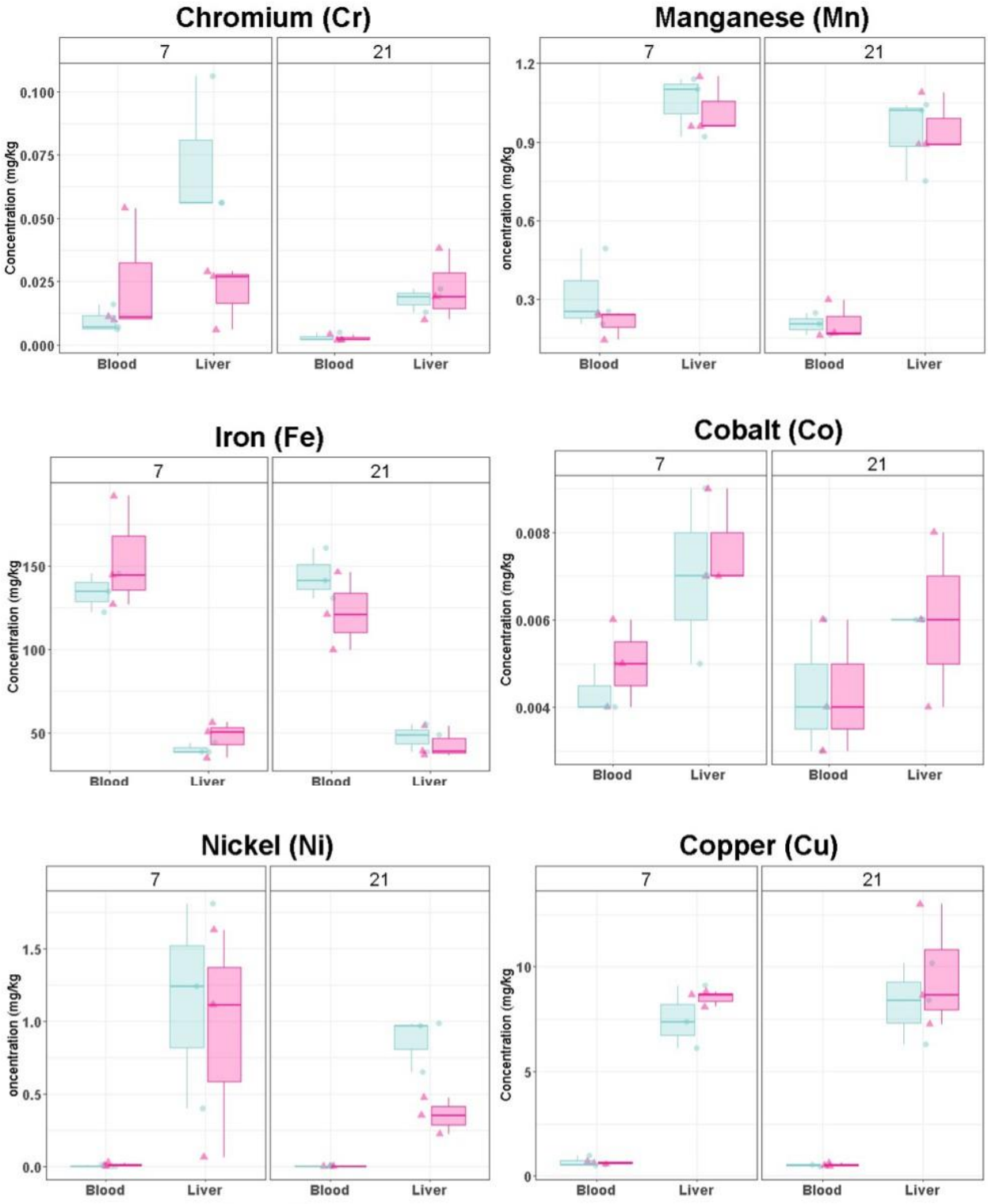


Figure 3.15: Correlation between number of crumb rubber and relative concentration and level of detected chemicals by LC/MS in in liver samples of exposed lumpfish (n=18).

3.5 Uptake of Metals by lumpfish

In addition to organic compounds, metals as Chromium (Cr); Manganese (Mn); Iron (Fe); Cobalt (Co); Nickel (Ni); Copper (Cu); Zink (Zn); Cadmium (Cd); Antimony (Sb) and Lead (Pb) were analyzed in liver samples (n=12) and blood samples (n=12) in control (n=6) and exposed (n=6) groups at day 7 and day 21. There were no significant (Mann-Whitney U test, $p > 0.05$) differences in concentrations of respective metals between treatments and timepoints (Figure 3.16 and Tables S3.12 and S3.15). As expected, metal concentrations differed between liver and blood.

For some metals control treatments tended to have higher average concentrations compared to the average concentrations of exposed groups. In liver samples of control lumpfish, manganese and nickel were found in higher concentration in both day 7 and day 21, chromium was found in higher concentration in day 7, zinc and cadmium were found in higher concentrations in day 21. In blood samples of control lumpfish, manganese was found in higher concentration in day 7, and iron and lead were found in higher concentration in day 21 (Figure 3.16 and Tables S3.14 – S3.15).


Chromium (Cr)
Manganese (Mn)
Iron (Fe)
Cobalt (Co)
Nickel (Ni)
Copper (Cu)
7
21
Blood
Liver
Concentration (mg/kg
oncentration (mg/kg

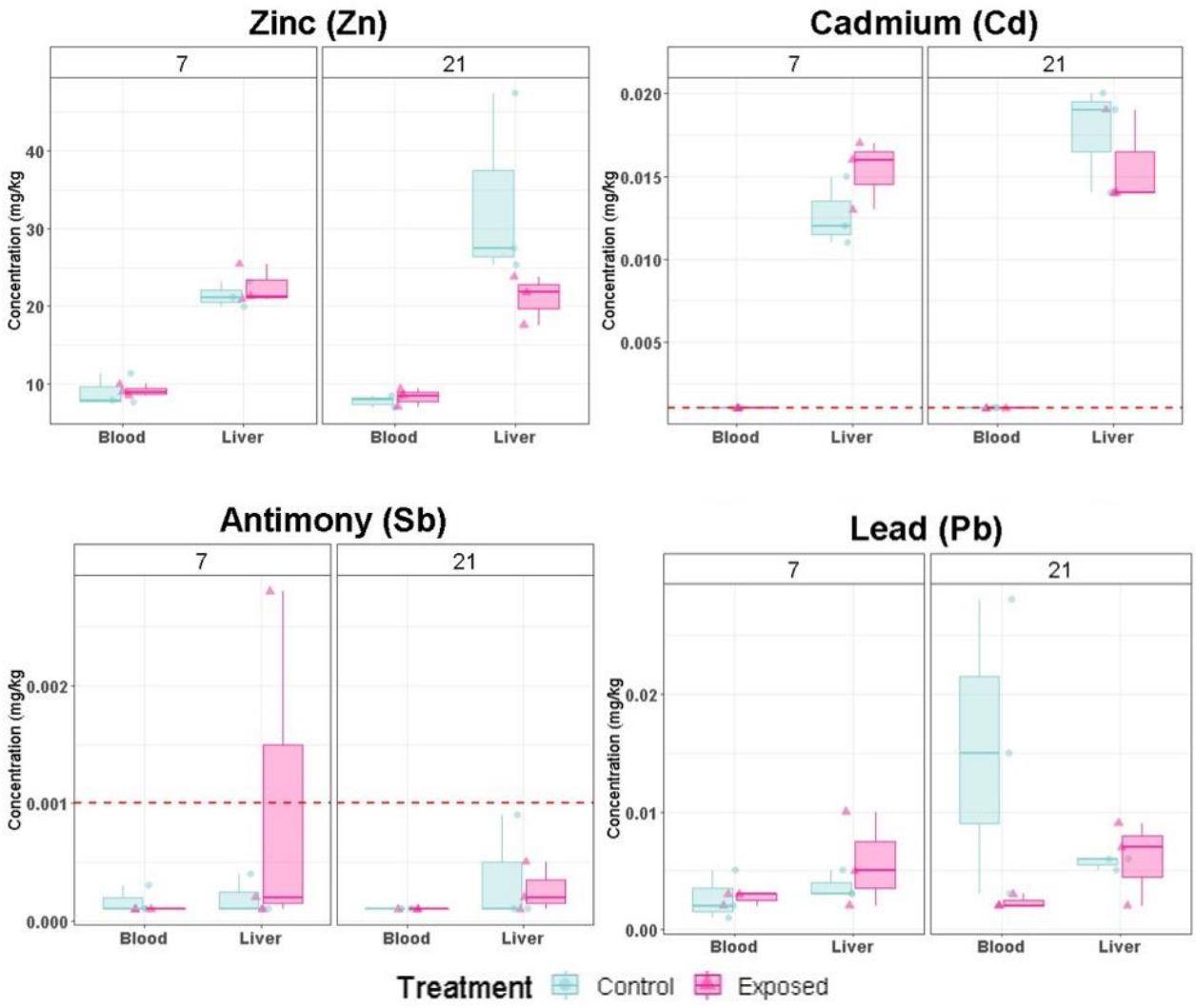


Figure 3.16: Concentration (mg/kg) of metals (Chromium (Cr); Manganese (Mn); Iron (Fe); Cobalt (Co); Nickel (Ni); Copper (Cu); Zink (Zn); and Lead (Pb)) in blood samples (n=12) and liver samples (n=12) of exposed Lumpfish (pink triangles) and control Lumpfish (blue circles) at different timepoints: day 7 in left side panel and day 21 in right side panel. Red dashed line indicates the LOD.

3.6 Ingestion of crumb rubber by northern shrimp

Pyrolysis-GC/MS was used to analyze CR ingestion by northern shrimps, due to the small particle size of the CR used in the exposure experiment and the difficulty to visually identify and count present CR. The pyrGC/MS is able to detect the presence of rubber particles on the basis of the chemical CR signature after pyrolyzing the digested and filtered shrimp samples (Figure 3.17). The results of the pyrolysis were inconclusive as the marker SBR-vinylcyclohexene was detected in all three treatments (control, low CR exposure and high CR exposure). The highest average concentration of SBR was detected in the low concentration

treatments on day 7 (283 µg/g; Figure 3.18 and Table S3.16), but there were no significant differences (Kruskal-Wallis test, $p > 0.05$) between treatments or time point (day 7 and 21; Figure 3.18 and Table 3.16 As a result, no conclusion can currently be drawn if the SBR signal shows ingestion of fine CR by northern shrimps in any of the treatments.

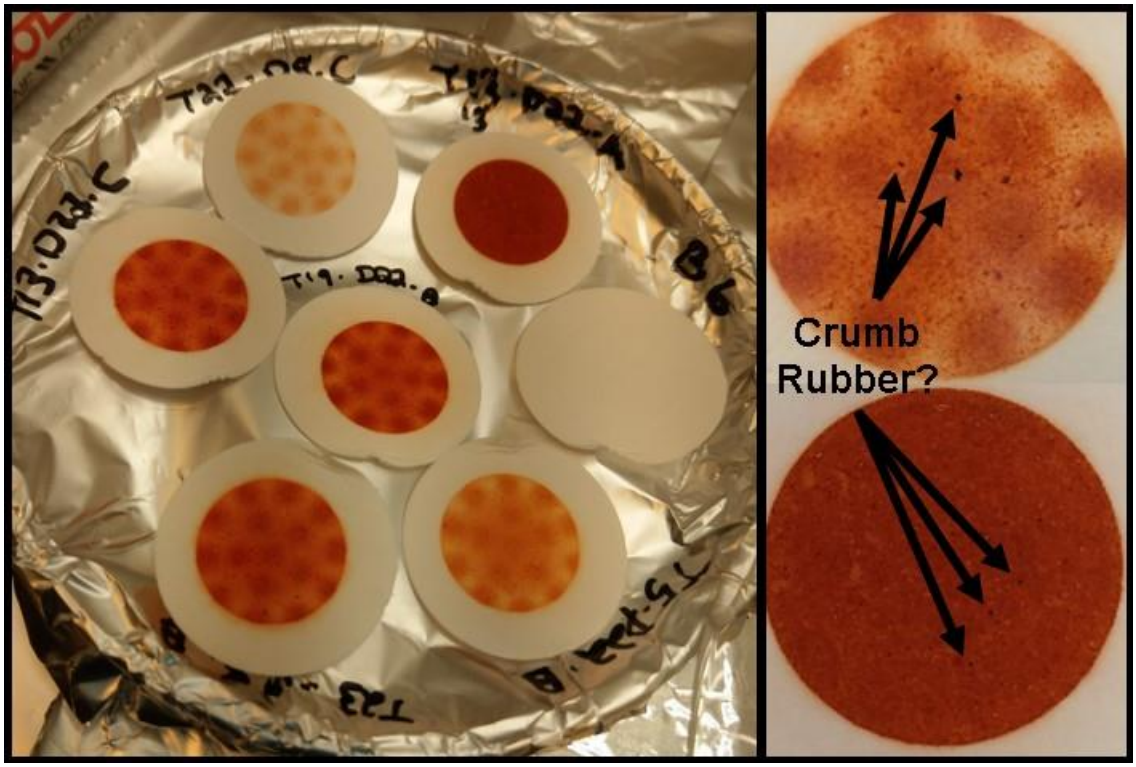


Figure 3.17: Glass fiber filters 2. Mm with digested and filtered northern shrimp GI-tract for analysis by PyrGC/MS (Left). Two glass fiber filters with potential CR particles on them (black arrows).

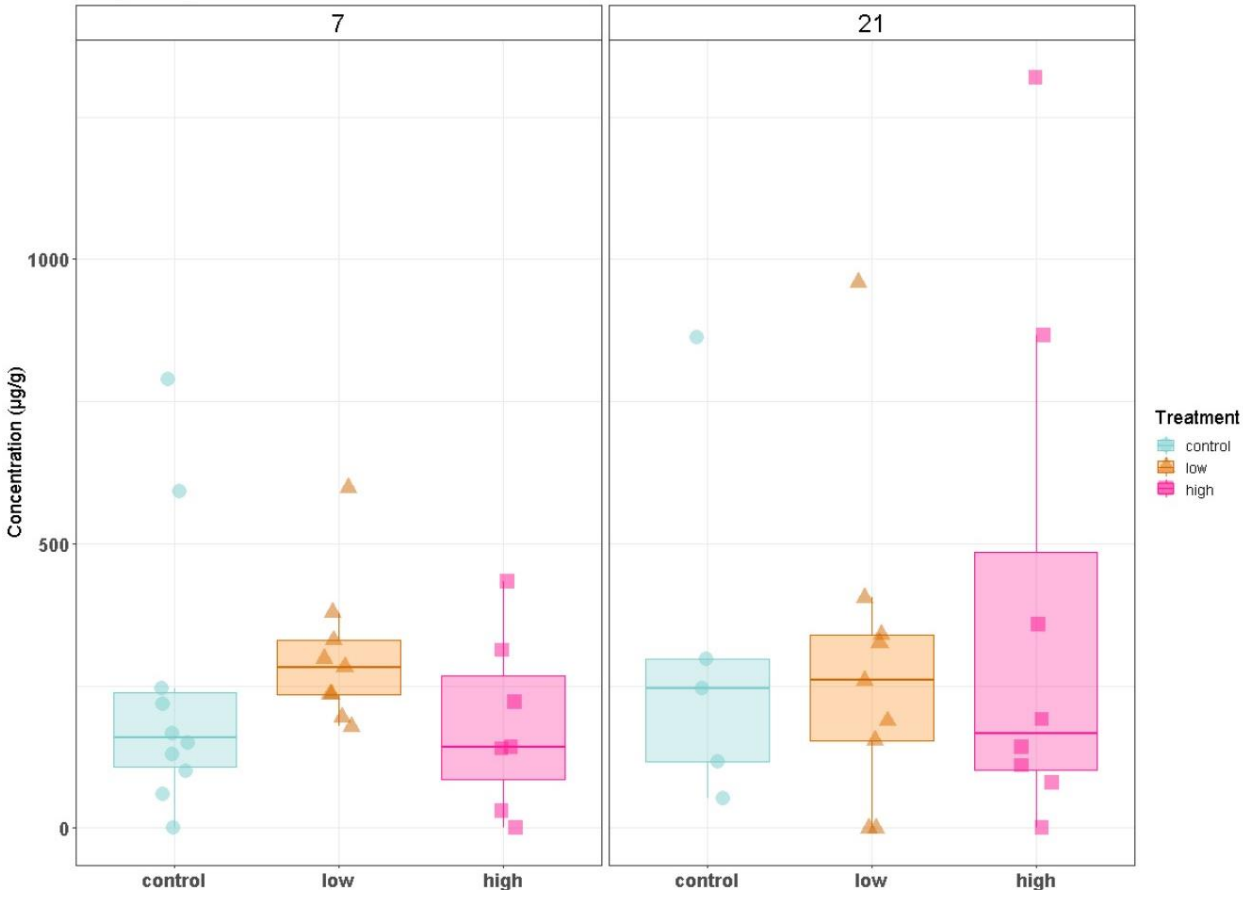


Figure 3.18: Results of PyrGC/MS of digested northern shrimp stomach and intestines for detection of the tyre rubber marker SBR-vinylcyclohexene (µg/g) in treatments (control, low CR exposure and high Cr exposure) and time (day 7 and 21). Blue circles represent control groups, orange triangles represent the low CR exposed groups and pink squares represents the high CR exposed groups.

3.7 Uptake of organic chemicals by northern shrimp

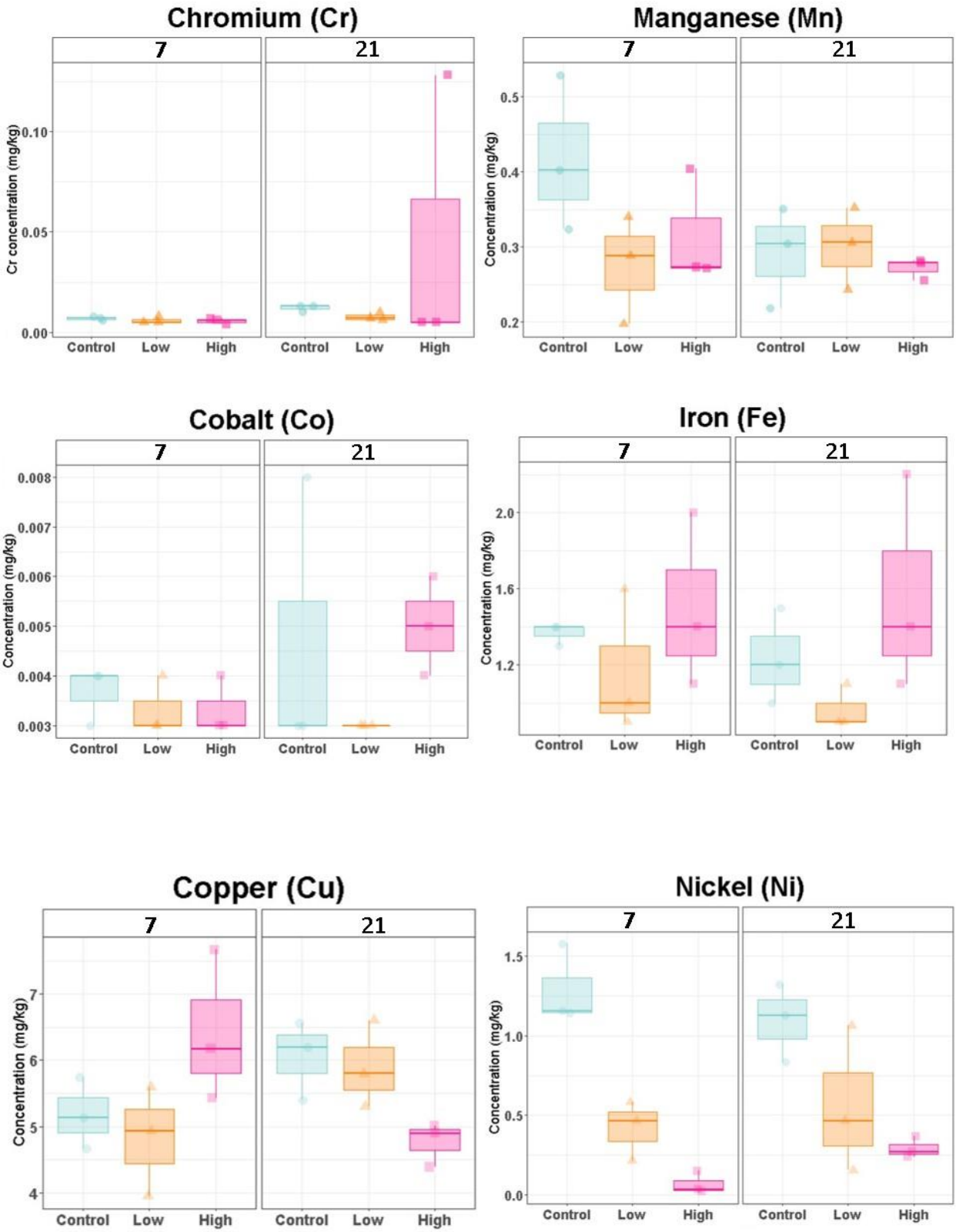
GC/MS-triple quadrupole was used to analyze northern shrimp tail muscle and hepatopancreas for tyre characteristic organic chemicals. To date, the results are inconclusive as further work and analysis is needed and the work is part of a larger project. See chapter 4.3.2 for continued discussions. Add description of findings of ISTD and 6PPD but no quinone.

3.8 Uptake of metals by northern shrimp

Chromium (Cr); Manganese (Mn); Iron (Fe); Cobalt (Co); Nickel (Ni); Copper (Cu); Zink (Zn); Cadmium (Cd); Antimony (Sb) and Lead (Pb) were analyzed in muscle samples (n=18) in controls (n=6), low CR exposed groups (n=6) and high CR exposed groups (n=6) on day 7

and day 21. There was a significant difference found for nickel between treatment groups (Kruskal-Wallis, $p < 0.05$) but not for time point ($p > 0.05$). There were no significant differences in concentrations for chromium, manganese, iron, cobalt, copper, zinc, cadmium, antimony and lead between treatments (control, low CR exposure and high CR exposure) or time (day 7 and 21; $p > 0.05$; Figure 3.19 and Table S3.18).

Zinc was the metal found in highest average concentration for all treatments (control, low CR exposure and high CR exposure) ranging between 11.9 – 13.4 mg/kg, while cobalt was the metal found in lowest concentrations for all treatments ranging between 0.003-0.005 (mg/kg). Cobalt and nickel average concentration increased over time (from day 7 to day 21) for the high CR exposed groups (from 0.003 to 0.005 mg/kg and 0.03 to 0.27 mg/kg, respectively). Whereas, the average concentration of nickel decreased over time (day 7 to 21) for the high CR exposed groups (from4.9 to 5.8 mg/kg). For the remaining metals (chromium, manganese, iron, nickel, copper, zinc, cadmium, antimony and lead) the average concentrations did not change (Figure 3.19 and Table S3.19).


Chromium (Cr)
7
21
Cr concentration (mg/kg)
0.10
0.05
0.00
Control
Low
High
Manganese (Mn)
Concentration (mg/kg)
0.5
0.4
0.3
0.2
Cobalt (Co)
0.008
0.007
0.006
0.005
0.004
0.003
Iron (Fe)
2.0
1.6
1.2
Copper (Cu)
7
6
5
4
Nickel (Ni)
1.5
1.0
0.5
0.0

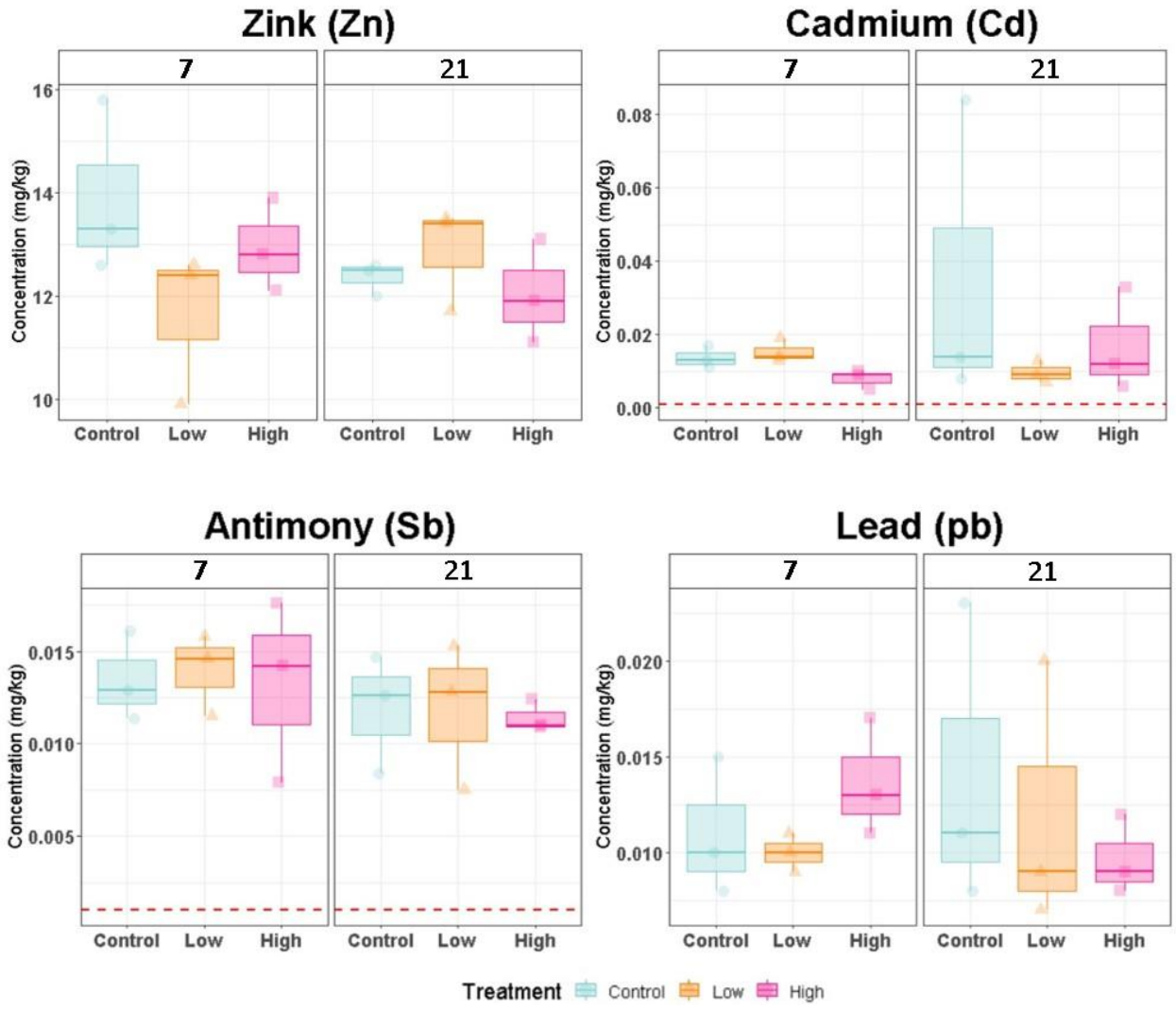


Figure 3.19: Concentration (mg/kg) of metals (Chromium (Cr); Manganese (Mn); Iron (Fe); Cobalt (Co); Nickel (Ni); Copper (Cu); Zink (Zn); and Lead (Pb)) in tail muscle samples (n=18) from northern shrimp of controls (blue circles), low CR exposed groups (orange triangles) and high CR exposed groups (pink squares) at different timepoints: day 7 in left side panel and day 21 in right side panel. Red dashed line indicates the LOD.

4. Discussion

Tyre rubber particles have been found in Arctic sediments (Adams *et al.*, 2021) and are known to be a major source for marine MP (Boucher and Friot, 2017) that are known to end up at the sea floor (Woodall *et al.*, 2014; Booth *et al.*, 2017b). In the current study the ingestion potential of CR particles and uptake of tyre-derived contaminants have been demonstrated.

4.1 Ingestion of CR by lumpfish and northern shrimp

Lumpfish did ingest CR particles when they were exposed to them through the food and/or the surrounding environment of the tank. Despite large individual variation in the number of CR particles per fish, there was a trend of increasing numbers of CR particles over time during the exposure (day 1-6) leading to a peak on day 8. CR were ingested in high numbers (maximum 84 particles) for some individuals, while some individuals contained low numbers of CR particles and some did not contain CR particles. CR particles were ingested on the first day of exposure (day 1; Figure 3.3). There were two days during the experiment where all fish had CR particles in the GI-tract, days 1 and 8 (Figure 3.4). The fact that CR accumulated in the GI-tract, leading to a peak in particle numbers on day 8 suggests a delay in the egestion, where the ingestion rate is greater than the egestion rate of CR. This led to an accumulation of ingested CR particles for several days in the GI-tract. CR was still found in the GI-tract of 6 fish at the end of the experiment suggesting that the gut retention time of CR can be longer than 14 days in some individuals. It is important to note, however, that time and amount of CR ingested and egested for individual fish is not known due to sampling mortality (the fish were sacrificed at the time of sampling). In addition, some CR may have been present in the tanks throughout the experimental period, being available for ingestion, including re-ingestion of egested particles. This may have been the case for the high number of particles observed in one individual on day 18 (56 CR particles). Thus, the results show the number of CR particles found in fish at the time of sampling and do not represent repeated measurements over time for an individual fish. However, the current results give a general overview of the ingestion pattern, showing that CR particles are both ingested, accumulated during exposure and then egested over varying periods of time.

The quantification of ingestion of CR for the northern shrimps was challenging. Finding the SBR marker (SBR-vinylcyclohexene) in the control groups was not expected as they were not exposed to CR. The contamination could be due to input of rubber into the control tanks during the experiment, as the rubber is easily dispersed and particles of the fine fraction difficult to see, or from other rubber materials used in the experimental set up, such as tubing used in the water supply to the tanks. The contamination could also have occurred throughout different steps of the experimental work, such as during the preparation of the rubber-contaminated food, the digestion of the GI-tract, or filter preparations. This last step could have been planned and carried out differently and more carefully to avoid potential cross-

contamination between samples: to improve the digestion and filter preparation phases, the controls should have been filtered first, followed by the low CR exposed and finally the high CR exposed with at least three digestion blanks per treatment. The filters should have been dried and folded separately by treatment and additional blanks could have been added.

Assuming the detected concentrations of SBR in control samples are naturally occurring concentrations from tyre rubber background contamination in the environment, concentration of SBR in the low CR exposed group are above these baseline levels, indicating that ingestion of the CR offered with the food had occurred in this treatment. The low levels of SBR marker in the fish exposed in the high concentration treatment are more difficult to explain. An explanation could be that the low CR concentration food was more attractive than the high concentration CR food, and thus was eaten more, while the high concentration food was avoided. The high concentration may have induced sensory responses, such as, taste, smell or both, that led to avoidance. In contrast, low concentration CR food balls may have been acceptable as food due to fewer CR particles and higher edibility. Shrimps have different external morphological structures (mandibles, maxillipeds and pereopod) that they can use for feeding (Shumway *et al.*, 1985). Thus, there is potential for shrimps to be selective in their feeding, in contrast to lumpfishes. Laboratory studies have shown that shrimps are selective in the size of the prey they feed upon in larval stages, preferring small prey in early stages compared to larger preys in later stages (Harvey and Morrier, 2003). However, it is unclear if adult shrimps are always selective in their feeding. Earlier stages of shrimp larvae can feed on the copepod *Calanus finmarchicus* (Harvey and Morrier, 2003) that are of sizes between 0.5 to 3 mm for copepodite stages I – IV and > 3 mm for adults (Marshall, Nicholls and Orr, 1934). Furthermore, an in situ study examining MP ingestion in Arctic and sub-Arctic benthos found that northern shrimp ingested MP predominately in size ranges between 0.8 to 1.5 mm and > 2.2 mm (Fang *et al.*, 2018). Hence, if adult shrimps can feed on similar or larger size classes of prey and can ingest MP > 2.2 mm, the fine CR (< 1.2 mm) used in the exposure in the current study should not be too large to ingest. Shrimps interacted with the food balls in the experiments, but were not directly observed to feed, in contrast to observations for the lumpfish. Thus, shrimp feeding was more difficult to observe due to the smaller CR particle size, both in direct observations and through indirect examination of the rubber marker in tissues.

The size and shape of ingested CR particles likely may have influenced both the gut retention time and the toxicity in terms of leaching of chemicals that can be taken up into tissues. CR particles tend to have an irregular shape that may increase the potential for CR to adhere to tissues, especially if they have sharp edges, prolonging the gut retention time and thus the time for leaching and the potential for uptake. However, surface to volume ratio is difficult to determine for CR because of the irregular shape, as every CR particle is unique in size and shape (Halsband *et al.*, 2020). If the size of the ingested CR particles varied to a large degree was not studied here and therefore the influence of CR particle size cannot be concluded. Although it would have been interesting to study the relationship between size and surface to volume ratio and chemical uptake into tissues, it was outside the scope of this project.

Lumpfish feed on small and benthic organisms such as crustaceans, cnidarians (Cox and Anderson, 1922; Davenport, 1985) and polychaetes (Cox and Anderson, 1922). Their feeding strategy has been described as sluggish and circumscribed, likely due to their habit of attaching to objects (Cox and Anderson, 1922). Stomach contents have indicated feeding both in the benthic and pelagic compartments (Cox and Anderson, 1922; Davenport, 1985). Hence, it seems that lumpfish are opportunistic feeders. The lack of selectivity in feeding provides high potential for tyre rubber ingestion. Lumpfish were indeed observed to ingest CR unselectively in the present study, both directly and indirectly through examination of their gut contents. Whereas, the northern shrimp is predominately a benthic opportunistic omnivorous feeder, both as predator and scavenger, with the addition of diel vertical migration to feed in the water column (Shumway *et al.*, 1985). Northern shrimps in Balsfjorden, Norway, have been shown to predominately feed on pelagic zooplankton as well as benthic species like polychaetes and detritus (Hopkins, Sargent and Nilssen, 1993). Thus, species residing and feeding on the seafloor may be more exposed to and interact more with tyre rubber than those feeding in the water column, because CR is heavier than seawater and sinks to the bottom.

The feeding strategy of Lumpfish does not change drastically throughout the developmental stages from juveniles to adults, but rather the same feeding strategies are utilized throughout development (Brown, 1986). Suggesting, that the exposure and effect could be similar throughout development and for different age groups in terms of exposure to CR materials under environmental conditions. In contrast, northern shrimp go through various stages of

development from larvae to adult shrimp. Northern shrimps are protandric hermaphrodites, meaning that they mature as males before going through a transition followed by maturing into females (Shumway, 1985). Northern shrimps seem to inhabit separate locations between age and size groups, larvae and juveniles residing further inshore and in shallower waters than adults (Ceccaldi, 1989). This dispersal of sex and age is attributed to spawning, something that is seen for lumpfish as well.

Ovigerous females of northern shrimps stop diel vertical migration and reside on the sea floor while carrying their eggs. This period can range from September to march and is also influenced by the temperature of the water as there is a correlation between egg hatching and temperature. Warmer water promotes earlier hatching (Shumway *et al.*, 1985). Thus, in northern regions, females and eggs would stay for a longer period of time on the sea floor. Similarly, lumpfish migrate shoreward in January-May to spawn in rocky seaweed covered shores. While the female migrates back to the open ocean after depositing the eggs, the male stays and cares for the eggs (Cox and Anderson, 1922; Davenport, 1985). Therefore, it is likely that male lumpfish and female shrimps and the respective eggs could be subjected to higher/prolonged exposure to CR near the shores that are in close proximity to urban areas and roads (Leads and Weinstein, (2019); Knight *et al.*, (2020); Goßmann, Halbach and Scholz-Böttcher, (2021).

Furthermore, during spawning for both lumpfish and shrimp, snow removed from roads are dumped into the ocean and could act as pathways for CR to the marine environment. Tyre rubber particles have been found in both snow in Norway (Rødland *et al.*, 2022) and rivers in North Carolina, U.S (Leads and Weinstein, 2019) and surface runoff have been suggested as one of the main routes for tyre rubber particles to enter the marine environment (Kole *et al.*, 2017) where the water flow may increase the transportation of tyre rubber particles (Leads and Weinstein, 2019). Furthermore, melt water from roadside snow contained high concentrations of TWP (76.0 – 14,500mg/L), suggesting accumulation of tyre rubber particles and an important pathway of both tyre rubber particles (Rødland *et al.*, 2022) and derivatives of tyre-derived chemicals (Seiwert *et al.*, 2022). This shows that marine organisms in coastal areas closer to urbanized areas and organisms that migrate and use the coastal areas as nurseries can be expected to be exposed to both higher abundance of CR particles as well as associated contaminants and potential derivatives. Furthermore, organisms with an

opportunistic feeding strategy can be exposed to tyre rubber particles and associated contaminants in higher degree than specialized and selective feeders. Feeding habitat is likely a key factor in the exposure of tyre rubber and derived contaminants with higher exposure in the benthic ecosystems.

4.2 Characterisation of tyre-derived organic chemicals

The crumb rubber used in the experiment contained several organic chemicals and metals were expected to be present based on previous work (Halsband et al. 2020). One of the largest peaks was 6PPD, shown in the GC/MS chromatogram of extracted fine size fractions of CR (Figure 3.5), similar to CR of several size fractions analyzed with GC/MS U.S. National Toxicology Program, 2019). That study also found that some chemicals in CR were bioavailable in different in vitro biofluids, but did not test ingestion and bioavailability in gastric fluid. It is known that benzothiazole and pyrene are major constituents of tyre rubber. What was done was a suspect list of various organic chemicals that are characteristic for tyre rubber (Table S3.20). Extraction of both new CR granulate and weathered CR from artificial turf showed moderate (12.1 - 50.7 mg/kg) and high (571 – 1479 mg/kg) concentrations of DPG and 6PPD, respectively. (Schneider *et al.*, 2020). Benzothiazole, pyrene, 6PPD and DCH were among the chemicals found in fine CR analyzed. Both DPG and 6PPD were detected in blood and liver from exposed lumpfish. Thus, it is shown that CR leached chemicals that were available for uptake into tissues of marine fish.

The CR used in these experiments was fresh, unweathered CR granulate normally used on artificial turfs. Organic chemical profiles of fresh CR have shown high concentrations of organic chemicals compared to CR from artificial turfs subjected to long periods of weathering, where these concentrations decrease with increasing CR age. This indicates that weathering processes remove leaching chemicals from the CR material over time. Similarly, indoor turfs contained higher concentrations of organic chemical than outdoor turfs, probably due to the lower weathering rates indoors (U.S. EPA and CDC/ATSDR, 2019; Celeiro, Armada, Dagnac, *et al.*, 2021; Celeiro, Armada, Ratola, *et al.*, 2021).

4.3 Uptake of tyre-derived organic chemicals into biological tissues

A number of organic chemicals have been shown to leach into the blood and liver of lumpfish that had ingested CR. Neither of the detected chemicals in blood and liver, with the exception

of $C_{13}H_{20}N_3$, seem to accumulate as the concentrations were higher on day 7 than on day 21. This suggests that the compounds, especially $C_{20}H_{20}N_2$, $C_{21}H_{26}N_2$, $C_{23}H_{26}N_2$ and $C_{19}H_{18}N_2$ that was not detected in tissues of exposed fish on day 21, are being excreted or metabolised within the timeframe of this experiment. In contrast, $C_{13}H_{20}N_3$ increased in concentration from day 7 to 21, indicating that this chemical is accumulating in both blood and liver tissue of exposed fish. The increase can also indicate a slower process of excretion and/or metabolisation for this chemical. For 6PPD, the compound does not seem to be metabolised by lumpfish at a considerable degree, as the degradation product of 6PPD, 6PPD-quinone, was not found in either blood or liver tissues. Since the concentrations of 6PPD decreased over time (from day 7 to day 21), it is either excreted or transferred to different tissues or both and can remain in the tissues 14 days in some individuals. However, there were still two fish with high concentrations of 6PPD on day 21 (280 and 680 pg/g), suggesting that 6PPD can be retained for several weeks without being excreted, metabolized or transferred. The high concentrations found on day 21 could indicate excretion of higher concentrations earlier in the experiment that would have been shown if this fish had been sampled earlier. Or simply that 6PPD has a slower process of being excreted, metabolized or transferred.

6PPD was identified by using a commercially available chemical standard and is a previously known constituent of tyre rubber. Both tyre rubber particles and leachate have been demonstrated as toxic to various aquatic organisms (Kellough, 1991; Day *et al.*, 1993; Nelson, Mueller and Hemphill, 1994; Nowaczyk and Domka, 1999; Wik and Dave, 2006, 2009b; Turner and Rice, 2010; Capolupo *et al.*, 2020; Halsband *et al.*, 2020; LaPlaca and van den Hurk, 2020; Tian *et al.*, 2020; Halle *et al.*, 2021; Hiki *et al.*, 2021; McIntyre *et al.*, 2021). Interestingly, toxicity testing of 6PPD and 6PPD-quinone have shown that freshwater organisms are sensitive to 6PPD but do not seem to be sensitive to 6PPD-quinone (Hiki *et al.*, 2021), unlike some marine organisms that are sensitive to 6PPD-quinone and less so to 6PPD (Tian *et al.*, 2020). From this study it can be concluded that lumpfish is not lethally short-term sensitive to 6PPD or any of the other chemicals detected as no lumpfish died during the experiment.

Because blood and liver samples analyzed by UFZ and blood samples analyzed by NILU were analyzed with different instruments (LC/MS-orbitrap vs GC/MS-orbitrap, respectively) different compounds were detected and the results are not directly comparable. The aim was

to analyze more volatile compounds by GC/MS compared to LC/MS that can detect non-volatile compounds in greater extent, to increase the number of detected organic chemicals. In terms of the blood samples, six amines were detected by the GC/MS that were not detected by LC/MS. This could be due to the different methods just mentioned but also due to the sensitivity of the different instruments. UFZ diluted the blood samples before analyzing them and thereby making it more difficult to detecting compounds like 6PPD with a low signal.

Chibwe *et al.*, (2021) stress the limited number of available suspect or non-target analyses of tyre rubber and the limited mass spectral libraries of tyre associated chemicals available and the need for shared high-resolution mass spectral libraries for tyre rubber and risk assessment of these. Our result demonstrates that both known and less known chemicals are taken up by marine organisms, indicating the need for shared libraries and sensitive instruments for sophisticated analyses.

A suspect screening for tyre-related chemicals in an urban creek and waste water treatment plant after heavy rain events found 1,3-DPG, M-DCH and DCH. This shows that in urban areas with combined sewer systems, tyre-related chemicals can reach surface waters untreated, releasing tyre-derived chemicals into the environment (Seiwert *et al.*, 2020). DPG and 6PPD-quinone have been found in surface waters of receiving roadway runoff. However, DPG was detected in samples from dry events, indicating that DPG may originate from other sources than tyre rubber or roadway runoff alone (Johannessen, Helm and Metcalfe, 2021). DPG, M-DCH and DCH were detected in both liver and blood of exposed lumpfish in this study. This shows that these chemicals leach from CR particles and are available for uptake into tissues and that they can be present in the environment.

Toxicity tests of CR leachate were conducted in three concentrations in another study (Halsband et al. 2020), where the low concentration (1-0.01 g/L) exposure would correspond to the current study (0.6 g/L; table S2.2)). Their results showed no increased mortality for pristine and pre-weathered CR in two marine copepod species compared with mortality in control groups. However, in exposure of copepods to low concentrations (1 -0.01) of weathered CR there were an observed increase in mortality for the concentration of 1 g/L. Although the observed increase in toxicity was not significantly different from the controls the observed increase in mortality could be attributed to the weathered state of the CR

particles (Halsband *et al.*, 2020). These results point to a lack of observed effect of low concentrations of CR leachate (1 g/L) that was higher than the approximate concentration of CR in the current study (0.6 g/L table S2.2).

Furthermore, the surrounding environment in the GI-tract will likely influence the potential for chemical leaching from CR once inside the organism. In solutions with varied pH basic conditions have been shown to promote higher leaching of CR compared to acidic conditions and least leaching was found in neutral conditions (Selbes *et al.*, 2015). Thus, leaching potential of CR particles likely varies both inter-species and intra-species as gastrointestinal environments vary both between species and individuals. As have been seen in Rainbow trout where the pH was different in different gastrointestinal fluids and changed over time during digestion (Bucking *et al.*, 2011).

It would have been interesting to look at leaching potential, size and the surface to volume ratio and a potential correlation between these, however, it was beyond the scope for this study. Individuals that did contain chemicals could have ingested smaller particles than individuals that did not contain chemicals, irrespective of the number of ingested CR. However, this not likely as the CR particles are quite uniform in the size and probably in distribution. Alternatively, indirect leaching could have bene an influencing factor. Since CR was mixed with the food and there could have been leaching of chemicals to the food or the surrounding water that was taken up into the tissues of the individuals. However, since chemicals were predominately detected in individuals that did contain CR this seems, in the current study, less likely.

The exposure concentrations used in the present study did not induce mortality in the lumpfish or the shrimps. Freshwater fish (*Dania rerio* and *Oryzias latipes*) and crustaceans *(Daphnia magna* and *Hyalella Azteca*) showed no toxicity or sensitivity of 6PPD-quinone (34 – 54 µg/L). Whereas, 6PPD (107 – 286 µg/L) induced > 80 % mortality in *O. latipes, D. magna and H. azteca*. 6PPD also delayed hatching rates, reduced the body length and caused spinal abnormalities in *D. rerio* and caused behavioral changes in *O. latipes* (Hiki *et al.*, 2021). The marine fish *Oncorhynchus kisutch* showed induced lethal toxicity in low concentrations of 6PPD-qunone (LC_{50} 0.82 µg/L) where mortality occurred within 2 – 6 hours, compared to 6PPD (LC_{50} 250 µg/L; Tian *et al.*, 2020). In contrast, the marine fish

Oncorhynchus keta exposed to 6PPD-quinone showed no sensitivity or toxicity (McIntyre *et al.*, 2021). In addition, exposed to pristine and weathered tire particle leachate of the freshwater amphipod (*Hyalella Azteca)* showed that particle suspension was more toxic than pure leachate and that pristine particles were more toxic than weathered. However, in a 48 h LC_{50} test (0.19 g/L, 364 ± 64 particles) on *H. Azteca* weathered particles were more toxic compared to pristine (0.91 g/L, 3073 ± 211 particles).

There are a lot of studies available on tyre leachate and roadway runoff exposure (Kellough, 1991; Day *et al.*, 1993; Nelson, Mueller and Hemphill, 1994; Skinner, Peyster and Schiff, 1999; Turner and Rice, 2010; McIntyre *et al.*, 2014, 2021; Young *et al.*, 2018; Capolupo *et al.*, 2020; Halsband *et al.*, 2020; Chibwe *et al.*, 2021; Halle *et al.*, 2021), however there are few studies available that look into the role of ingestion in terms of toxicity and effect. CR exposure (38 − 355 μm) of the marine fish mummichog (*Fundulus heteroclitus*) and the freshwater fish fatheaded minnow (*Pimephales promelas*) in different concentrations (0, 0.3, 1.9, 6.0 g/L crumb rubber) showed that both mummichog and fathead minnow did ingest CR. There were an 40 % mortality of the fathead minnows in the high concentration exposure (6.0 g/L), no mortality in the mummichog experiment. They found a significant difference between liver enzymatic activity with increased EROD assays in exposed individuals of both species, indicating toxicity response(LaPlaca and van den Hurk, 2020). This agrees with our observations of no mortality in the marine lumpfish. However, it is worth mentioning that the concentrations in the current study was well below their high concentration groups and would be more relatable to the lower concentrations. Furthermore, sampling and examination of EROD assays was not performed in the current study and thus cannot be discussed.

Freshwater amphipod (*Hyalella azteca)* exposed to tyre particles and leachate showed that *H. azteca* did ingest particles with a gut retention of 24 – 48 h. The authors found leachate to be more toxic in low concentration compared to particles that were more toxic in higher concentrations. Suggesting a role of chemical delivery by the particles into organisms through ingestion rather than leaching in surrounding water in ambient temperature. In a 21-day exposure (0.58 g/L, 2000 particles/mL) the mortality was 92.5 % and the reproduction decreased from an average offspring output of 18.9 in control groups to 2. 4 in exposed groups. Growth rate reduced as well from 18 to 6.7 net growth rate over the exposure period. The mortality, decreased reproduction and growth rate increased with increased tire particle

concentration (Halle *et al.*, 2021). Similarly, estuarian fish and shrimp species showed behavioural changes in swimming abilities and reduced growth when exposed to tyre particles (< 1 μm and 1-20 μm), where higher concentration of particles entailed increased ingestion. The observed effects of tyre particles may have long-term effect on a populational level of organisms, pointing to the importance of evaluating and address the effect of tyre particles in the environment (Siddiqui *et al.*, 2022).

Both raw and chemically-modified rubber vulcanizate were found to be toxic to bacteria (*Thiobacillus ferrooxidans)*. The toxicity of particular components of the rubber was determined and the most toxic agent was found to be the anti-ageing Dusantox 6PPD (N-(l,3-dimethyl-butyl)-N-phenyl-p-phenylenediamine) (Nowaczyk and Domka, 1999). 6PPD has been suggested as potential for biomagnification due to lipophilicity (log $K_{OW} = 5 - 5.5$[15]; Hiki *et al.*, 2021). However, from the results in this study when 6PPD were observed to be excreted and not bioaccumulate the potential for biomagnification is less likely. Although, more research is needed both on the fate and transport as well as the toxicity and effect of 6PPD and other tyre-derived contaminants. Environmental concentrations of 6PPD-quinone have been measured in roadway runoff in regions of Seattle, US, between 0.3 – 19 μg/L. During storm events three receiving watersheds showed < 0.3 - 3.2 μg/L in areas where mortality events of *O. kisutch* was known to have occurred. Environmental concentrations of 6PPD-quinone have been measured in roadway runoff in regions of Los Angeles, US, between 4.1 – 6.1 μg/L and in regions of San Francisco between 1.0 – 3.5 μg/L (Tian *et al.*, 2020). 6PPD can be a source for other transformation products than solely 6PPD-quinone. There is a diverse range of transformation products just experimentally revealed from different oxidation reactions of 6PPD, showing that 6PPD-quinon is only one of many more. Furthermore, 6PPD-quinone does not necessarily have to be the end product but can give rise to further transformation products. Increasing polarity of the transformation products along the reactive pathways indicate the importance for other transformation products in aquatic environments (Seiwert *et al.*, 2022). Considering the quite species-specific sensitivity of 6PPD-quinone to Coho salmon, the toxicity of other transformation products of 6PPD and 6PPD-quinone, as well as other tyre-derived chemicals and related transformation products, needs to be considered. In addition, due to the physiochemical properties of different compounds the fate and transport needs to be further studies in order to better predict the effects and environments and species affected. Environmental concentrations for 6PPD-

quinone exceeds the limits for lethal toxicity for coho salmons (Tian *et al.*, 2020; Johannessen *et al.*, 2021).

Derivatives of other tyre-related chemicals then the ones detected and discussed in the current study are diverse and abundant. Johannessen et al (2021) stress the importance of continued research on derivatives of tyre-derived chemicals and monitoring and of urban receiving aquatic environments and the toxicological effect and mitigation of these chemicals and derivatives. Furthermore, they point out the lack and importance of available commercial standards for analytical enhancement (Johannessen 2021). Furthermore, 6PPD-quinone and DPG was detected in samples from snow melt events, indicating the potential for these compounds to sequester in snow. There were a positive correlation between the mass load (g) and amount of precipitation for both 6PPD-quinone and DPG and even small rain events could mobilize 6PPD-quinone and DPG (Johannessen *et al.*, 2021). DPG is a vulcanization agent widely used in tyre manufacturing. DPG was detected in the so called Coho mortality signature and one of the three largest peaks in mortality regions and one of the 10 largest peaks in roadway runoff (Peter *et al.*, 2018).

Because of the difficulties in cleaning the hepatopancreas and tail muscle samples from shrimp, no conclusion can be made. The samples were cleaned/purified by solid phase extraction (SPE) for enhanced analysis. This process did not go as planned. In order to evaluate if there were any organic chemicals in the samples and how much, the samples had to be combined with the wash fraction from the clean-up step in order to evaluate if analyte and internal standard were washed out and how much ended up in the sample and how much ended up in the wash fraction. The standards and analytes need to be re-analyzed for signal and retention time to be able to evaluate if these are found in the sample or wash fraction. Further analysis of the existing samples from this study is needed to provide quantification of uptake of organic chemicals in northern shrimp tissues. The benefit of the clean-up step is the removal of large biological molecules that would interfere with the analysis, while keeping the analyte, if any, in the sample. During the clean-up we suspect that the analyte was washed out along with the internal standard. Therefore, we could not determine how much was in the samples. This is to some extent in accordance to the results of Johannessen et al (2021) that reported < 30% recovery of 6PPD-quinone in solid phase extraction by HLB cartridges,

similar method as the one used here. Furthermore, the standards seemed to be very sensitive. They showed to be light and temperature sensitive as well as easily evaporated. Therefore, the standards needed to be freshly prepared, only used once and kept in dark GC/MS-glass vials. Moreover, the signal of the analytes and internal standards in the current study are weak. Hence, samples need to be spiked with sufficient amount of internal standard and sophisticated instruments with high sensitivity is needed to detect them. Hence, for interpretable results the methods need to be revised and further research is needed on the behavior of 6PPD and 6PPD-quinone to obtain higher recoveries. Decapod crustaceans like the Northern shrimp lack teeth in their mouth, instead they have a gastric mill. The gastric mill is located in the stomach and constitutes of sets of teeth operating as chewing parts. The teeth are mobile and grind down the food into a soup, only fine particles < 1 nm can pass through the filter structure of the stomach and be digested. Particles not able to pass through to digestion becomes faeces while the particles that passes the filter enters the hepatopancreas for metabolisms (Ceccaldi, 1989). Initially, the idea was to see if ingested CR particles accumulated in the stomach as shrimps have a filter structure that would not allow for particles > 1 nm to pass. Furthermore, if CR particles were somewhat grinded, enhancing the potential for chemical leaching from CR. Since it was not possible conclude anything based on the results of both CR quantification and chemical uptake into shrimp tissues, these questions cannot be answered to date.

4.4 Correlation between number of ingested CR particles and chemical uptake

There is a positive linear correlation between number of CR particles and concentrations of chemicals. This indicates that the number of ingested CR particles influences the amount of leaching and/or uptake of chemicals into blood and liver of fish.

Predominately fish with a high number of CR particles in the GI-tract were found with higher concentrations of organic chemicals, although there were exceptions. One fish contained 3 CR particles but had a high concentration of 6PPD in the blood (Figure 3.13) and DPG in the liver (Figure 3.15), something that was not seen for the other chemicals. There was one fish that ingested 33 particles but had low concentrations of some chemicals ($C_{21}H_{26}N_2$, $C_{20}H_{20}N_2$, $C_{23}H_{26}N_2$, 6PPD, $C_{19}H_{18}N_2$, $C_{12}H_{15}N$ in the blood and M-DCH, DCH and TBT (Figure 3.13)). Individuals with high numbers of CR particles in the GI-tract and low concentrations of chemicals may have ingested particles large in size that leached less chemicals that could be

taken up, or the particles were ingested late in the exposure and had a shorter period of leaching within the fish. The individual with only 3 CR particles in the GI-tract and high concentration of 6PPD, but not the other chemicals, especially amines, is more difficult to explain. This fish may have ingested a high number of CR particles early in the exposure period. Early ingestion could have prolonged the time for chemical leaching and uptake into tissues where 6PPD accumulated, while the CR particles were egested before sampling of the fish. It could also be the case that chemicals were transferred to other tissues by the blood and 6PPD was the only chemical left to detect in high concentrations. Finally, 6PPD may not be homogenously distributed in the tissues and be located in high concentrations in certain 'hot-spots'.

4.5 Uptake of metals into biological tissues

The lack of difference in metal concentrations in tissues (lumpfish blood and liver and northern shrimp tail muscle) between treatments and time points is in accordance with findings of Kellough, (1991) who exposed rainbow trout (*Oncorhynchus mykiss*) to tyre leachate and found no difference in metal concentrations between treatments. Metals are known to readily leach from CR especially Zinc (Zn) is abundant and have been shown to leach (Halsband *et al.*, 2020). However, metals have to be bioavailable for uptake into tissues. Metals have been reported as being generally low in bioavailability (~ 3 %; U.S. EPA and CDC/ATSDR, 2019) which would agree with the current results showing no evidence for uptake into tissues by either lumpfish shrimp.

O'Mara (2019) found that School prawns (*Metapenaeus macleayi*) had very low uptake of Cadmium (Cd), Manganese (Mn) and Zinc (Zn) over a 7-day exposure through the water. Although, the negligible concentrations of these metals, they found a difference in uptake of Mn and Zn between molted and non-molted individuals. Molted individuals had higher concentrations compared to non-molted individuals. The higher concentration may be due to substitution of calcium ions with ions of similar chemistry in the exoskeleton (O'Mara *et al.*, 2019). Copper (Cu) periodically stored and released from the hepatopancreas is used in hardening of the exoskeleton in crustaceans after molting and repair (Rao and Anjaneyulu, 2008). The marine fish sand whiting (*Sillago ciliate*) had no detectable uptake of dissolved Cadmium (Cd), Manganese (Mn) and Zinc (Zn) after 6 days of exposure, even with increased concentrations of dissolved metals. School prawns and sand whiting were also exposed to

metals through the diet, showing interspecies differences in uptake and accumulation as well as differences between metal retention in different diets. Dietary exposure of metals was higher for prawns and fish compared to dissolved metals (O'Mara *et al.*, 2019), something that was not found in the current study. Although, it is important to note that O'Mara (2019) exposed fish and prawns directly with metal isotopes dissolved through the water or spiked through the food, whereas the current study exposed both fish and shrimps to CR through the food. O'Mara (2019) also speculated that prawns, by molting, purified themselves from metal contaminants. In contrast to the findings of O'Mara (2019), exposure experimental studies of of zinc and copper through water showed bioaccumulation of both metals in muscle tissue of a marine fish (*Anabas testudineus).* Both Zinc and Copper steadily increased in concentrations over time in muscle tissue until reaching a steady-state. Zinc were accumulated in higher rate compared to Copper (Majed *et al.*, 2019). No uptake or accumulation of metals were observed in this study.

Metal characterization of CR from synthetic turf fields have shown metal concentrations similar to that of fresh CR from manufacturing plants. Furthermore, the bioavailability of metals were generally low with ~ 3% in gastric fluid and < 1 % in saliva and sweat (in vitro biofluids; U.S. EPA and CDC/ATSDR, 2019). The low leachability and bioavailability would explain the current results where there was no difference between treatments or time in metal concentrations. However, metals are known to leach from CR (Li *et al.*, 2010; Halsband *et al.*, 2020), which seems, to some extent, be in contrast to the results obtained by EPA and CDC/ATSDR (2019) regarding the similar metal content of fresh and turf CR. Halsband et al (2020) suggested that the observed increased toxicity of weathered CR was due to changed properties of weathered CR can increase the surface area enhancing the potential for leaching of contaminants (Halsband *et al.*, 2020).

Although, the leachate was not evaluated or measured in the current study, it has been studied by Li *et al.*, 2010 and Halsband *et al* (2020). Leachability of metals from crumb rubber material (0.84 – 2.0 mm) were found in decreasing order Zn > Mn > Cu > Ni > Pb > Cr > Cd (Li *et al.*, 2010). Another study found that metals leached from CR particles into sea water and that the concentrations in the leachates corresponded to the metal profile of CR materials. Zinc was the metal found in highest concentration in CR leachate, followed by in decreasing order Fe > Mn > Cu > Co. Remaining metals Cr, Ni, Cd, Sb and Pb) were found < 10 ug/L

(Halsband *et al.*, 2020). Concentrations of metals found in blood from lumpfish is quite similar to the profile of metals found in leachate by Halsband *et al* (2020). Although there was no significant difference for the metals in blood samples, except Cr, the metals were found in similar concentrations in both exposed and unexposed groups in decreasing order Fe > Zn > Cu > Mn > Cr. Cr. Co, Cd and pb were found in concentrations < ~ 0.01 and Sb was < LOD. Considering, blood samples, Fe was expected to be the metal in highest concentration. Metal concentrations in liver were found in decreasing order Fe > Zn > Cu > Mn > Ni > Cr. Co, Cd and Pb were found < ~ 0.01 and Sb were < LOD.

Zn being the metal found in second highest concentration would make sense if not found in controls as well, as Zn is the predominant metal found in tyre rubber and also the corresponding predominant metal found in tyre rubber leachates. Either an additional exposure source was present, or the metals were not bioavailable for uptake into tissues, or metals did not accumulate in the tissues selected for analyses. If metals were bioavailable and accumulative, even if there were additional sources of exposure, these would likely be similar over treatments and tanks and exposed individuals should have exhibited higher metal concentration, as the potential case for ingested CR in northern shrimp.

Since, metal concentration increased for some metals (Mn, Ni) in control groups, this suggest another source for these metals, besides the CR. However, the increase was not seen in both treatments for the same metals, as described in above section. Thus, the difference in concentration is likely not due to other sources of metal contamination but potentially individual variation. Since both lumpfish and shrimps were kept in flow through systems, ocean water was pumped through the tanks, which we could not control for contaminants. Water samples from the flow through systems could have been taken an analysed as water blanks. This would have enhanced the method and analysis to some extent. Detected metal concentrations in both lumpfish and shrimp did not exceed the thresholds for the Environmental Quality Standard (EQS)or the Annual Acceptable Environmental Quality Standard (AA EQS) for priority substances and river basin specific pollutants (Table 4.1; Grung *et al.*, 2021).

Table 4.1: Environmental Quality Standard (EQS) concentrations and Annual Acceptable Environmental Quality Standard (AA EQS) concentrations for Zn, Cu, Cd, Pb, Ni and Cr and average concentrations of the same metals found in lumpfish liver and shrimp muscle. EQS and AA EQS from Grung *et al* (2021).

	EQS (mg/kg dry weight)	AA EQS (µg/L)	Control Lumpfish liver (mg/kg)	Exposed Lumpfish liver (mg/kg)	Control Shrimp muscle (mg/kg)	Low CR exposed Shrimp muscle (mg/kg)	High CR exposed Shrimp muscle (mg/kg)
Zinc (Zn)	139	3.38	27.36	21.81	13.14	12.25	12.49
Copper (Cu)	84	2.6	7.90	9.07	5.62	5.36	5.59
Cadmium (Cd)	2.5	0.2	0.015	0.016	0.025	0.012	0.012
Lead (Pb)	150	1.3	0.0045	0.0059	0.013	0.011	0.012
Nickel (Ni)	42	8.6	1.01	0.64	1.19	0.49	0.19
Chromium (Cr)	660	3.4	0.045	0.021	0.009	0.007	0.026

Metal uptake by the marine fish Mangrove snapper (*Lutjanus argentimaculatus)* exposed to dissolved radiolabelled Zn and Cd showed an increased uptake over a 2 -day exposure into gill and viscera (the tissue layer covering the inside of organs, such as the inner walls of the GI-tract) tissues. The concentration factor (radioactivity in fish divided by the radioactivity in water) was highest in gills and viscera for cadmium whereas it was higher for zinc in the remining body and whole body. In exposure to radiolabelled Zn and Cd through the food the metals levelled of in a 1-day depuration(Xu and Wang, 2002). Here, they showed that zinc and cadmium was taken up both through the water and the diet. Something that was not seen in the current study. Xu and Wang (2002) used dissolved metals and perhaps potential metals that leached from the CR in this study was not dissolved enough and thus not bioavailable for uptake.

Environmental concentrations of Cu, Cr, Cd, Ni and Pb in the marine grey mullet (*Mugil auratus*) showed consistently higher concentration of Cu, Cd and Pb in liver tissues compared to muscle tissue where concentrations ranged between (0.49-1.30; 0.15-0.50; 060-1.21, µg/g WW, respectively). Whereas, Cr and Ni in both liver and muscle were < LOD in all samples (Filazi *et al.*, 2003). Liver concentrations in lumpfish in this study for Cu, Cd and Pb (8, 0.01, and 0.003-0.007 mg/kg, respectively). Interestingly, Cu concentrations were well above the concentrations found in wild fish, even for control fish, whereas concentrations for Cd and Pb were lower in this study compared to environmental levels found in the grey mullet. Environmental concentrations of Cu, Cd and Pb in sediments of a Norwegian urban fjord were < EQS (environmental quality standard; Grung *et al.*, (2021)). Therefore, if there was no uptake of metals from CR, the low concentrations of Cd and Pb agrees with the low environmental concentrations. The quite high concentrations of Cu suggest another source for this metal in the current study.

These results indicate an overall low bioavailability of metals but the bioavailability needs to be further studied in a wide range of organisms (U.S. EPA and CDC/ATSDR, 2019) and there seems to be a large knowledge gap in the leaching, bioavailability, uptake and accumulation of metals, seeing as the results varies widely between studies

5. Conclusion

Marine fish can ingest high numbers of CR particles that can remain in the digestive system for 14 days or longer, during which they leach of tyre-derived contaminants that are taken up into tissues. Chemicals detected in this study did not show signs of accumulation, although some chemicals can take longer than others to depurate.

Marine organisms residing in benthic and coastal areas close to urbanized regions are likely to be exposed to high abundance of both tyre rubber particles and associated contaminants. When these habitats are utilized for spawning and as nurseries, adults and developing embryos can be subjected to tyre particles and derived contaminants. Some of these chemicals have been shown to induce adverse effects on the development. The exposure risk is likely influenced by feeding strategy, where organisms with an opportunistic feeding strategy pose a

higher risk than specialized and selective feeders. Furthermore, feeding habitat is likely a key factor in the exposure of tyre rubber and derived contaminants, with higher exposure in the benthic ecosystems.

Despite increasing research in the field of tyre rubber particles during the past years, it is evident that knowledge gaps remain in the toxicity and long-term effects of tyre rubber particles but also in analyzing these. Far from all chemicals have been identified and evaluated for their toxicological effect, not to mention the effect of chemical mixtures.

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S2. Material and methods

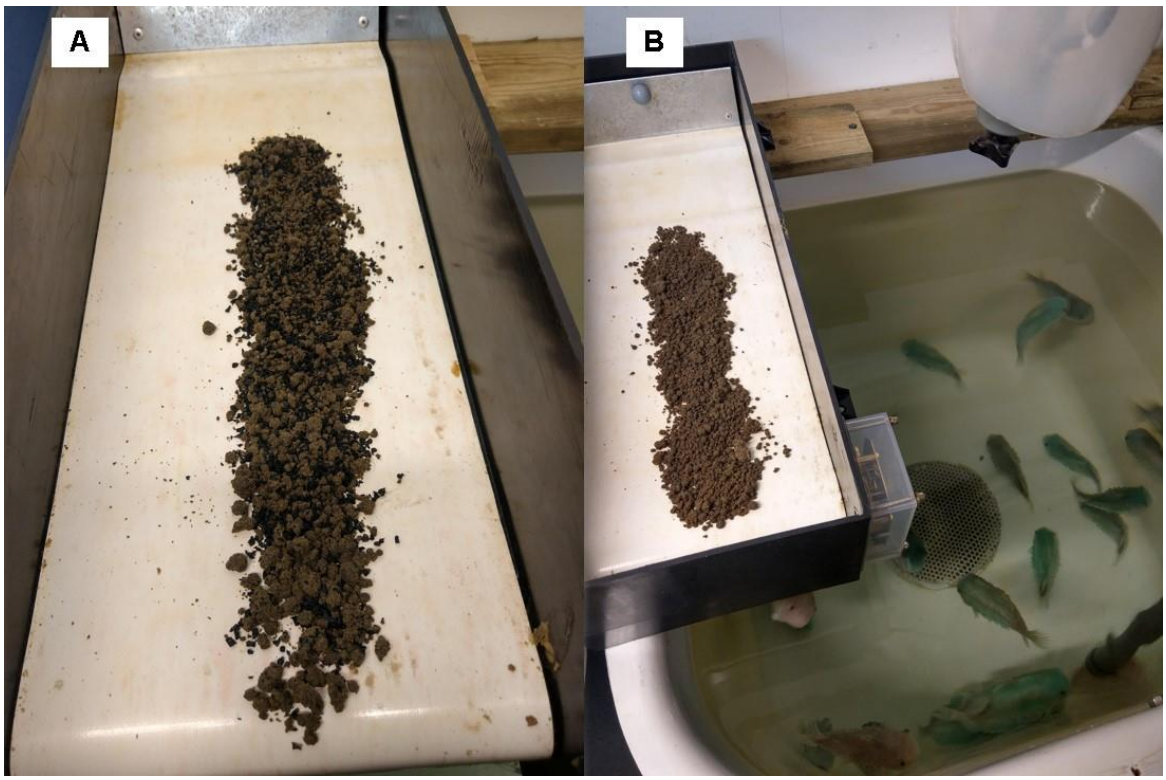

Figure S2.1: Experimental set up of lumpfish exposure with 240 l tanks, flow through systems and automatic feeders.

Figure S2.2: Experimental set up of shrimp exposure with 45 l tanks and flow through systems.

Table S2.1: Food distribution per tank for the exposure period (first week) based on the mean biomass per tank.

Day	Mean biomass per tank (g)	Food per tank (g)
0	3600	72
1	3240	64.8
2	2880	57.6
3	2880	57.6
4	2530	50.4
5	2520	50.4
6	2520	50.4

Mean weight of 20 CR particles ~ 0.126 g (10 replicates measured)

$$\frac{430\ g}{0.126g} = \sim 3\ 413\text{g}$$

$$3413 * 20 = \sim 68\ 254\ CR\ particles$$

Table S2.2: Amount of food and CR used in the exposure of lumpfish. Amount and weight of food used per treatment and tanks. Approximate concentrations in food and water are calculated.

	Crumb rubber	Food	Water
Weight (g)	430.5	1200	
Volume (L)	1	1.5	
Per/tank (g)	143	403	
Per/treatment (g)	430	1209	
Total	430 g	2418 g	240 L
Concentration/tank		0.36 g/g	0.6 g/L

Table S2.3: Number of CR particles and approximate concentration of CR in food and water

	Total	Per/tank	Concentration food (particles/g)	Concentration water (particles/L)
Number of CR particles	68 254	22 751	57	95
Weight of CR particles	1200	403		

Table S2.4: Diet and number of food balls required for one week calculated for one shrimp, one tank, one treatment group and all tanks and treatment groups.

	Shrimp/week	Tank/week	Tanks/treatment/week	Total
Food (g)	5	15	90	270
Number of food balls (1.6 – 1.8 g)	3	9	54	162

Table S2.5: Amount of food, CR and approximate CR concentration in the food and water used in the exposure of shrimps.

	Low exposure			High exposure		
	Total	Per/tank	Per/shrimp	Total	Per/tank	Per/shrimp
Food (g)	90	4.8 - 5.4	1.6 – 1.8	90	4.8 - 5.4	1.6 – 1.8
Crumb rubber (g)	0.09	0.015	0.005	0.9	0.15	0.05
Concentration food ball(g/g)			0.003			0.03
Concentration water (g/g)		0.0001			0.001	

Extraction protocol for lumpfish and shrimp samples

Extraction of blood and liver

Lab blanks were prepared by adding Milli-Q water (0.25 mL) to empty glass vials. Acetonitrile (0.5 mL), Hexane (1.5 mL) and internal standard (0.05 mL) were added to the samples. The samples were vortexed for 15 – 20 seconds before 30 minutes of sonication. The process was repeated, samples were vortexed for 15 – 20 seconds before 30 minutes of sonication. The samples were horizontally shaken over night for 15 – 20 hours following 10 minutes of centrifugation at 1500 rpm. The upper hexane layer (100 µl) of the samples were transferred to GC/MS glass vials (0.4 mL) before adding recovery standard (20 µl; 6PPD-quinone-$^{13}C_6$; 0.1 ng/ µl). All samples and remaining extracts were frozen until analysis

Extraction of tail muscle

Lab blanks were prepared by adding Milli-Q water (0.15 mL) to empty tubes. Three ceramic beads, Acetonitrile (0.3 mL), Hexane (0.9 mL) and internal standard (0.05 mL) were added to the samples (0.3 g). The samples were run in a Precellys shaker (program 3: 3x20 seconds) before 30 minutes of sonication. The process was repeated, samples were run on Precellys shaker (program 3: 3x20 seconds) before 30 minutes of sonication. Samples were put in a vortex shaker for 25 minutes before they were centrifuged at 1500 rpm for 10 minutes. The

upper hexane layer (0.3 mL) of the samples were transferred to GC/MS vials (0.3 mL). All samples and remaining extracts were frozen until analysis.

Extraction from hepatopancreas

Samples (0.5 g) were measured and transferred to glass vials (7 mL). Samples < 0.5 g had Milli-Q water added accordingly: 0.2 g of sample, 0.15 mL Milli-Q added; 0.3 g of sample, 0.10 mL Milli-Q added; 0.4 g sample, 0.05 mL Milli-Q added. Lab blanks were prepared by adding Milli-Q water (0.25 mL) to empty glass vials (7 mL). Acetonitrile (0.5 mL), Hexane (1.5 mL) and internal standard (0.05 mL) were added to the samples. The samples were vortexed for 25 minutes following 30 minutes of sonication. The process was repeated, samples were vortexed for 25 minutes following 30 minutes of sonication. The samples were horizontally shaken overnight for 15 – 20 hours. The upper hexane layer (0.3 mL) of the samples were transferred to GC/MS vials (0.3 mL). All samples and remaining extracts were frozen until analysis.

Analysis of 6PPD and unknown substances in the lumpfish blood extracts by GC-MS(Orbitrap)

GC-MS instrument was Q Exactive GC equipped with Trace 1310 Gas chromatograph and TriPlus RSH autosampler.

Injector was split/splitless operating at splitless mode at 280°C.

The column was Thermo scientific TG-5SILMS, length – 30m, internal diameter – 0.25mm, film thickness – 0.25mm.

Temperature program was as follows: 60°C for 2 minutes, then 20°C/min to 300°C, hold for 16 min.

Transfer lines were held at 280°C, ion source at 250°C.

Mass-spectrometer operated at 40eV ionization energy in full-scan mode, mass range was selected m/z 180-330, Mass resolution was set at 120000.

Sample preparations an analysis of shrimp hepatopancreas and tail muscle

Hepatopancreas and tail muscle samples were removed of biological molecules to enhance the analysis of the samples. Samples were added procedural internal standards (*d*6-Phenol 250 ug/mL, *d*8-pCresol 1.14 ug/mL, *d*12-4-n-Propylphenol 1.32 ug/mL, *d*8-Naphthalene 10 ng/mL, *d*10-Phenanthrene 50 ng/mL, *d*12-Chrysene 50 ng/mL, and *d*12-Perylene 50 ng/mL, all from Sigma Aldrich, Norway) prior to solid phase extraction (SPE). The SPE-cartridges (Waters Oasis HLB, 150 mg) were activated with methanol (6 mL) (Supelco HPLC grade, Merck Life Science AS, Norway) and conditioned with milliQ water (6 mL; Merck Millipore, 18 μΩ). Samples were loaded onto the column and rinsed with milliQ (2 x 4 mL), and dried under vacuum prior to elution with methanol (2 x 4 mL). The eluate was evaporated to dryness under nitrogen and reconstituted with dichloromethane (400 uL, Rathburn Chemicals Ltd, UK) and added a procedural recovery standard mix (100 uL, *d*10-Acenaphthene, 100 ng/mL, and *d*10-Fluorene, 100 ng/mL, both from Sigma Aldrich, Norway. Samples were stored in freezer until analysis.

The extraction procedure did not seem suitable to retain the target analytes. Thus, an effort was made to reconstruct the sample by combining extracts of the wash fraction (milliQ) and the original methanol extract (which was reconstituted with dichloromethane). The water fraction was extracted with 50 % dichloromethane in hexane, 3 x 2 mL, shaken and centrifuged at 2000 rpm at 20 ^{o}C between each addition of organic solvent. The organic layer after each centrifugation addition was transferred to a Kimax tube. The organic extract was dried down under nitrogen to a volume of 500 uL.

Metal analysis protocol

Metal concentrations in the CRG extracts and tissue extracts were determined for different experiments at NILU, Kjeller using ICP-MS approaches. Briefly, analysis was performed using an Agilent 8800 Triple Quadrupole ICP-MS (ICP-QQQ) fitted with a SPS 4 Autosampler. In the second approach, samples were digested in 5 mL HNO_3 and 3 mL

deionized water at 250 °C for 65 min, followed by dilution in MilliQ and addition of ^{115}In as an internal standard. Analysis was performed using an Agilent 7700x ICP-MS.

Protocol sample preparations and analysis by UFZ

Lumpfish liver samples (n=36) and laboratory blanks (n=3) were analysed by UFZ Leipzig, Germany. Below is a protocol for the sample preparation conducted by UFZ before analysis.

Sample pretreatment:

The extracts have been diluted 1:10 with a MilliQ and MeOH solution (1:1).

RPLC-HRMS. For the reversed-phase analysis, an ACQUITY ultra performance liquid chromatography (UPLC) connected to a Xevo G2-XS quadrupole time-of-flight (QTof) mass spectrometry (Waters, Eschborn, Germany) was used. The injection volume was 10 µL. The UPLC separation was achieved using an ACQUITY UPLC HSS T3 column (100 × 2.1, 1.7 µm) at a flow rate of 0.45 mL min^{-1}. The column temperature was set to 45 °C. The mobile phase consisted of (A) water (0.1% formic acid) and (B) methanol (0.1% formic acid). The following gradient was applied: 0–0.25 min, 2% B; 12.25–15 min, 99% B; 15.1–17 min, 2% B.

Samples were analysed using above instruments in positive and negative electrospray ionization modes (separate runs) following the same HRMS parameters. A lock-spray containing leucine enkephalin was continuously infused during measurement. The source settings include capillary voltage of 0.7 kV in positive and -2 kV in negative ionisation modes, source temperature at 140 °C, and desolvation temperature at 550 °C. The sampling cone voltage and source offset were set as 20 V and 50 V, respectively. Nitrogen and argon were used as cone and collision gases, respectively. The desolvation gas flow was 950 L h^{-1}. The data was recorded in sensitivity mode (resolution approx. 20000) as centroid data with a 0.15 s scan time over the mass range *m/z* 50 to *m/z* 1200. The MS^E acquisition was performed to simultaneously collect two data sets: a low-collision-energy scan (4 eV) to obtain parent ion information and an elevated-collision-energy scan (15–35 eV) to get all fragment ions.

MarkerLynx and TargetLynx were used for data analysis. With MarkerLynx a peak picking was performed (time: 1 to 12 min, retention time difference 0.1 min, exact mass error of 0.01 Da) and a matrix with RT-m/z and intensities in the different samples were generated. These

matrixes were screened for peaks that only appear in the treated samples (non-target screening). Furthermore, a suspect screening was performed to screen for known tire related compounds based on previous studies of the UFZ by TargetLynx. In case standards have been available – the identity was checked by retention time fit (<0.01 min) exact mass fit of the molecular ion and fragment ions (<5ppm). The integration of the peak areas was done by TargetLynx. Areas are reported when they fulfil the criteria to be >3 times the S/N and the quantification was done based on external calibration in MeOH/MillQ water (1:1).

S3. Results

INGESTION OF CR PARTICLES

Table S3.1: Descriptive statistics over crumb rubber findings in the GI-tract of exposed lumpfish (n=90). Table shows median, interquartile range, 1st and 3rd quartile, minimum, maximum and variance over crumb rubber found in Lumpfish for sampling days (n= 9 per sampling day).

Day	Median	Interquartile range	1st quartile	3rd quartile	Minimum	Maximum	Variance
1	5	10	4	14	2	21	53,1
2	7	30	1	31	0	60	471
4	3	2	1	3	0	5	3,52
7	18	48	9	57	0	59	691
8	28	7	22	29	3	57	244
9	16	35	4	39	0	84	1072
11	9	13	4	17	0	34	125
14	0	6	0	6	0	23	62,5
18	0	1	0	1	0	56	342
21	3	8	0	8	0	33	112

Table S3.2: Descriptive data over number of CR retrieved from exposed (n=90) lumpfish over tanks (2, 4 and 6) and days (1, 2, 4, 7, 8, 9, 11, 14, 18, 21).

Tank	Day	Median number of CR	IQR	1st quartile	3rd quartile	Minimum	Maximum	Variance
2	1	5	7,5	5	12,5	5	20	75
2	2	7	19,5	4,5	24	2	41	450
2	4	0	1,5	0	1,5	0	3	3
2	7	17	9	8,5	17,5	0	18	102
2	8	28	3,5	25	28,5	22	29	14,3
2	9	0	2	0	2	0	4	5,33
2	11	0	8,5	0	8,5	0	17	96,3
2	14	0	5,5	0	5,5	0	11	40,3
2	18	1	28	0,5	28,5	0	56	1027
2	21	3	4	1,5	5,5	0	8	16,3
4	1	14	8,5	9	17,5	4	21	73
4	2	1	0,5	0,5	1	0	1	0,33
4	4	2	1	1,5	2,5	1	3	1
4	7	9	28	4,5	32,5	0	56	904
4	8	29	17,5	25,5	43	22	57	343
4	9	81	34	48,5	82,5	16	84	1476
4	11	21	15	12,5	27,5	4	34	226
4	14	1	3	0,5	3,5	0	6	10
4	18	1	1,5	0,5	2	0	3	2,33
4	21	10	15	6,5	21,5	3	33	246

6	1	4	2,5	3	5,5	2	7	6,33
6	2	31	24	21,5	45,5	12	60	584
6	4	5	1	4	5	3	5	1,33
6	7	57	1	57	58	57	59	1,33
6	8	8	16	5,5	21,5	3	35	296
6	9	34	13	23,5	36,5	13	39	190
6	11	9	2,5	7	9,5	5	10	7
6	14	0	11,5	0	11,5	0	23	176
6	18	0	0	0	0	0	0	0
6	21	0	0,5	0	0,5	0	1	0,33

BLOOD CHEMICALS

Table S3.3: Mann-Whitney U test of differences between treatment groups of control and exposed in concentration for $C_{21}H_{26}N_2$; $C_{20}H_{20}N_2$; $C_{23}H_{26}N_2$; 6PPD; and $C_{19}H_{18}N_2$ and difference in response for $C_{12}H_{15}N$. Table show the test statistics, p-values and 95 % confidence interval for respective chemicals. $\alpha = 0.05$, * indicates p-value < α.

Chemical	$C_{21}H_{26}N_2$	$C_{20}H_{20}N_2$	$C_{23}H_{26}N_2$	6PPD	$C_{19}H_{18}N_2$	$C_{12}H_{15}N$
W	76.5	56	80	54.5	88	10
P-value	**0.020***	**0.001***	**0.015***	**0.007***	**0.032***	**< 0.0001***
Confidence interval (95 %)	-6.03e+0 3.748e-06	-42.7 - 0	-13.9 - 0	-1.50e+02 - 4.14e-05	-9.42e-05 0.0	-355719 - 80567

Table S3.4.1: Descriptive data of median, interquartile range, first and third quartile, minimum and maximum values and variance for $C_{20}H_{20}N_2$ (pg/g) in analyzed blood samples (n=36) from control lumpfish (n=18) and exposed Lumpfish (n=18).

Treatment	Day	median.C20H20N2	IQR.C20H20N2	First.Q.C20H20N2	ThirdQ.C20H20N2	min.C20H20N2	max.C20H20N2	var.C20H20N2
control	7	0	0	0	0	0	0	0
control	21	0	0	0	0	0	0	0
exposed	7	384	665	59,5	724	0	789	134410
exposed	21	0	66	0	66	0	85,47	1370

Table S3.4.2: Descriptive data of median, interquartile range, first and third quartile, minimum and maximum values and variance for 6PPD (pg/g) in analyzed blood samples (n=36) from control lumpfish (n=18) and exposed Lumpfish (n=18).

Treatment	Day	median.6PPD	IQR.6PPD	First.Q.6PPD	ThirdQ.6PPD	min.6PPD	max.6PPD	var.6PPD
control	7	20	10	20	30	0	60	362
control	21	20	20	20	40	20	120	1878
exposed	7	330	320	155	475	20	700	64427
exposed	21	40	80	20	100	20	680	47800

Table S3.4.3: Descriptive data of median, interquartile range, first and third quartile, minimum and maximum values and variance for $C_{21}H_{26}N_2$ (pg/g) in analyzed blood samples (n=36) from control lumpfish (n=18) and exposed Lumpfish (n=18).

Treatment	Day	median.C21H26N2	IQR.C21H26N2	First.Q.C21H26N2	ThirdQ.C21H26N2	min.C21H26N2	max.C21H26N2	var.C21H26N2
control	7	0	0	0	0	0	0	0
control	21	0	0	0	0	0	64	455
exposed	7	139	95,2	107	202	0	603	44117
exposed	21	0	0	0	0	0	121	1615

Table S3.4.4: Descriptive data of median, interquartile range, first and third quartile, minimum and maximum values and variance for $C_{23}H_{26}N_2$ (pg/g) in analyzed blood samples (n=36) from control lumpfish (n=18) and exposed Lumpfish (n=18).

Treatment	Day	median.C23H26N2	IQR.C23H26N2	First.Q.C23H26N2	ThirdQ.C23H26N2	min.C23H26N2	max.C23H26N2	var.C23H26N2
control	7	0	0	0	0	0	0	0
control	21	0	0	0	0	0	0	0
exposed	7	111	118	45,1	163	0	428	23723
exposed	21	0	0	0	0	0	0	0

Table S3.4.5: Descriptive data of median, interquartile range, first and third quartile, minimum and maximum values and variance for the response of $C_{19}H_{18}N_2$ (peak area) in analyzed blood samples (n=36) from control lumpfish (n=18) and exposed Lumpfish (n=18).

Treatment	Day	median.C19H18N	IQR.C19H18N	First.Q.C19H18N	ThirdQ.C19H18N	min.C19H18N	max.C19H18N	var.C19H18N
control	7	0	0	0	0	0	0	0
control	21	0	0	0	0	0	0	0
exposed	7	43,3	87,4	8,36	95,7	0	115	2624
exposed	21	0	0	0	0	0	0	0

Table S3.4.6: Descriptive data of median, interquartile range, first and third quartile, minimum and maximum values and variance for the response of $C_{12}H_{15}N$ (peak area) in analyzed blood samples (n=36) from control lumpfish (n=18) and exposed Lumpfish (n=18).

Treatment	Day	median.C12H15N	IQR.C12H15N	First.Q.C12H15N	ThirdQ.C12H15N	min.C12H15N	max.C12H15N	var.C12H15N
control	7	6153	16321	2177	18498	250	72375	5,36E+08
control	21	17362	24469	7258	31727	1210	52510	3,3E+08

exposed	7	399789	388989	228362	617351	25756	1791067	3,05E+11
exposed	21	92978	29443	82851	112294	31063	354674	1,18E+10

Table S3.5: Mann-Whitney U test for comparison of difference in chemical levels for M-DCH, DCH, DPG, TBT, $C_{16}H_{16}N_3O$ and $C_{13}H_{20}N_3$ in blood samples between treatment groups (control and exposed). $\alpha = 0.05$, * indicates p-value < α.

Compound	M-DCH	DCH	DPG	$C_{16}H_{16}N_3O$	TBT	$C_{13}H_{20}N_3$
W	36.5	18	29.5	80.5	52	48.5
P-value	**< 0.0001***	**< 0.000001***	**< 0.00001***	**0.0104***	**0.00053***	**< 0.0001***
95 % Confidence interval	-228 -12	-27700 -3760	-24892 -7088	-3837 -405	-1735 -362	-843 -19

Table S3.6.1: Descriptive data of median, interquartile range, first and third quartile, minimum and maximum values and variance for M-DCH in analyzed blood samples (n=36) from exposed lumpfish (n=18) and unexposed (n=18).

Treatment	Day	Median	IQR	1st quartile	3rd quartile	Minimum	Maximum	Variance
Control	7	36	4	32	36	32	40	9,78
Control	21	36	8	32	40	28	40	24
Exposed	7	308	692	80	772	0	1592	286460
Exposed	21	48	24	40	64	36	184	2186

Table S3.6.2: Descriptive data of median, interquartile range, first and third quartile, minimum and maximum values and variance for DCH in analyzed blood samples (n=36) from exposed lumpfish (n=18) and unexposed (n=18).

Treatment	Day	Median	IQR	1st quartile	3rd quartile	Minimum	Maximum	Variance
Control	7	0	0	0	0	0	0	0
Control	21	0	0	0	0	0	0	0
Exposed	7	29068	78668	3980	82648	0	154408	3,53E+09
Exposed	21	4616	3412	3712	7124	0	10352	13924264

Table S3.6.3: Descriptive data of median, interquartile range, first and third quartile, minimum and maximum values and variance for DPG in analyzed blood samples (n=36) from exposed lumpfish (n=18) and unexposed (n=18).

Treatment	Day	Median	IQR	1st quartile	3rd quartile	Minimum	Maximum	Variance
Control	7	0	0	0	0	0	128	1820,444
Control	21	0	0	0	0	0	0	0
Exposed	7	12436	23268	252	23520	0	48332	3,19E+08
Exposed	21	11276	19940	7088	27028	0	57456	3,42E+08

Table S3.6.4: Descriptive data of median, interquartile range, first and third quartile, minimum and maximum values and variance for $C_{16}H_{16}N_3O$ in analyzed blood samples (n=36) from exposed lumpfish (n=18) and unexposed (n=18).

Treatment	Day	Median	IQR	1st quartile	3rd quartile	Minimum	Maximum	Variance
Control	7	1246	302	1089	1391	0	2248	398398
Control	21	1234	978	921	1899	366	2206	401408
Exposed	7	3950	6162	756	6918	0	9080	10670114
Exposed	21	2537	2338	1753	4091	20	6008	4209212

Table S3.6.5: Descriptive data of median, interquartile range, first and third quartile, minimum and maximum values and variance for TBT in analyzed blood samples (n=36) from exposed lumpfish (n=18) and unexposed (n=18).

Treatment	Day	Median	IQR	1st quartile	3rd quartile	Minimum	Maximum	Variance
Control	7	992	204	886	1090	305	1126	64880
Control	21	1063	293	791	1084	304	1133	71001
Exposed	7	2166	3172	1223	4395	0	11272	12210352
Exposed	21	1646	885	1339	2224	917	3196	599108

Table S3.6.6: Descriptive data of median, interquartile range, first and third quartile, minimum and maximum values and variance for $C_{13}H_{20}N_3$ in analyzed blood samples (n=36) from exposed lumpfish (n=18) and unexposed (n=18).

Treatment	Day	Median	IQR	1st quartile	3rd quartile	Minimum	Maximum	Variance
Control	7	0	0	0	0	0	0	0
Control	21	0	0	0	0	0	35	136
Exposed	7	286	770	0	770	0	2225	640949
Exposed	21	790	599	529	1128	0	3243	978856

LIVER CHEMICALS

Table S3.7: Mann-Whitney U test for comparison of difference in chemical levels for M-DCH, DCH, DPG, TBT, $C_{16}H_{16}N_3O$ and $C_{13}H_{20}N_3$ in liver samples between treatment groups (control and exposed). $\alpha = 0.05$, * indicates p-value < α.

Compound	M-DCH	DCH	DPG	$C_{16}H_{16}N_3O$	TBT	$C_{13}H_{20}N_3$
W	0	0	20	36	36	27
P-value	**<0.000001***	**<0.000001***	**<0.000001***	**<0.00001***	**<0.0001***	**<0.00001***
95 % Confidence interval	-184 -34	-1920 -553	-2402 -930	-1249.170 - 196.515	-148 -52	-234 -96

Table S3.8.1: Descriptive data for M-DCH (pg/g) in analyzed liver samples (n=36).

Treatment	Day	Median	IQR	1st quartile	3rd quartile	Minimum	Maximum	Variance
Control	7	6	0	6	6	0	7	4.53
Control	21	6	6	0	6	0	7	9.61
Exposed	7	140	124	98	222	9	342	11840
Exposed	21	44	62	32	94	8	361	12909

Table S3.8.2: Descriptive data for DCH (pg/g) in analyzed liver samples (n=36)

Treatment	Day	Median	IQR	1st quartile	3rd quartile	Minimum	Maximum	Variance
Control	7	15	4	14	18	13	21	7.19
Control	21	16	6	14	20	13	28	24.4
Exposed	7	1685	1790	1418	3208	74	6689	4865497
Exposed	21	800	1075	439	1514	58	2061	529273

Table S3.8.3: Descriptive data for DPG (pg/g) in analyzed liver samples (n=36).

Treatment	Day	Median	IQR	1st quartile	3rd quartile	Minimum	Maximum	Variance
Control	7	0	0	0	0	0	0	0
Control	21	0	0	0	0	0	22	53
Exposed	7	2145	1617	930	2547	0	3882	1935570
Exposed	21	1155	1247	518	1765	82	5930	3147165

Table S3.8.4: Descriptive data over the response (peak area) of $C_{16}H_{16}N_3O$ in analyzed liver samples (n=36).

Treatment	Day	Median	IQR	1st quartile	3rd quartile	Minimum	Maximum	Variance
Control	7	0	0	0	0	0	0	0
Control	21	0	0	0	0	0	0	0
Exposed	7	1249	1115	520	1635	0	2431	678108
Exposed	21	245	427	102	529			

Table S3.8.5: Descriptive data over the response (peak area) of TBT in analyzed liver samples (n=36).

Treatment	Day	Median	IQR	1st quartile	3rd quartile	Minimum	Maximum	Variance
Control	7	33	12	24	36	14	58	193
Control	21	29	23	19	42	11	47	187
Exposed	7	289	268	131	399	40	495	29177
Exposed	21	91	83	50	133	17	174	2863,5

Table S3.8.6: Descriptive data over the response (peak area) of $C_{13}H_{20}N_3$ in analyzed liver samples (n=36).

Treatment	Day	Median	IQR	1st quartile	3rd quartile	Minimum	Maximum	Variance
Control	7	0	0	0	0	0	0	0
Control	21	0	0	0	0	0	0	0
Exposed	7	150	205	85	290	0	439	23670
Exposed	21	203	124	110	234	0	507	27183

CORRELATION OF CR PARTICLES WITH CONCENTRATION

Table S3.9: R^2 and p-value for the F-statistics explaining the correlation between the number of CR particles counted in the GI-tract and the concentration of chemicals detected by GC/MS in the blood of exposed lumpfish. Compounds with a strong correlation are marked with bold R^2 -values.

Compound	$C_{21}H_{26}N_2$	$C_{20}H_{20}N_2$	$C_{23}H_{26}N_2$	6PPD	$C_{19}H_{18}N_2$	$C_{12}H_{15}N$
R^2	0.54	**0.75**	0.62	0.44	**0.73**	0.64
F	35.7	90.1	49.51	24.1	81.1	63.21
p-value	**< 0.00001**	**<0.000000001**	**<0.0000001**	**< 0.0001**	**<0.000000001**	**<0.0000001**

Table S3.10: R^2 and p-value for the F-statistics explaining the correlation between number of particles and concentration of chemicals detected by LC/MS in blood from exposed lumpfish. Compounds with a strong correlation is marked with bold R^2 -values.

Compound	M-DCH	DCH	DPG	$C_{16}H_{16}N_3O$	TBT	$C_{13}H_{20}N_3$
R^2	**0.70**	**0.77**	**0.77**	0.62	0.57	0.19
F	83.9	115	115	58.4	47.6	9.05
p-value	**<0.001**	**<0.001**	**<0.001**	**<0.001**	**<0.001**	0.005

Table S3.11: R^2 and p-value for the F-statistics of the correlation between the number of CR particles and the concentration of chemicals detected by LC/MS in liver tissue of exposed lumpfish. Compounds with a strong correlation are marked with bold R^2 -values.

Compound	M-DCH	DCH	DPG	$C_{16}H_{16}N_3O$	TBT	$C_{13}H_{20}N_3$
R^2	0.66	**0.81**	0.61	**0.86**	**0.87**	0.51
F	67.4	146	55.6	203	233	37.2
p-value	**<0.001**	**<0.001**	**<0.001**	**<0.001**	**<0.001**	**<0.001**

METALS IN LUMPFISH BLOOD AND LIVER

Table S3.12: Mann-Whitney U test (α = 0.05). The Mann-Whitney test statistic (W), the p-value and the 95 % confidence interval for concentrations of various metals in Lumpfish blood and liver by treatment (control and exposed). *p-value < α.

Metal	Chromium (Cr)	Manganese (Mn)	Iron (Fe)	Cobalt (Co)	Nickel (Ni)	Copper (Cu)	Zinc (Zn)	Cadmium (Cd)	Antimony (Sb)	Lead (Pb)
W Blood	16	24	20	15	11.5	14	11		21	24
p-value Blood	0.81	0.38	0.81	0.68	0.30	0.63	0.30		0.41	0.36
Confidence Interval (95%) Blood	-0.04 0.005	-0.05 0.19	-31.3 33.7	-0.002 0.001	-0.02 0.001	-0.16 0.29	-2.00 1.40		0 8.75e-05	-0.001 0.03
W Liver	25.5	20	17	14	26	13	24	16.5	13	15
p-value Liver	0.26	0.81	0.94	0.57	0.23	0.47	0.38	0.87	0.44	0.69
Confidence Interval (95%) Liver	-0.01 0.07	-0.17 0.18	-15.6 9.60	-0.002 0.002	-0.46 1.02	-2.81 1.37	-1.90 21.9	-0.005 0.005	-0.002 0.0004	-0.005 0.003

Table S3.13: Mann-Whitney U test (α = 0.05). The Mann-Whitney test statistic (W), the p-value and the 95 % confidence interval for concentrations of various metals in lumpfish blood and liver between time points (days) for both treatments (control and exposed). *p-value < α

Metal	Chromium (Cr)	Manganese (Mn)	Iron (Fe)	Cobalt (Co)	Nickel (Ni)	Copper (Cu)	Zinc (Zn)	Cadmium (Cd)	Antimony (Sb)	Lead (Pb)
W Blood	36	22	21	22	26.5	30	25		21	12
p-value Blood	**0.004 ***	0.58	0.69	0.56	0.17	0.06	0.30		0.41	0.36
Confidence Interval (95%) Blood	0.004 0.05	-0.05 0.19	-18.9 45.8	-0.002 0.002	- 0.00005 0.02	- 0.00005 0.4	-0.60 2.90		0 0.00009	-0.02 0.001

W Liver	28	27	16.5	28	26	16	10	9	18	11.5
p-value Liver	0.13	0.17	0.87	0.12	0.23	0.81	0.23	0.17	1	0.33

Table S3.14: Descriptive data over Lumpfish blood samples (n=12) analyzed for Chromium (Cr); Manganese (Mn); Iron (Fe); Cobalt (Co); Nickel (Ni); Copper (Cu); Zink (Zn); and Lead (Pb) at two timepoints (day 7 and 21).

Metal	Treatment	Day	Median	Interquartile range	1st Quartile	3d Quartile	Minimum	Maximum	Variance
Chromium (Cr)	Control	7	0,007	0,005	0,007	0,01	0,006	0,02	0.00003
	Control	21	0,002	0,002	0,002	0,004	0,002	0,005	0,000003
	Exposed	7	0,01	0,02	0,01	0,03	0,01	0,05	0,0006
	Exposed	21	0,002	0,001	0,002	0,003	0,002	0,004	1,33333E-06
Manganese (Mn)	Control	7	0,25	0,14	0,23	0,37	0,20	0,49	0,024
	Control	21	0,21	0,042	0,19	0,23	0,16	0,25	0,002
	Exposed	7	0,24	0,05	0,19	0,24	0,14	0,25	0,003
	Exposed	21	0,17	0,07	0,17	0,23	0,16	0,30	0,006
Iron (Fe)	Control	7	135	11,6	128	140	122	145	1334
	Control	21	141	15,1	136	151	131	161	233
	Exposed	7	144	32,5	136	168	127	192	1132
	Exposed	21	121	23,4	110	134	99,4	146	546
Cobalt (Co)	Control	7	0,004	0,0005	0,004	0,005	0,004	0,005	3,33333E-07
	Control	21	0,004	0,0015	0,0035	0,005	0,003	0,006	2,33333E-06
	Exposed	7	0,005	0,001	0,005	0,006	0,004	0,006	0,000001

	Exposed	21	0,004	0,0015	0,004	0,005	0,003	0,006	2,33333E-06
	Control	7	0,004	0,004	0,004	0,008	0,004	0,01	2,13333E-05
	Control	21	0,004	0,0005	0,004	0,005	0,004	0,005	3,33333E-07
Nickel (Ni)	Exposed	7	0,007	0,01	0,006	0,02	0,005	0,03	0,000177333
	Exposed	21	0,004	0,001	0,004	0,005	0,004	0,006	1,33333E-06
	Control	7	0,53	0,24	0,52	0,76	0,5	0,98	0,07
	Control	21	0,52	0,03	0,50	0,53	0,47	0,53	0,001
Copper (Cu)	Exposed	7	0,63	0,07	0,10	0,66	0,56	0,69	0,004
	Exposed	21	0,49	0,08	0,48	0,56	0,47	0,63	0,008
	Control	7	7,9	1,85	7,8	9,65	7,7	11,4	4,33
	Control	21	8	0,8	7,45	8,25	6,9	8,5	0,67
Zinc (Zn)	Exposed	7	9	0,75	8,75	9,5	8,5	10	0,58
	Exposed	21	8,5	1,15	7,8	8,95	7,1	9,4	1,34
	Control	7	0,001	0	0,001	0,001	0,001	0,001	0
	Control	21	0,001	0	0,001	0,001	0,001	0,001	0
Cadmium (Cd)	Exposed	7	0,001	0	0,001	0,001	0,001	0,001	0
	Exposed	21	0,001	0	0,001	0,001	0,001	0,001	0
	Control	7	0,0001	0,0001	0,0001	0,0002	0,0001	0,0003	1,33333E-08
Antimony (Sb)	Control	21	0,0001	0	0,0001	0,0001	0,0001	0,0001	0

	Exposed	7	0,0001	0	0,0001	0,0001	0,0001	0,0001	0
	Exposed	21	0,0001	0	0,0001	0,0001	0,0001	0,0001	0
	Control	7	0,002	0,002	0,0015	0,0035	0,001	0,005	4,33333E-06
	Control	21	0,02	0,0125	0,009	0,0215	0,003	0,028	0,000156333
Lead (Pb)	Exposed	7	0,003	0,0005	0,0025	0,003	0,002	0,003	3,33333E-07
	Exposed	21	0,002	0,0005	0,002	0,0025	0,002	0,003	3,33333E-07

Table S3.15: Descriptive data over Lumpfish liver samples (n=12) analyzed for Chromium (Cr); Manganese (Mn); Iron (Fe); Cobalt (Co); Nickel (Ni); Copper (Cu); Zink (Zn); and Lead (Pb) at two timepoints (day 7 and 21).

Metal	Treatment	Day	Median	Interquartile range	1st Quartile	3d Quartile	Minimum	Maximum	Variance
	Control	7	0,06	0,03	0,06	0,08	0,06	0,11	0,0008
	Control	21	0,02	0,005	0,02	0,02	0,01	0,02	0,00002
Chromium (Cr)	Exposed	7	0,03	0,01	0,02	0,03	0,006	0,03	0,0002
	Exposed	21	0,02	0,01	0,02	0,03	0,01	0,04	0,0002
	Control	7	1,1	0,11	1,01	1,12	0,92	1,14	0,01
	Control	21	1,02	0,15	0,87	1,03	0,75	1,04	0,03
Manganese (Mn)	Exposed	7	0,96	0,10	0,96	1,06	0,96	1,15	0,01
	Exposed	21	0,89	0,1	0,89	0,99	0,89	1,09	0,01
	Control	7	38,4	2,7	38,4	41,1	38,3	43,7	9,54

Iron (Fe)	Control	21	48,5	8,35	43,4	51,8	38,3	55	70,9
	Exposed	7	50,5	10,7	42,7	53,3	34,8	56,1	122
	Exposed	21	38,9	8,7	37,8	46,5	36,6	54	89,34
Cobalt (Co)	Control	7	0,007	0,002	0,006	0,008	0,005	0,009	0,000004
	Control	21	0,006	0	0,006	0,006	0,006	0,006	0
	Exposed	7	0,007	0,001	0,007	0,008	0,007	0,009	1,33E-06
	Exposed	21	0,006	0,002	0,005	0,007	0,004	0,008	0,000004
Nickel (Ni)	Control	7	1,24	0,70	0,82	1,53	0,40	1,81	0,50
	Control	21	0,97	0,17	0,81	0,98	0,65	0,98	0,04
	Exposed	7	1,11	0,78	0,60	1,37	0,07	1,63	0,64
	Exposed	21	0,36	0,13	0,29	0,42	0,23	0,48	0,02
Copper (Cu)	Control	7	7,36	1,49	6,74	8,23	6,12	9,09	2,23
	Control	21	8,4	1,955	7,34	9,29	6,27	10,2	3,83
	Exposed	7	8,65	0,37	8,37	8,73	8,08	8,81	0,15
	Exposed	21	8,64	2,87	7,95	10,8	7,25	13	8,97
Zinc (Zn)	Control	7	21,1	1,6	20,5	22,1	19,9	23,1	2,61
	Control	21	27,5	11	26,4	37,4	25,3	47,3	147
	Exposed	7	21,3	2,25	21,1	23,4	20,9	25,4	6,20
	Exposed	21	21,8	3,1	19,7	22,8	17,6	23,8	10
	Control	7	0,01	0,002	0,01	0,01	0,01	0,02	4,33E-06

Cadmium (Cd)	Control	21	0,02	0,003	0,02	0,02	0,01	0,02	1,03E-05
	Exposed	7	0,02	0,002	0,02	0,02	0,01	0,02	4,33E-06
	Exposed	21	0,01	0,003	0,01	0,02	0,01	0,02	8,33E-06
Antimony (Sb)	Control	7	0,0001	0,0002	0,0001	0,0003	0,0001	0,0004	3E-08
	Control	21	0,0001	0,0004	0,0001	0,0005	0,0001	0,0009	2,13E-07
	Exposed	7	0,0002	0,001	0,0002	0,002	0,0001	0,003	2,34E-06
	Exposed	21	0,0002	0,0002	0,0002	0,0004	0,0001	0,0005	4,33E-08
Lead (Pb)	Control	7	0,003	0,001	0,003	0,004	0,003	0,005	1,33E-06
	Control	21	0,006	0,0005	0,006	0,006	0,005	0,006	3,33E-07
	Exposed	7	0,005	0,004	0,004	0,008	0,002	0,01	1,63E-05
	Exposed	21	0,007	0,004	0,005	0,008	0,002	0,009	0,00001

SHRIMP DATA

INGESTION OF CR BASED ON SBR-VINYLCYCLOHEXENE DETECTION

Table S3.16: Descriptive data over pyrolysis of Shrimp stomach and intestines for detection of the tyre rubber marker SBR-vinylcyclohexene (µg/g) in treatments (control, low CR exposure and high Cr exposure) and time (day 7 and 21).

Treatment	Day	Median	Interquartile range	1st quartile	3rd quartile	Minimum	Maximum	variance
control	7	158	132	107	239	0	790	62439
control	21	245	181	117	297	52,8	862	103106
high	7	143	183	84,6	267	0	433	23575
high	21	166	383	102	485	0	1330	217142
low	7	283	96	234	330	178	598	16303
low	21	260	186	154	340	0	959	82878

Clean up of Shrimp hepatopancreas and muscle samples

Hepatopancreas and tail muscle samples were removed of biological molecules to enhance the analysis of the samples. Samples were added procedural internal standards (*d*6-Phenol 250 ug/mL, *d*8-pCresol 1.14 ug/mL, *d*12-4-n-Propylphenol 1.32 ug/mL, *d*8-Naphthalene 10 ng/mL, *d*10-Phenanthrene 50 ng/mL, *d*12-Chrysene 50 ng/mL, and *d*12-Perylene 50 ng/mL, all from Sigma Aldrich, Norway) prior to solid phase extraction (SPE). The SPE-cartridges (Waters Oasis HLB, 150 mg) were activated with methanol (6 mL) (Supelco HPLC grade, Merck Life Science AS, Norway) and conditioned with milliQ water (6 mL; Merck Millipore, 18 µΩ). Samples were loaded onto the column and rinsed with milliQ (2 x 4 mL), and dried under vacuum prior to elution with methanol (2 x 4 mL). The eluate was evaporated to dryness under nitrogen and reconstituted with dichloromethane (400 uL, Rathburn Chemicals Ltd, UK) and added a procedural recovery standard mix (100 uL, *d*10-Acenaphthene, 100 ng/mL, and *d*10-Fluorene, 100 ng/mL, both from Sigma Aldrich, Norway. Samples were stored in freezer until analysis.

METALS IN MUSCLE TISSUE

Table S3.18: Kruskal-Wallis test (α = 0.05). The Kruskal-Wallis chi-square test statistic and the p-value for concentrations of various metals in high CR exposed groups (n=6), low CR exposed groups (n=6) and control groups (n=6) of northern shrimp tail muscle tissue. *p-value < α

Metal	Chromium (Cr)	Manganese (Mn)	Iron (Fe)	Cobalt (Co)	Nickel (Ni)	Copper (Cu)	Zinc (Zn)	Cadmium (Cd)	Antimony (Sb)	Lead (Pb)
Kruskal-Wallis chi-square	3.78	2.05	5.27	3.28	12.6	0.22	0.56	2.39	0.57	0.56
p-value	0.15	0.36	0.072	0.19	**0.002***	0.90	0.75	0.30	0.75	0.76

Table 3.19: Descriptive data over northern shrimp tail muscle samples (n=18) from high CR exposed groups (n=6), low CR exposed groups (n=6) and control groups (n=6) analyzed for Chromium (Cr); Manganese (Mn); Iron (Fe); Cobalt (Co); Nickel (Ni); Copper (Cu); Zink (Zn); and Lead (Pb) at two timepoints (day 7 and 21)

Metal	Treatment	Day	Median	IQR	1st Quartile	3d Quartile	Minimum	Maximum	Variance
Chromium (Cr)	Control	8	0,007	0,001	0,007	0,008	0,006	0,008	0,000001
	Control	22	0,02	0,002	0,012	0,01	0,01	0,01	0,000003
	Low	8	0,005	0,002	0,005	0,007	0,005	0,008	0,000003
	Low	22	0,007	0,002	0,007	0,009	0,006	0,01	4,33E-06
	High	8	0,006	0,002	0,005	0,007	0,004	0,007	2,33E-06
	High	22	0,005	0,06	0,005	0,07	0,005	0,13	0,005043
	Control	8	0,40	0,10	0,36	0,47	0,32	0,53	0,01

Manganese (Mn)	Control	22	0,30	0,07	0,26	0,33	0,22	0,35	0,005
	Low	8	0,29	0,07	0,24	0,31	0,20	0,34	0,005
	Low	22	0,31	0,06	0,28	0,33	0,24	0,35	0,003
	High	8	0,27	0,07	0,27	0,34	0,27	0,40	0,006
	High	22	0,28	0,01	0,27	0,28	0,26	0,28	0,0002
Iron (Fe)	Control	8	1,4	0,05	1,35	1,4	1,3	1,4	0,003
	Control	22	1,2	0,25	1,1	1,35	1	1,5	0,06
	Low	8	1	0,35	0,95	1,3	0,9	1,6	0,1
	Low	22	0,9	0,1	0,9	1	0,9	1,1	0,01
	High	8	1,4	0,45	1,25	1,7	1,1	2	0,21
	High	22	1,4	0,55	1,25	1,8	1,1	2,2	0,32
Cobalt (Co)	Control	8	0,004	0,0005	0,004	0,004	0,003	0,004	3,33E-07
	Control	22	0,003	0,003	0,003	0,006	0,003	0,008	8,33E-06
	Low	8	0,003	0,0005	0,003	0,004	0,003	0,004	3,33E-07
	Low	22	0,003	0	0,003	0,003	0,003	0,003	0
	High	8	0,003	0,0005	0,003	0,004	0,003	0,004	3,33E-07
	High	22	0,005	0,001	0,005	0,006	0,004	0,006	0,000001
Nickel (Ni)	Control	8	1,15	0,22	1,15	1,37	1,14	1,58	0,06
	Control	22	1,13	0,24	0,98	1,23	0,84	1,32	0,06
	Low	8	0,46	0,19	0,34	0,52	0,21	0,58	0,04

	Low	22	0,47	0,46	0,31	0,77	0,15	1,06	0,21
	High	8	0,03	0,07	0,03	0,09	0,02	0,15	0,005
	High	22	0,27	0,07	0,25	0,32	0,24	0,37	0,005
	Control	8	5,14	0,54	4,91	5,44	4,67	5,74	0,29
	Control	22	6,2	0,58	5,8	6,38	5,4	6,56	0,35
Copper (Cu)	Low	8	4,93	0,82	4,44	5,26	3,95	5,59	0,68
	Low	22	5,8	0,65	5,55	6,2	5,3	6,6	0,43
	High	8	6,17	1,12	5,8	6,92	5,43	7,67	1,30
	High	22	4,9	0,31	4,65	4,96	4,39	5,01	0,11
	Control	8	13,3	1,6	13	14,6	12,6	15,8	2,83
	Control	22	12,5	0,3	12,3	12,6	12	12,6	0,10
Zinc (Zn)	Low	8	12,4	1,35	11,2	12,5	9,9	12,6	2,26
	Low	22	13,4	0,9	12,6	13,5	11,7	13,5	1,02
	High	8	12,8	0,9	12,5	13,4	12,1	13,9	0,82
	High	22	11,9	1	11,5	12,5	11,1	13,1	1,01
	Control	8	0,01	0,003	0,01	0,02	0,01	0,02	9,33E-06
	Control	22	0,01	0,04	0,01	0,05	0,008	0,08	0,002
	Low	8	0,01	0,003	0,01	0,02	0,01	0,02	1,03E-05
Cadmium (Cd)	Low	22	0,009	0,003	0,008	0,01	0,007	0,01	9,33E-06
	High	8	0,009	0,003	0,007	0,01	0,005	0,01	0,000007
	High	22	0,01	0,01	0,009	0,02	0,006	0,03	0,0002

Antimony (Sb)	Control	8	0,01	0,002	0,01	0,02	0,01	0,02	5,76E-06
	Control	22	0,01	0,003	0,01	0,01	0,008	0,02	1,03E-05
	Low	8	0,02	0,002	0,01	0,02	0,01	0,01	4,92E-06
	Low	22	0,01	0,004	0,01	0,01	0,008	0,02	1,59E-05
	High	8	0,01	0,005	0,01	0,02	0,008	0,02	2,42E-05
	High	22	0,01	0,0008	0,01	0,01	0,01	0,01	7,03E-07
Lead (Pb)	Control	8	0,01	0,004	0,009	0,01	0,008	0,02	0,00001
	Control	22	0,01	0,008	0,01	0,02	0,008	0,02	0,00006
	Low	8	0,01	0,001	0,01	0,01	0,009	0,01	0,000001
	Low	22	0,009	0,007	0,008	0,02	0,007	0,02	0,00005
	High	8	0,01	0,003	0,01	0,02	0,01	0,02	9,33E-06
	High	22	0,009	0,002	0,009	0,01	0,008	0,01	4,33E-06

Table S.3.20: Chemical characterization of fine CR extracts. The chemicals in table were analyzed for in the blood samples from lumpfish, plotted and analyzed statistically.

Chemical name or formula
Bisnorabietatriene
Fluoranthene
Pyrene
OctadecanoicAcid
Naphthylaniline
C21H26N2
C15H30N6O6
C24H36N2
CPPD
C19H24N2O
DPPD
C19H18N2
C23H26N2
C27H34N2
C20H20N2
6PPD-Quinone-D5 INTERNAL STSANDARD
6-PPD-Quinone
17144-C14H23
17823-C14H23
6PPD
Benzothiazole

Dicyclohexylamine

C12H15N

Phthalimide

0894

Benzothiazolone

C18H20

10880

HexadecanoicAcid

C13H9NS

Fluoranthene

C15H30N6O6

13856-Alkyl

C24H36N2

C23H27N2

14254-Alkyl

C19H24N2O

14490-C24H30N2

14530-C24H30N2

C15H39N6O6

C24H30N2

14670-Alkyl

14819-Alkyl

C19H18N2

C23H26N2

C27H34N2

C20H20N2

15134-Alkyl

15656-Alkyl

16100-C27H34N2

16102-C27H34N2

16264-Alkyl

16978-Alkyl

17144-C14H23

17823-C14H23

18756-C14H23

18848-Alkyl

NTS-26840-C12H16NX