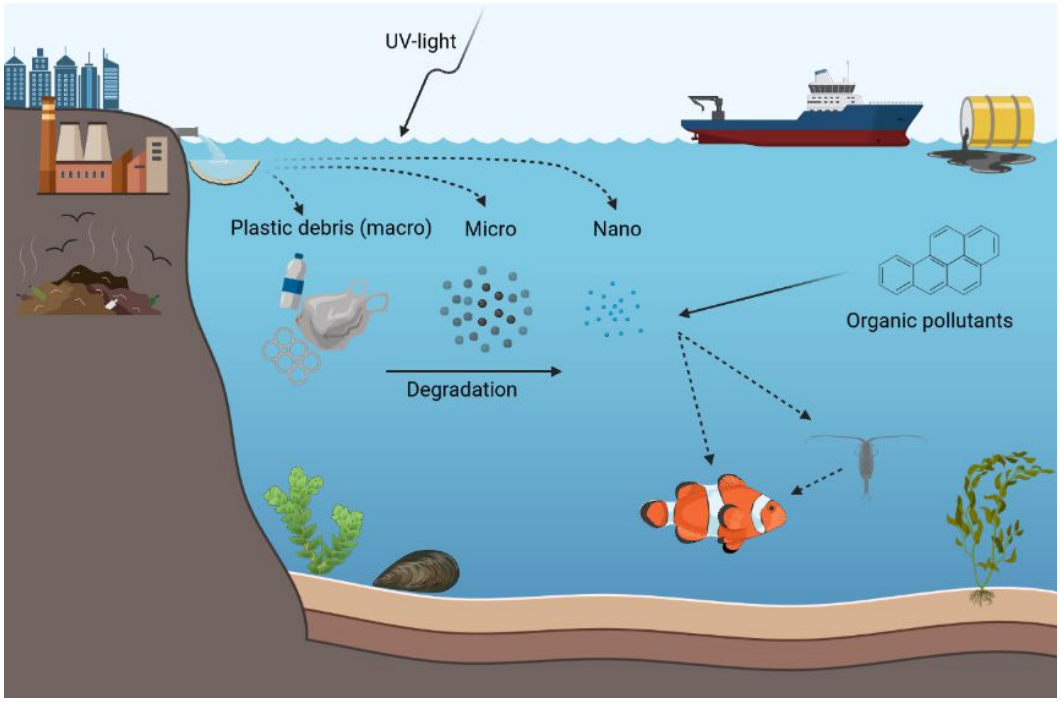
UNIVERSITY OF GOTHENBURG

DEPARTMENT OF BIOLOGICAL AND ENVIRONMENTAL SCIENCES

SINGLE AND COMBINED EXPOSURE OF POLYSTYRENE NANOPARTICLES AND NORTH SEA MARINE OIL IN TWO *IN VITRO* FISH MODELS

Assessing acute toxicity and sensitive endpoints of toxicity



Monica Olsson

Degree project for Master of Science (120 hec) with a major in molecular biology
BIO727, Physiology and cell biology, 60 hec
Second cycle
Semester/year: Autumn 2021-Spring 2022
Supervisors: Joachim Sturve, Department of biological and environmental sciences
Marianne Brookman-Amissah, Department of biological and environmental sciences
Examiner: Kristina Sundell, Department of biological and environmental sciences

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Abstract

The awareness of micro-and nanoplastic pollution and its impact on the marine ecosystem has increased over the last decade. Nanoplastics may affect aquatic organisms since their small size and high surface-to-volume ratio increases their bioavailability and ability to cross biological membranes. An enhanced understanding of interactions between nanoplastics and other environmental pollutants is needed for risk assessment and management. This study aims to examine possible toxic effects associated with single and combined exposures of polystyrene nanoparticles (PsNPs) and water-accommodated fractions (WAF) of North Sea marine oil, by measuring acute toxicity and exploring sensitive *in vitro* assays on two *in vitro* fish models. Rainbow trout gill- and liver cell lines were exposed to 25 nm PsNPs (1-100 μg/mL), WAF (10-90%) or a mixture of them for 24 h. AlamarBlue and Neutral Red assays revealed a dose-dependent decrease in cell metabolic activity and lysosomal stability after WAF exposure. 80%-90% WAF reduced cell membrane integrity significantly in the CFDA-AM viability assay. PsNPs alone did not significantly affect the cytotoxicity. However, higher concentration of PsNPs in the mixtures significantly decreased cell viability, indicating an additive effect. Generation of intracellular reactive oxygen species measured with DCFH-DA assay increased over a 6 h period for all treatments. No significant differences among single exposures of PsNPs or of different PsNP concentrations in the mixtures were found, even if a slightly protective effect can be discussed. A trend of increasing induction of CYP1A dependent EROD-activity was observed in cells exposed to 20-50% WAF, and to mixtures with higher PsNP concentration. Sublethal- and lethal effects in zebrafish embryos tended to increase with higher concentration of WAF alone, and with higher PsNP concentration in the mixtures. To conclude, PsNPs alone were generally not found to be toxic but may increase toxicity of organic pollutants found in crude oil.

Keywords: Polystyrene nanoparticles; crude oil; combined toxicity; cytotoxicity; oxidative stress

Sammanfattning

Medvetenheten om mikro-och nanoplastföroreningar och deras påverkan på det marina ekosystemet har ökat under det senaste decenniet. Nanoplast kan påverka vattenlevande organismer eftersom deras lilla storlek och höga yt-till-volym-förhållande ökar deras biotillgänglighet och förmåga att passera biologiska membran. En utökad förståelse av interaktioner mellan nanoplast och andra miljöföroreningar är nödvändig för vidare riskbedömning och riskhantering. Denna studie syftar till att undersöka möjliga toxiska effekter associerade med enkel och kombinerad exponering av polystyren-nanopartiklar (PsNPs) och vattenfraktioner (WAF) av Nordsjöolja, genom att mäta akut toxicitet och utforska känsligare *in vitro*-analysmetoder med hjälp av två *in vitro*-fiskmodeller. Gäl-och leverceller från regnbågsöring exponerades för antingen 25 nm PsNPs (1–100 μg/mL), WAF (10–90%) eller en blandning av dessa under 24 h. AlamarBlue- och NeutralRed-analyserna uppvisade en dosberoende minskning i metabol cellaktivitet och lysosomal stabilitet efter WAF-exponering. Även cellmembransintegriteten uppmätt med CFDA-AM-analys reducerades signifikant efter exponering för 80–90% WAF. Enkelexponering för PsNPs hade ingen toxisk effekt på cellerna. Däremot reducerade en högre koncentration av PsNPs i blandningarna cellviabiliteten signifikant, vilket indikerar en additiv effekt. Genereringen av intracellulära reaktiva syreföreningar uppmätta med proben DCFH-DA ökade över en 6 h period efter alla behandlingar. Ingen signifikant skillnad observerades mellan enkelexponeringarna av PsNPs, WAF eller av olika PsNP-koncentrationer i blandningarna, även om en lätt skyddande effekt kan diskuteras. En trend mot ökande induktion av CYP1A-beroende EROD-aktivitet observerades i celler exponerade för 20–50% WAF och för blandningar med högre PsNP-koncentration. Subletala-och letala effekter i zebrafiskembryos tenderade att öka med högre WAF-koncentration enskilt, och med högre PsNP-koncentration i blandningarna. Sammanfattningsvis, PsNPs var generellt inte toxiska vid enkelexponering även om de möjligtvis kan öka toxiciteten av organiska föroreningar från råolja.

Abbreviations

3R Replace, reduce, refine

AB AlamarBlue

AhR Aryl hydrocarbon receptor

CF 5-carboxyfluorescein

CFDA-AM 5-carboxyfluorescein diacetate acetoxymethyl ester

CNS Central nervous system

CYP1A Cytochrome P4501A

DCF 2', 7' –dichlorofluorescein

DCFH-DA 2',7' –dichlorofluorescin diacetate

DMSO Dimethyl sulfoxide

EDTA Ethylenediaminetetraacetic acid

ER 7-ethoxyresorufin

EROD Ethoxyresorufin-O-deethylase

FBS Fetal bovine serum

FET Fish embryo toxicity

HOC Hydrophobic organic compounds

Mp Microplastic

MSNM Manufactured silica nanomaterials

MWCNT Multi-Walled Carbon Nanotube

Np Nanoplastic

NR Neutral Red

PAH Polycyclic aromatic hydrocarbon

PCB Polychlorinated biphenyls

PHH Planar halogenated hydrocarbon

PsNP Polystyrene nanoparticle

ROS Reactive oxygen species

RTgill-W1 Rainbow trout gill cell line

RTL-W1 Rainbow trout liver cell line

t-BHP Tert-butyl hydroperoxide

WAF Water-accommodated fraction

ZFEM Zebrafish embryo medium

β-NF β-naphthoflavone

L-15 Lei-bovits-15

Introduction

Marine pollution

One of the widely documented sources of marine pollution are plastic debris, which is of emerging concern since it has the potential to challenge the function, diversity, and productivity of aquatic ecosystems (Peng et al., 2020). Globally distributed plastic debris is posing a growing threat to organisms like fish, mussels, and algae (Napper and Thompson, 2019; Fred-Ahmadu et al., 2020), for example through entanglement or ingestion (Li et al., 2016), and has become the focus of attention for many researchers.

Another known source to marine pollution is crude oil. The widespread crude oil exploitation, exploration and storage have led to a prevalent pollution of petroleum hydrocarbons in the environment. Crude oil consists of a complex mixture of several different types of compounds, such as heavy metals, polycyclic aromatic hydrocarbons (PAHs), hetero-polycyclic aromatic hydrocarbons, resins, and saturated hydrocarbons. Mortality, disease, and recruitment failure of aquatic organisms are associated with crude oil exposure (Leggieri et al., 2017).

Several studies suggest that plastic debris in the ocean can act as a vector for the transport or absorption of chemical pollutants (e.g., PAHs) from invertebrates to higher trophic levels (Li et al., 2016; Teuten et al., 2009). However, the knowledge about interactions between micro- and nano sized plastics and marine pollutants such as compounds from crude oil is limited, and the possible toxic effects of this needs to be studied further.

Plastic pollution

The promotion of new materials has been triggered by the technological developments during the last century. During the 1940s, the production of plastics started at an industrial scale (Al-Salem et al., 2010). The commercial and industrial usage of plastics increased due to their low cost, versatility, and favorable characteristics (lightweight, durable, strong, water and corrosion-resistant) (Wang et al., 2020), and later also due to both the new demand in emerging economies such as China and the growing demand in developed economies such as the USA, Canada, and EU (Schirinzi et al., 2017). This expansion has generated large quantities of plastic waste, of which 13 million tons is estimated to be released into rivers and oceans each year (Enfrin et al., 2019; Li et al., 2020). Increased consumption, irresponsible waste handling, combined with the limited biodegradable nature of plastic have resulted in an widespread and rapid accumulation in both aquatic and terrestrial ecosystems (Jambeck et al., 2015).

Land based sources of plastic debris such as industrialized or densely populated areas contributes to most of the plastics entering the marine environment, due to solid waste disposal, littering (Derraik, 2002), accidental spillage of raw manufacturing materials (Redford et al., 1997), refuse site leachate and wastewater effluent (Browne et al., 2010). Some examples are food wrappings, bottles, plastic straws, cosmetic products, cartons, and other macro items (Waring et al., 2018). Other contributors are ocean-based sources like commercial fishing (e.g., discarded fishing gear) (Li et al., 2016), oil- and gas platforms or shipping activities (Waring et al., 2018).

Plastics can be categorized based on which polymeric material they are composed of. Common types are polyvinyl chloride, polypropylene, polyamide, polyethylene, and polystyrene (Vianello et al., 2013; Digka et al., 2018). Physical- and chemical processes like ultraviolet light (photodegradation), mechanical action, heat (thermal degradation), ionizing radiation (radio degradation) or bacteria, algae, fungi, yeasts, and their enzymes (biodegradation) can cause degradation of plastic polymers (Yousif & Haddad, 2013). This generates so called secondary microplastics (Mps) and nanoplastics (Nps), in this article defined as plastic particles/fragments <5 mm and <100 nm respectively (Hartmann et al., 2019;

Karbalaei et al., 2018; Liu et al., 2016; Waring et al., 2018). Primary (manufactured) Mps/Nps are on the contrary produced to have a function in different materials and give products specific characteristics (Jeong et al., 2021). Mps and Nps small sizes enable spreading of them in the environment by water drainage, wind and ocean currents.

Nanoparticles

The small sizes of nanoparticles (1-100 nm) contribute to a high surface to volume ratio, which give nanomaterials unique physical and chemical properties that differ from their corresponding bulk material at macro-scale (Farré & Barceló, 2012; Nel et al., 2006). The development in nanotechnology during the last years has made it possible to produce nanomaterials in a controlled manner (Gustafsson, 2014). This has contributed to new materials and opportunities for both consumer- and industrial products, which are applicable in nanomedicine, personal care products (Marchesan & Prato, 2012), electronics (Sanchez et al., 2011), textiles, sport equipment (Contado, 2015), food packaging, food additives and household products (Eleftheriadou et al., 2017; Ranjan et al., 2014). Their capabilities like reactivity, optical sensitivity and conductivity cause a possible risk for detrimental interactions with the environment and biological systems, which potentially may induce toxicity (Nel et al., 2006). The behavior, environmental fate (Nickel et al., 2014) and ecotoxicological effects of nanoparticles have been evaluated in several studies (Fu et al., 2014; Yan et al., 2011) using both *in vitro* and *in vivo* approaches. The number of toxicity tests showing that nanoparticles can cause adverse effects are increasing. However, this field is still in an initial phase of development and the knowledge about the molecular mechanisms that explain possible effects is limited (Handy et al., 2012).

Nanoplastics

The field of plastic ecotoxicology has developed during the last years (González-Pleiter, 2019). Mps/Nps are produced for use in paints, cosmetics, drug delivery formulations, air blasting technologies, toothpastes, and cleaners (Cole et al., 2011; Sharma & Chatterjee, 2017). Other potential sources are automobile tires, clothing, fishing gears and cigarette filters (Toussaint et al., 2019). Major contributors to the release of Mps/Nps in the environment are manufacturing processes, agriculture or wastewater treatment plants which discard their effluents into aquatic systems or distribute their sludge on agricultural land (Waring et al., 2018). Nps can affect marine biota since their high surface-to-volume ratio make them bioavailable and their small size make it possible for them to cross biological barriers such as cell membranes (Jeong et al., 2021). In general, factors inducing cellular toxicity are small size, high dose, positive charge, and presence of pollutants or toxic additives in the Nps, by causing genotoxicity, membrane damage, oxidative stress, or immune responses (Banerjee & Shelver, 2021).

Ingestion, inhalation, skin contact, and injection (blood circulation) are possible exposure routes for nanomaterials (Banerjee & Shelver, 2021; Oberdörster et al., 2005). Micro/nanoparticles (and presumably, Nps) have in several studies shown to be able to reach systemic circulation by crossing the intestinal barrier (Banerjee & Shelver, 2021). The uptake of Mps/Nps through intestinal cells is mostly associated with endocytotic mechanisms (Fröhlich & Roblegg, 2012). The transport across membranes *in vivo* may be affected by a corona or shell of biological constituents since nanoparticles can interact with carbohydrates, lipids, or proteins (Waring et al., 2018). Once nanoparticles are in the systemic circulation the distribution is widespread and includes heart, brain, liver, kidney, and placenta (Waring et al., 2018). Nanoparticles can cross the blood/brain barrier and it is hypothesized that they may affect the central nervous system (CNS). This is supported by studies that report behavioral changes in fish and shrimps induced by polystyrene nanoparticles (PsNPs) delivered through the food chain, which potentially can affect ecosystem functioning (Cedervall et al., 2012; Mattsson et al., 2017). Cedervall et al. (2012) also observed that PsNPs had “devastating effects on the lipid

metabolism of top-consumer", in their case fish.

The polymer type may influence the toxicity of plastics. Based on hazard classification of monomers, the most hazardous out of 55 different polymers investigated were styrene-based, polyvinyl chloride, polyurethane, epoxy resins and polyacrylonitriles (Lithner et al., 2011). Additionally, an increasing number of studies shows that secondary Nps are generated under laboratory conditions, which implies that there may be substantially amounts of them in the environment (Jeong et al., 2021). However, the ecological impact of Nps in the environment and the distribution of them is still insufficiently understood (González-Pleiter et al., 2019) since the detection and quantification methods are limited (Jeong et al., 2021).

Sorption of organic pollutants (e.g., PAHs) to micro-and nanoplastics and combined toxicity

The process in which chemical species from a fluid phase adhere to the surface or in the bulk of a solid phase, plastic particles for example, is called *sorption* (Menéndez-Pedriza and Jaumot, 2020; Yu et al., 2021). Both adsorption and absorption are included in this term (Fred-Ahmadu et al., 2020). Adsorption refers to when "chemical contaminants stay on the interface between the fluid and the solid phases" (Yu et al., 2021), in contrast to absorption where "molecules penetrate and are retained within the matrix of the sorbent" (Yu et al., 2021). Mechanisms involved in the adsorption on solid surfaces are for example van der Waals forces, steric or covalent bonds and ion interactions (Borkovec and Papastavrou, 2008; Yu et al., 2021). The interactions between Mps or Nps and organic pollutants are mostly based on adsorption (Yu et al., 2021).

Mps and Nps are efficient sorbents for hydrophobic organic chemicals like polycyclic aromatic hydrocarbons (PAHs) and polychlorinated biphenyls (PCBs), due to their high surface area, amorphous structure, porosity (Wang et al., 2019; Ziccardi et al., 2016) and the hydrophobic features of the polymer, which consist of >92% carbon (Liu et al., 2016). The concentration of PCBs held by microplastics can be one million-fold higher than in the surrounding water (Betts, 2008). Sorptive carbon nanomaterials may therefor play a significant role in transport, partition, and toxicity of other contaminants (Karickhoff et al., 1979; Lohmann et al., 2005; Zhou and Rowland, 1997). The sorption of hydrophobic organic compounds (HOCs) to solid phases largely determines their fate and effects in the environment. Some examples on processes that depend on the sorption of HOCs in the situation of interest are biodegradation by microorganisms, accumulation by benthic animals and HOC release from contaminated sediment beds to lake waters and coastal seawaters (Lohmann et al., 2005; Schwarzenbach et al., 2003).

Polycyclic aromatic hydrocarbons (PAHs) are molecules with in between two and seven fused aromatic rings, on which aromatic- and alkyl groups might be added. Incomplete burning of petroleum, coal and wood contributes to their production (Newman, 2015). They are widely spread and found in the environment in high quantities both in soil, water, and the air (Bussolaro et al., 2019; Newman, 2015; Sogbanmu et al., 2016). PAHs that come from oil spills and other petrochemical sources are named petrogenic PAHs. They are slowly created during petrogenesis in low temperatures and are often alkylated. One example is 2,6-dimethylnaphthlene that has an added alkane group (Newman, 2015). PAHs have the potency to be mutagenic and carcinogenic. The direct exposure of PAHs to organisms may be increased by Nps that carry hydrophobic organic chemicals since they might be absorbed by organisms after ingestion and/or penetrate their membranes. The PAHs may have higher sorption coefficients compared to nonplanar PCBs with the same hydrophobicity, since PAHs are planar molecules (Liu et al., 2016).

The knowledge about combined ecotoxicity of carbon-based nanomaterials and co-contaminants is today inadequate (Schirinzi et al., 2017). Since the carbon nanomaterials or

nano-sized plastics can interact with the co-occurring contaminants in a complex way, this can change their toxicity in an antagonistic or synergistic way, by changing the bioavailability (Sanchís et al., 2016). Earlier studies looking at the role of carbon nanomaterials as vectors for other contaminants are for example Sanchís et al. (2016) that exposed *Daphnia magna* to binary mixtures of fullerene soot and malathion. Malathion was unexpectedly released inside living organisms and nanomaterials aggregates acted as a vector. The toxicity increased proportional to the size of the carbon nanomaterials aggregates. Baun et al. (2008) studied how C60 fullerene aggregates influenced aquatic toxicity of other contaminants. The toxicity was determined by the type of contaminants, where the use of pentachlorophenol and phenanthrene changed the toxicity but not the use of methyl parathion and atrazine as contaminants. In another study the presence of Multi-Walled Carbon Nanotube (MWCNT) increased the toxicity of diuron to *Chlorella vulgaris* (Schwab, 2013). In a third, MWCNT enhanced the bioavailability of phenanthrene to *Oryzias latipes* (Su, 2013), while MWCNT and Single-Walled Carbon Nanotube decreased the bioaccumulation of pyrene in earthworms (Petersen, 2009). However, more studies need to be done on the sorption of hydrophobic organic chemicals to Nps and how this can affect aquatic organisms. During assessment of the hazard of both nanoparticles and of hydrophobic organic chemicals, like PAHs, there is a need to think about the effects of a combination of these two.

Nanotoxicity and reactive oxygen species

One of the important mechanisms of nanotoxicity is the cytotoxicity and apoptosis that research suggests is caused by generation of reactive oxygen species (ROS) and damage to DNA (Thit et al., 2017). An increased generation of ROS can cause serious damage to the cell membrane since oxidation of membrane lipids can lead to lipid peroxidation where peroxides are formed through a radical chain reaction. Additionally, oxidation of proteins can inactivate receptor functions and enzyme activity (Halliwell, 1987). Earlier studies have reported that generation of ROS have an essential role in both growth and proliferation of cancer cells (Manda et al., 2015; Poillet-Perez et al., 2015; Tang et al., 2011). Polypropylene has been shown to cause oxidative stress in human neuronal cell lines and Waring et al. (2018) proposed that high concentration of plastic contamination in the CNS might lead to inflammatory responses, plaque deposition or depression, which has been linked to nanoparticle toxicity in the CNS. Although they conclude that serious toxicity from plastic contamination of the food chain is unlikely as long low levels of contamination are found in human tissues. However, it is not impossible under special circumstances such as a permeable blood-brain barrier, a leaky gut or long-term ingestion of contaminated food (Waring et al., 2018).

CYP1A gene activation

Several fish biomarkers have become well-established to evaluate the health of aquatic ecosystems. These biomarkers are useful since a reliable indication of past pollution events generally not are provided by direct measurements of environmental hydrocarbon concentrations. One biomarker used for predicting environmental impacts of persistent organic pollutants on different aquatic model organisms is 7-ethoxyresorufin O-deethylase (EROD), which is catalyzed by Cytochrome P4501A (CYP1A), a member of the cytochrome P450 enzymes (Agus et al., 2015; Fisher et al., 2006; Mortensen et al., 2006). CYP1A levels in tissues like gills, liver and kidney correlates with EROD activities (Ortiz-Delgado et al., 2008). An increase in CYP1A activity is used as a detector for petroleum pollution (Bucheli & Fent, 1995; Oris & Roberts, 2007; Whitehead et al., 2012) and as a well-established biomarker for PAHs (Goksøyr & Förlin, 1992; Stegeman & Hahn, 1994). Components of crude oil, like PAHs (aryl hydrocarbon receptor (AhR) agonist) (Clark et al., 2010), upregulates expression of CYP1A gene which normally are low (Uno et al., 2012). This results in increased transcription of mRNA that are translated into active proteins with the function to up-regulate the detoxification

pathway in different organs (Levine & Oris, 1999).

Induction of liver CYP1A caused by compound mixtures from crude oil has been shown in many fish species such as Atlantic salmon *Salmo salar* (Gagnon & Holdway, 1999), rainbow trout *Oncorhynchus mykiss* (Ramachandran et al., 2004) and others (Danion et al., 2014; Frantzen et al., 2015; Jung et al., 2009). Since Rainbow trout is introduced in various water bodies around the world and is sensitive to chemicals, it is a useful model for toxicological studies (Bailey et al., 1996; Buhler & Wang-Buhler, 1998). Toxic oil constituents are mainly metabolized in the liver and standardized laboratory bioassays of liver CYP1A induction is routinely used to estimate PAH exposure (Hodson et al., 1996). Studies have recently shown that EROD activity in the gills, which are the primary routes of PAH uptake (Levine & Oris, 1999), can provide a good indication of petroleum hydrocarbons exposure in marine fishes, like polar cod *Boreoga dussaida* (Nahrgang et al., 2010) and Atlantic cod *Gadus morhua* (Abrahamson et al., 2008; Holth et al., 2014).This may indicate that part of the adsorbed PAHs are metabolized by gills CYP1A before it reaches liver and kidney (Jönsson et al., 2006).

In vitro assays

In vitro assays play a key role in ecotoxicological effects and risk evaluations of pollutants. *In vitro* assays with cell lines allow to study the effects of a chemical inside a cell or on cell surface, where the interaction initially takes place. Responses to a stressor at a cellular level can be seen as an "early warning" since they occur before effects are seen at an organism level. These responses can provide knowledge about the mechanisms of toxic action of the stressor or "indicate disturbance of homeostasis and the physiological status of the organisms" (Thit et al., 2017). The fish embryo toxicity (FET) test, also considered as *in vitro* <120 h after fertilization, is a promising alternative to classical acute toxicity test with live fish (Lammer et al., 2009). Both *in vitro* models are valuable 3Rs tools which focus on the "replacement, refinement and reduction of animals used for *in vivo* experimentation" (Bussolaro et al., 2019).

Measurements of cytotoxicity

AlamarBlue is a well establish assay in environmental and biological studies based on the ability of viable and metabolically active cells to reduce and convert the active compound resazurin, which is a cell permeable, virtually non-fluorescent and blue molecule compound, to the fluorescent, pink product resorufin (Bols et al., 2005). Since this reduction occur continuously in metabolically active cells, viability (and cytotoxicity) can be measured quantitatively based on the fluorescence intensity that are emitted.

The plasma membrane integrity in fish cell line cultures has been assayed in different ways, where the esterase substrate 5-carboxyfluorescein diacetate acetoxymethyl ester (CFDA-AM) is one that for long has been used most often (Bols et al., 2005; Schirmer et al., 1997; Shirmer et al., 1998; Shirmer et al., 2000). Non-specific esterases of living cells convert the non-fluorescent and non-polar CFDA-AM dye that has diffused into the cells to a fluorescent, polar 5-carboxyfluorescein (CF) dye, which slowly diffuses out of the cells (Bols et al., 2005).

The neutral red assay is used to measure lysosomal activity and relies on the ability of viable cells to accumulate the neutral red dye, which specifically occurs in the lysosomes (Hammond et al., 1980).

Measurements of oxidative stress (ROS generation)

2',7' –dichlorofluorescin diacetate (DCFH-DA) is a cell permeant reagent used to quantitatively assess peroxyl, hydroxyl and other reactive oxygen species (ROS) activity in live cell samples. The DCFH-DA assay protocol is based on the ability of ROS containing cells to convert DCFH-DA into the fluorescent product 2', 7' –dichlorofluorescein (DCF). The DCFH-DA diffuses into the cell where cellular esterases deacetylate it to a non-fluorescent compound, which in turns is oxidized into DCF by ROS, which can be detected by fluorescence spectroscopy.

Aim and objectives of the study

The knowledge about ecotoxicity of co-exposure of Nps and co-contaminants (pollutants) found in the aquatic environment is limited. Therefore, this study aims to investigate possible toxic effects associated with single and combined exposure of PsNPs and water-accommodated fractions (WAF) of North Sea marine oil (containing organic pollutants, for example polyaromatic hydrocarbons (PAHs)).

To achieve this aim, acute toxicity is measured with standardized *in vitro* methods and sensitive endpoints of toxicity is explored and optimized in two *in vitro* fish models. Firstly, toxicity evaluations of single exposures are conducted in rainbow trout (*Oncorhynchus mykiss*) cell lines and in zebrafish *(Danio rerio)* embryos, to attain knowledge about the risks associated with the single exposures alone. Secondly, toxicity studies with the mixtures are carried out (with the same *in vitro* models) and a comparative analysis is done to establish a relationship between the single and mixture exposure toxicity, and to gain an overall picture of the risks associated with interactions between PsNPs and WAF.

Materials and Methods

This project was divided into two parts: The first part focused on single exposure *in vitro* experiments where rainbow trout (*Oncorhynchus mykiss*) cells and zebrafish (*Danio rerio*) embryos (considered as *in vitro* <120 h post fertilization) were exposed to either PsNPs or WAF of North Sea marine oil in various concentrations. It involved measurements of cytotoxicity and generation of intracellular reactive oxygen species (ROS) in rainbow trout gill cells, and induction of CYP1A-dependent EROD activity in rainbow trout liver cells. Additionally, acute toxicity test was conducted in zebrafish embryos. The second part focused on mixture exposure experiments, where the same measurements were done with mixtures of PsNP and WAF, using selected concentration also used in the single exposure experiments.

Chemicals, solutions and materials

Cell culture phenol red-free Leibovitz's L-15 medium (L-15), trypsin, fetal bovine serum (FBS), Penicillin and Streptomycin were purchased from Gibco, ThermoFisher Scientific, Sweden. Acetonitrile, β-naphthoflavone (β-NF), ethanol (EtOH), 7-ethoxyresorufin (ER), ethylenediaminetetraacetic acid (EDTA), Fluorescamine, 3-Amino-7-dimethylamino-2-methylphenazine hydrochloride (Neutral Red) solution, copper sulphate ($CuSO_4$) and Tert-butyl hydroperoxide (t-BHP) in decane solution were bought from Sigma-Aldrich, Sweden. AlamarBlue (AB) solution was purchased from Invitrogen, Sweden and 5-carboxyfluorescein diacetate acetoxy methyl ester (CFDA-AM) was from ThermoFisher Scientific, Sweden). Plain polystyrene microspheres (25 nm, 10% solids (100 mg/mL)) was purchased from Bangs Laboratories, Inc. 6-carboxy-2′ 7′ -dichlorofluorescein diacetate (DCFH-DA) powder from Sigma Aldrich, Sweden, was dissolved in Dimethyl sulfoxide (DMSO) or ethanol.

L-15/ex saline buffer stock (Solution A: NaCl, KCl, $MgSO_4x7H_2O$, $MgCl_2x6H_2O$, Solution B: $CaCl_2$ x $2H_2O$, Solution C: Na_2HPO_4 x $12H_2O$, KH_2PO_4, Solution D: Sodium pyruvate (Sigma P5280), Solution E: Galactose (Sigma G5388) (Schirmer et al., 1997), artificial freshwater ($NaHCO_3$, KCL, $CaCl_2$ x $2H_2O$, $MgSO_4$ x $7H_2O$, pH 6.2±0.1) acidified ethanol solution (1% glacial acetic acid: 50% ethanol: 49% Milli-Q water solution), 0.2% ethylenediaminetetraacetic acid (EDTA) in phosphate-buffered saline (PBS) solution and Zebrafish embryo medium ($MgSO_4x7H_20$, KH_2PO_4, Na_2HPO_4 (anhydrous), $CaCl_2x2H_2O$, KCl, NaCl) (Sigma Aldrich, Sweden) were prepared in our lab according to standard protocols. Slide-A-Lyzer G2 Dialysis Cassette (3 mL capacity, 10 kDa molecular weight cut offs (gamma irradiated)) was bought from ThermoFisher Scientific, Sweden. 96-well cell culture plates, 6-well plates, and cell culture flasks (75 m^3) were purchased from Sarstedt, Germany.

Rainbow trout gill Waterloo 1 (RTgill-W1) cell line was purchased from ATCC, Sweden. Rainbow trout liver Waterloo 1 (RTL-W1) cell line was a gift from Prof. Dr. Kristin Schirmer, Head of department of Environmental toxicology, Eawag – Swiss Federal Institute of Aquatic Science and Technology.

Preparation of exposure chemicals

Preparation of polystyrene nanoparticles

In preparation for experimental assays, plain polystyrene microspheres (in nanosize and therefor referred to as PsNPs) were dialyzed to prevent the interference of sodium azide with the assays, using Slide-A-Lyzer G2 Dialysis Cassette (gamma irradiated). Cassettes were soaked in MilliQ-water for 30 s to activate the membranes before the sample of PsNP solution (25 nm, 100 mg/mL) was introduced with a pipette. The cassette was placed in a 2 L glass bottle with MilliQ-water on a magnetic stirrer for 72 h. MilliQ-water was changed after 24, 48 and 60 h to ensure all the sodium azide preservative had been removed. The dialysis cassette was put in a smaller beaker with MilliQ-water meanwhile the water was changed. After 72 h, the dialysis cassette was blotted on a paper tissue and the PsNP solution was transferred to a falcon tube with a syringe, and thereafter aliquoted and stored at 4 ℃.

Preparation of Water-accommodated fraction

The use of Water accommodated fraction (WAF) is one of the most common methods for preparing oil samples for aquatic toxicity testing. To 100 mL of North Sea Marine oil, 900 mL of sterile freshwater was added in a 1 L glass flask and shaken in the dark for 48 h at 35 rpm with an orbital shaker. This was done to attain functional saturation of soluble compounds from the oil in the aqueous phase following a modified protocol from Singer et al. (2000). The mixture was poured into a separatory funnel to allow the oil- and aqueous phase to settle and separate before the aqueous phase was collected into a 1 L bottle.

Preparation before cell experiments

Single exposures: PsNPs (1-100 µg/mL) and WAF (10-90%) were diluted in L-15 medium on the day of exposure. Mixture exposures: nine different mixture solutions (C1-C9, see result section) were prepared in falcon tubes covered with aluminum foil one day prior to exposure. The mixtures were put on an orbital shaker, shielded from light for 24 h until exposure of the cells.

Preparation before zebrafish experiments

Zebrafish embryo medium (ZFEM) stock (20x) was prepared by adding 700 mL dH_20 to a clean 1 L glass flask which was placed on a magnetic stirrer. Following chemicals was weighed as precisely as possible and added in the listed order: 4.9 g $MgSO_4x7H_20$, 0.41 g KH_2PO_4, 0.12 g Na_2HPO_4 (anhydrous), 2.9 g $CaCl_2x2H_2O$, 0.75 g KCl, 17.5 g NaCl. The solution was stirred until the salts were dissolved, thereafter diluted to 1 L and stored in fridge. Before the medium was used for experiments, the stock was diluted 1:20 in MilliQ water in a clean 1 L glass bottle which was autoclaved (121°C) and cooled down to room temperature before use.

Single exposures

200-220 mL/concentration of PsNPs (50 µg/mL and 100 µg/mL) single exposures and WAF (10%, 50% and/or 80%) single exposures was serially diluted in ZFEM (exposure day one). Not all concentrations were used in all experiments due to limited number of healthy eggs.

Mixture exposures

Four different mixtures of WAF and PsNP (C1a-C4a, see results section) were diluted in ZFEM and prepared one day prior to exposure and put on an orbital shaker for 24 h. The 80% WAF were excluded since sublethal- or lethal effects were observed in almost all zebrafish embryos

exposed to this concentration in the single exposure experiments (performed prior to the mixture experiments).

Cell culture and experimental animals

Cell culture

The experiments were carried out with either rainbow trout gill Waterloo 1 (RTgill-W1) or rainbow trout liver Waterloo 1 (RTL-W1) cell lines. Both cell lines were cultured in 75 m^3 culture flask containing Leibovitz's (1X) L-15 medium supplemented with 5% FBS and maintained/incubated at 19 °C (fig. 1). 1% Penicillin and Streptomycin were added to L-15 medium used for culturing RTL-W1 cells to prevent contamination. The culture medium was replaced every 5-9 days, and cells were sub-cultured by splitting the culture flasks 1:2 or 1:3 to form a confluent monolayer (Schirmer et al., 1997).

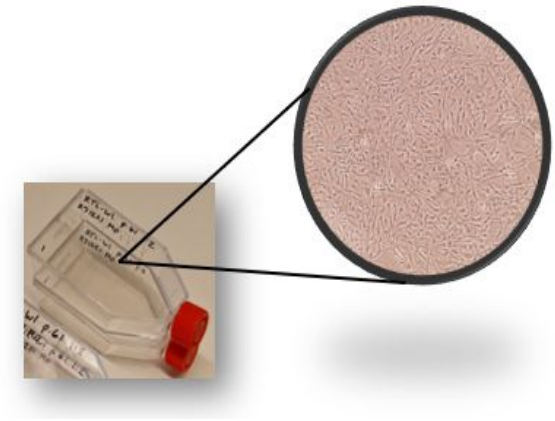

Fig. 1. Confluent monolayer of rainbow trout cell line. Cultured in 75 m^3 culture flask with L-15 medium +5% FBS and inspected in microscope before cell seeding.

Seeding of cell lines

All solutions were brought to room temperature prior to cell culture and exposure. A sterile environment was maintained by working on an LAF bench and by using 70% ethanol when needed. The cells were inspected in microscope to check for confluency (and if any contamination had occurred this could also be observed) (fig. 1 and 2). Thereafter the culture medium in the cell culture flask was aspirated with an autoclaved glass Pasteur pipette connected to a vacuum pump. The cells were washed once with 5 mL EDTA, which was left for 3 min before it was aspirated. Then 1.5 mL trypsin solution was added, and the flask was gently rotated to ensure that the trypsin completely covered the bottom. The trypsin was left for approximately 1-2 min, to let the cells detach from the flask bottom which was confirmed using a microscope. Thereafter 7 mL culture (L-15) medium with 5% FBS was added to inactivate trypsin. The cell suspension was gently pipetted up and down several times (~10x) to obtain a homogenous cell suspension and transferred into a sterile falcon tube. A Burker-Turk counting chamber was used together with an inverted microscope (100x magnification) to count the cell density, which was adjusted to a seeding density of 50 000 cells/well. The cell suspension was uniformly mixed (pipetted up and down) and then seeded into 96-multiwell plates, with 100 µL of the cell suspension per well. Plate was sealed with parafilm to avoid evaporation and incubated 24 h in 19 °C, cell seeding process illustrated in figure 2.

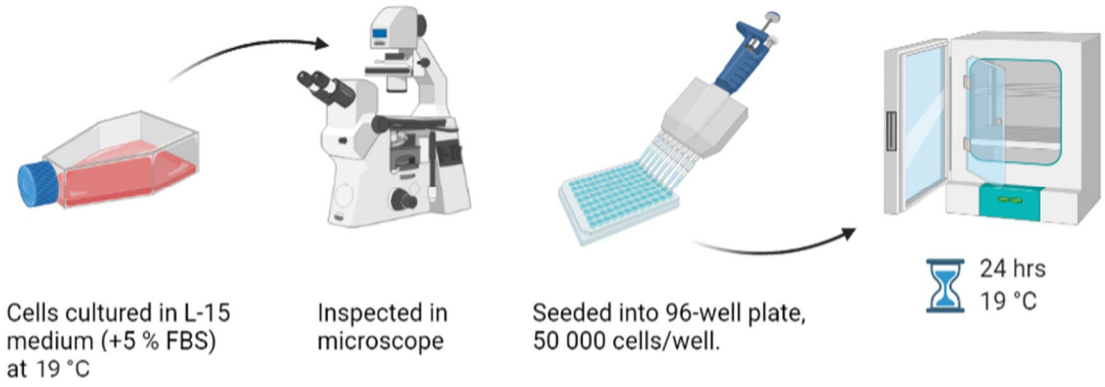

Fig. 2. Experimental set up for cell seeding. Created with Biorender.com.

Fish Husbandry

Adult zebrafish (*Danio Rerio*) (wildtype, strain AB) were kept in 30 L tanks (20 fish/tank) in a temperature-controlled room (26-30 °C) with 14 hours light and 10 hours dark photoperiods, at the animal facility at Zoologen, University of Gothenburg. They were fed ad libitum once a day with artemia and pellets. Animal use was approved by the Swedish Board of Agriculture (ethical permit 15986-2018).

Cytotoxicity assays

To detect toxicity, the probes alamarBlue, 5-carboxyfluorescein diacetate acetoxy methyl ester (CFDA-AM) and Neutral Red were used in fluorescence-based assays, experimental set up illustrated in figure 3. Initial pilot tests were performed measuring cytotoxicity in RT-Gill cells after the use of different kind of test medium; Cell culture phenol red-free Lei-bovits-15 medium (L-15) with 5% fetal bovine serum (FBS), cell culture phenol red-free L-15 medium without FBS and L-15/ex saline buffer. This was conducted together with a range of WAF and PsNPs single exposure concentrations which was diluted in one of the three test media. L-15 medium was considered as a suitable and more sensitive test medium and was therefore used in further cell experiments, also the concentrations used for mixture exposure concentrations were based on observation from the pilot experiments.

Exposure of cells

RT-gill cells that were seeded in a 96-well plate the day before was taken out of the incubator and observed in an inverted microscope to make sure that all seeded cells looked similar, and then placed into a sterile environment. The culture media in the plate was carefully removed using a multi-pipette and sterile pipette tips. 100 µL of exposure solution was added to each well and the plate was sealed with parafilm. Cells were exposed for approximately 24 h in 19 °C (fig. 3). Copper sulphate [200 µL/mL] was used as positive control and L-15 medium without FBS was used as negative control.

AlamarBlue and CFDA-AM assay

AlamarBlue and CFDA-AM assays were performed on the same set of cell replicates 24 h after either single exposure of PsNP and WAF or a mixture of both (fig. 3), following a modified protocol (Lammel, 2013). 125 µL of the commercial alamarBlue solution was diluted in 10 mL L-15/ex buffer solution in a falcon tube covered with aluminum foil, to obtain a final concentration of 1.25% (v/v). 10 µL of CFDA-AM stock (4 mM) prepared in DMSO was diluted in the same 10 mL L-15/ex to obtain a final concentration of 4 µM. The exposure medium in the 96-well plate was removed with a multichannel pipette, and the cells were rinsed once with 150 µL /well of L-15/ex buffer solution. All solutions were carefully added from the

top to the center of the monolayer to avoid cell detachment from the bottom. 100 µL of the alamarBlue/CFDA-AM solution was added to each well containing cells (60 inner wells) and to one column with wells that did not contain cells (blank control). The plates were incubated at 19 °C for 30 min at dark conditions. After incubation, fluorescence intensity in each well was measured using a Spectramax Gemini M microplate reader at excitation/emission wavelengths of 532/590 nm (alamarBlue assay) and 485/535 nm (CFDA-AM assay).

Neutral red assay

The cells were visually inspected in a microscope after the alamarBlue/CFDA-AM readout, to see if any cell detachment had occurred. The same set of cells were then washed once with 150 µL L-15/ex buffer solution, to remove previous probe solution (fig. 3). 100 µL of Neutral Red (NR) was diluted in 10 mL L-15/ex to obtain a concentration of 0.03 mg/mL. 100 µL of the diluted Neutral Red solution was added to each well before the plates were incubated at 19 °C for 1-2 h in dark conditions. After incubation, the cells were washed with 150 µL L-15/ex solution. To extract the NR retained inside the cells, 150 µL acidified ethanol solution was added to each well and the 96-well plate were placed in a box covered with aluminum foil which was put on an orbital shaker for 10 min. NR fluorescence intensity was measured at 530/645 nm excitation/emission wavelengths, using a Spectramax Gemini M microplate reader. All cytotoxicity assays fluorescence values were corrected with the cell free control "background" (fluorescence values from all wells were subtracted with the mean of fluorescence values from empty wells). The fluorescence intensity emitted from exposed cells was divided by the mean of fluorescence intensity emitted from the negative control (cells exposed to L-15-medium) and multiplied by 100 to obtain the relative fluorescence in percentage of the negative control, which is proportional to the relative cell viability. Following formula was used to calculate the relative cell viability (%):

$$\textbf{\textit{Relative cell viability}}\ (\%) = \frac{(Fluorescence\ intensity\ exposed\ cells\ - Background)}{(Fluorescence\ intensity\ negative\ control - Background)} \times 100$$

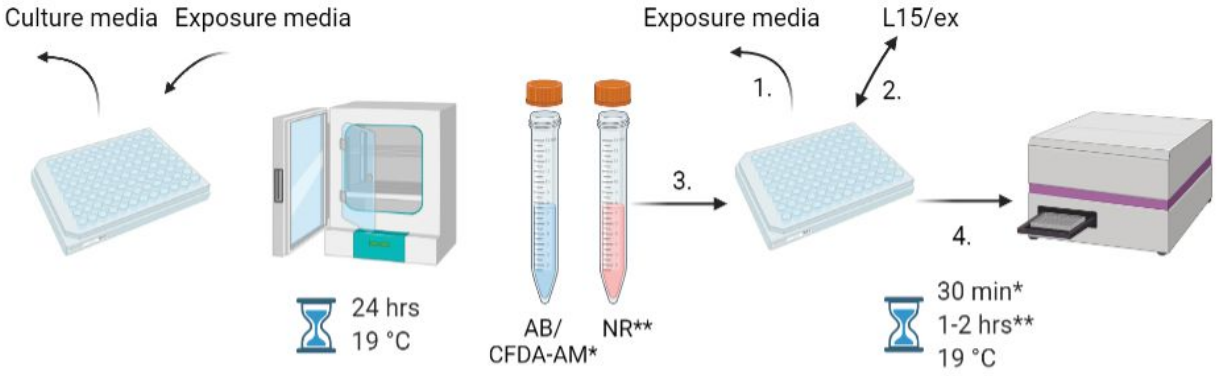

Fig. 3. Experimental set up for single or combined exposure of PsNPs and/or WAF followed by the three cell viability assays: AlamarBlue (AB), CFDA-AM and Neutral Red (NR) performed on the same set of cells. Illustration created with BioRender.com

Generation of reactive oxygen species DCFH-DA assay

The probe of 6-carboxy-2′ 7′ -dichlorofluorescein diacetate (DCFH-DA) was used to measure oxidative stress and detect the generation of reactive oxygen species. A stock (20 mM) DCFH-DA was dissolved in DMSO or ethanol, transferred to Eppendorf tubes and stored in a freezer. The DCFH-DA stock was diluted in L-15 medium to a final concentration of 20 µM. The cell culture medium was removed from the wells in the 96-well plate after the 24 h incubation period, using a multichannel pipette. 100 µL of the DCFH-DA (20 µM) loading solution was added to all 96 wells. The cells were incubated at 19 °C in dark conditions for 30-45 min to allow the DCFH-DA to diffuse into the cell and be converted to DCF. After the incubation, the loading solution was removed with a multichannel pipette and replaced with 100 µL of either single exposures of WAF or PsNP, or a mixture of them both. Cells exposed to 100 µl of 1 M tert-butyl hydroperoxide (t-BHP) was used as positive control and cells exposed to L-15 medium as negative control, one column was left empty to serve as background/blank, and L-15 medium was added to another column to serve as medium control. The kinetics of fluorescence intensity was monitored every 30 min for 6 or 18 h directly after the treatment and measured at 485 nm/535 nm excitation/emission wavelengths, using a Spectramax Gemini M microplate reader. Fluorescence values were corrected with the cell-free control (fluorescence values from all wells were subtracted by the average emitted fluorescence from empty wells, referred to as "background light"), since we want to see the effect of the cells and not from the plate itself.

CYP1A enzyme activity assay

Exposure of cells

RTL-W1 cells that were seeded in a 96-well plate the day before was taken out of the incubator and observed in an inverted microscope to make sure that all seeded cells looked similar, notes were taken if any deviation was observed. The culture media in the plate was carefully removed using a multi-pipette and sterile pipette tips, working on a LAF bench. 100 µl/well of exposure solution were carefully added and the plate was sealed with parafilm and exposed for approximately 24 h in 19 °C.

CYP1a dependent EROD-activity

After the incubation period, the exposure media was removed and cells were washed with 150 µL L15/ex buffer solution, using a multi-pipette and sterile pipette tips. 20 µl 7-ethoxyresorufin in DMSO stock [2 mM] was diluted 1:1000 in 19,98 mL autoclaved L15/ex solution to a final concentration of 2 µM in a falcon tube. 100 µL of the ethoxyresorufin-solution was gently added to each well and the plate was directly placed in the plate reader. Since the reaction starts immediately, this step was performed fast. The fluorescence was measured at 530/590 nm (high gain) excitation/emission wavelengths using Spectramax Gemini M microplate reader. The resorufin-content (emitted fluorescence) was measured every minute, for 10 minutes. EROD activity was calculated using appropriate standard curves for resorufin and normalized against cells/well. Following formula was used to calculate the EROD activity [ρmol/min]:

$$1.\ EROD\ activity\ (\rho mol) = \frac{Resorufin\ formation - Resorufin\ standard\ y\ intercept}{Resorufin\ standard\ slope}$$

$$2.\ EROD\ activity\ per\ unit\ time\ (\rho mol/\min) = \frac{EROD\ activity\ t10 - EROD\ activity\ t0}{10}$$

The total substrate (ethoxyresorufin) turnover, which equals the amount of produced resorufin [ρmol], was calculated by subtracting the y-intercept value (t [FU]=151.34) calculated from the resorufin standard curve from the emitted fluorescence values measured in the EROD assay (resorufin formation) and divided by the slope value (a=2.65) from the resorufin standard curve (1). The function y=ax+t was used for the resorufin standard curve, where y= fluorescence intensity, a= slope, x=ρmol resorufin per well, and t=y-intercept. The produced resorufin [ρmol] after 10 min (t=10) was subtracted by the starting resorufin content (at t=0) and divided by 10 to get the total substrate turnover per unit time [ρmol/min] (2).

Fish embryo acute toxicity test

Breeding and egg handling

Fish embryo acute toxicity (FET) test, OECD test guideline (TG) 236, was performed to determine the acute or lethal toxicity on embryonic stages of zebrafish. 8 breeding tanks were placed into the zebrafish aquariums (1 breeding tank/aquarium) during the afternoon the day before collection of the eggs. The zebrafish were allowed to breed, and the newly fertilized eggs were collected during the following morning. The water together with the eggs from the breeding tanks water was poured through a filter and eggs were gently rinsed with zebrafish medium (ZFEM, see salts under "preparation before zebrafish experiments") and transferred to petri dishes with ZFEM. Thereafter egg selection was immediately done where healthy eggs with the development stage of four cells and above were selected with the help of a microscope. Coagulated and non-fertilized eggs were discarded.

Exposure of zebrafish embryos

In the first pilot experiment the selected eggs were placed into a glass petri dish (20 eggs/Petri dish, 2 petri dishes/exposure, 30 mL exposure solution/petri dish. For the following experiments 6-well culture plates were used instead (5 eggs/well, 1 plate/exposure, 8 mL exposure solution/well) due to limited number of eggs with good quality in some batches and since it simplified the registration of the lethal and sublethal effects. The exposure medium consisted of either single exposures of PsNPs or WAF or a mixture of both, see concentrations under "preparation before zebrafish embryo experiment". ZFEM was used as medium in the negative control. The zebrafish embryos were incubated in 28-30 °C and exposed for 96 h. The exposure medium was changed after 24, 48 and 72 h to keep the exposure concentration constant. The number of coagulated eggs were counted with a microscope and discarded before each medium change, both to notice if any healthy egg had been discarded by mistake and to register when and how many eggs were coagulated. Three observations were recorded with a stereo microscope (leica EZ4HD) as indicators of lethality at the end of the exposure period (after 96 h exposure), including: coagulation of fertilized eggs, unhatched and non-viable. Three observations were recorded as sublethal effects: scoliosis, oedema and lack of inflated swim bladder. Acute toxicity and sublethal effects were determined based on a positive outcome in any of the recorded observations mentioned above and calculated as percentage of total amount

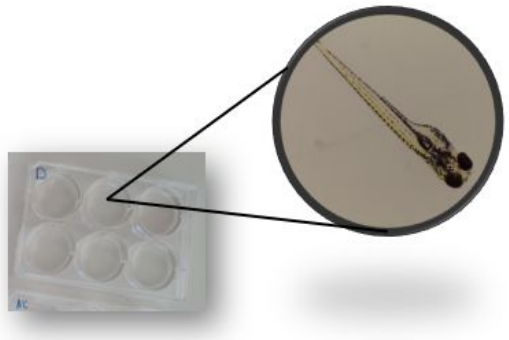

Fig. 4. Zebrafish embryo inspected in microscope after 96 h of exposure. One plate/exposure concentration with 5 eggs/well and 8 mL exposure solution/well.

of embryos in each plate/exposure concentration. No LC50 value was calculated due to limited amount of data.

Statistical analysis

All statistical tests were performed using the IBM SPSS statistics 28.0 software for windows. Sine a one-way ANOVA test requires certain assumptions, a Shapiro-Wilk test was used to test for normality (to test if the dependent variable´s values were a simple random sample from a normal distribution), and Levene´s test was performed to test for homogeneity of variances (to test if the variance of the dependent variable was equal over all subpopulations). If the data passed these assumptions, a standard one-way ANOVA was performed to test for statistically significant ($p<0.05$) differences between group means, and a Tukey post hoc test to test which groups differed from each other (multiple comparison). If Levene's test was statistically significant ($p<0.05$), the null hypothesis of equal population variances was rejected (data failed the assumption of homogeneity of variances), and a Welch ANOVA was performed since it is robust to violation of the homogeneity assumption, followed by a Games-Howell post hoc test. All data sets passed Shapiro-wilk test for normality.

Results

Measurement of cytotoxicity in rainbow trout gill cell line

Cytotoxicity in response to PsNPs and WAF single exposures

Cytotoxicity was measured in RTgill-W1 cells after 24 h exposure to various concentrations of PsNPs (25 nm, 1-100 µg/mL) or WAF (10-90%) single exposures, using alamarBlue, CFDA-AM and Neutral Red fluorescence-based assays (see fig. 3 for experimental set up). Mean relative cell viability for all plates (n=4) was calculated and represented as percentage of the negative control ± SEM (fig. 5).

Metabolic activity

Cell viability, or more specific metabolic activity, measured with the alamarBlue assay decreased in a dose-dependent manner after WAF single exposures and was statistically significantly reduced in cells exposed to 40% (79.88±3.61, $p<0.05$), 70% (55.06±4.77, $p<0.01$), 80% (12.18±5.46, $p<0.001$) and 90% (3.81±0.43, $p<0.01$) WAF compared to the 10% WAF (106.69±7.48) (fig. 5). Cytotoxicity in cells exposed to different concentrations (1-100 µg/mL) of PsNP single exposures was not significantly different, in other words, PsNP single exposures did not have a statistically significant effect on metabolic activity. However, the relative cell viability (metabolic activity) was slightly lower in cells exposed to the highest (100 µg/mL) PsNP concentration compared to cells exposed to 1 µg/mL PsNPs (fig. 5), although not significant ($p=0.801$).

Cell membrane integrity and enzymatic activity

The cell-permeant viability probe 5(6)-CFDA-AM which is an esterase substrate, was used to measure both cell membrane integrity (needed for retention of its fluorescent product) and enzymatic activity (required to activate the fluorescence of the substrate). The relative cell viability (cell membrane integrity and enzymatic activity) was significantly reduced in cells exposed to 80% (22.26±15.79, $p<0.05$) and 90% (2.17±1.12, $p<0.001$) WAF compared to 10% WAF (105.55±6.29) single exposure (fig. 5). However, it did not reveal as strong dose-dependent pattern in the cells exposed to 20-60% WAF as in the alamarBlue assay. PsNP single exposures did not have a significant effect on the cell membrane integrity and enzymatic activity, even if it was slightly lower ($p=0.982$) in the highest (100 µg/mL) PsNP concentration compared to cells exposed to 1 µg/mL PsNPs, in a similar manner as in the alamarBlue assay (fig. 5).

Lysosomal activity

Neutral Red assay was used to quantify induced cytotoxicity since it measures the ability of viable cells to incorporate and bind the weak cationic dye neutral red in their lysosomes. The cell viability (lysosomal activity) decreased in a dose-dependent manner in cells exposed to 40-90% WAF (fig. 5). The lysosomal activity was significantly reduced in cells exposed to 70% (53.98±11.97, $p<0.01$), 80% (9.63±11.88, $p<0.001$) and 90% (2.10±0.57, $p<0.001$) WAF compared to 10% WAF single exposure (103.12±16.22). PsNP single exposures did not influence lysosomal activity (fig. 5).

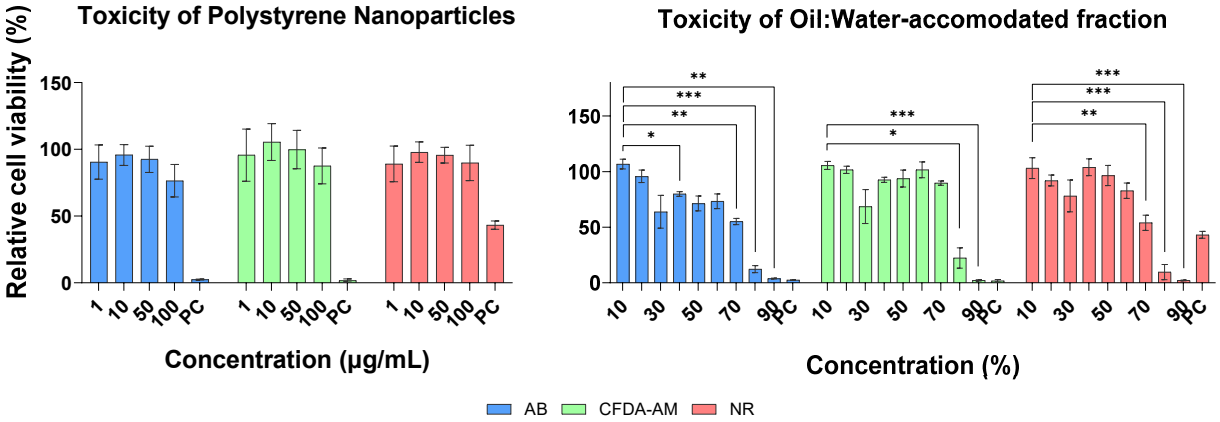

Fig. 5. Relative cell viability in rainbow trout gill (RTgill-W1) cells after 24 h single exposures of polystyrene nanoparticles (PsNPs, 1-100 µg/mL) or water-accommodated fractions (WAF, 10-90%) of North Sea marine oil, measured with alamarBlue (metabolic activity), CFDA-AM (cell membrane integrity) and Neutral Red (lysosomal function) cell viability indicator dyes. Cells were cultured in L-15 medium (+5% FBS) prior to exposure and cell viability is expressed as fluorescence units relative to the negative control (cells treated with L-15 medium). Each bar shows the mean value of four biological replicates ± SEM. Significance level *p<0.05, **p<0.01, ***p<0.001.

Cytotoxicity in response to PsNPs and WAF mixture exposures

RTgill-W1 cells were exposed to nine different mixtures of PsNPs and WAF (C1-C9) for 24 h. Low WAF mixtures consisted of following concentrations; C1: 10% WAF and 10 µg/mL PsNPs, C2: 10% WAF and 50 µg/mL PsNPs, C3: 10% WAF and 100 µg/mL PsNPs. Mid WAF mixtures consisted of; C4: 50% WAF and 10 µg/mL PsNPs, C5: 50% WAF and 50 µg/mL PsNPs, C6: 50% WAF and 100 µg/mL PsNPs. High WAF mixtures; C7: 80% WAF and 10 µg/mL PsNPs, C8: 80% WAF and 50 µg/mL PsNPs, C9: 80% WAF and 100 µg/mL PsNPs. Biological replicates with different passage numbers were used for all cytotoxicity assays with the mixtures to include biological variation. Mean relative cell viability for all plates (n=5) was calculated and represented as percentage of the negative control ± SEM (fig. 6).

Metabolic activity

Metabolic activity in cells exposed to a mixture of PsNPs and WAF was measured with the alamarBlue assay. A similar pattern was revealed as in the cells exposed to WAF single exposures, with a dose-dependent decrease in relative cell viability with increasing WAF concentration. To study the combined toxicity of PsNPs and WAF, the effect of addition of PsNPs to the mixtures was examined by comparing the relative cell viability in cells exposed to the different concentration of PsNPs within the same WAF concentration. There was a significant reduction ($p<0.001$) of cell viability in cells exposed to the highest concentration (100 µg/mL) of PsNPs compared to the lowest concentration (10 µg/mL) in all WAF mixtures. The average relative cell viability (%) ± SEM in cells exposed to 100 µg/mL PsNPs was 67.28±4.55 compared to 96.93±10.31 in cells exposed to 10 µg/mL PsNPs in 10% WAF mixtures, 57.77±6.9 (100 µg/mL PsNPs) compared to 95.63±6.67 (10 µg/mL) in 50% WAF

mixtures, and 10.62±4.82 (100 µg/mL PsNPs) compared to 40.63±3.37 (10 µg/mL) in 80% WAF mixtures. The cell viability tended to decrease also in cells exposed to 50 µg/mL PsNPs in all WAF mixtures, where a significant ($p<0.01$) decrease could be observed in cells exposed to the 50% WAF mixtures (mean relative cell viability (%) =77.24±5.74). 10% and 50% WAF mixture exposures revealed a similar pattern, while cells exposed to the 80% WAF mixture had lower metabolic activity (fig. 6).

Cell membrane integrity and enzymatic activity

In the CFDA-AM assay there was a slight decrease in relative cell viability (cell membrane integrity and enzymatic activity) in RTgill-W1 cells exposed to high (80%) WAF mixtures, even if it was not as distinct as in the alamarBlue assay. The addition of the highest concentration of PsNPs (100 µg/mL) significantly affected the cell viability in cells exposed to 10% WAF (75.04±3.0, $p<0.05$) and 80% WAF (24.65±7.31, $p<0.001$) mixtures compared to cells exposed to 10 µg/mL PsNPs in 10% WAF (98.66±6.80) and 80% WAF (75.15±3.02) mixtures. The combination of the highest WAF (80%) and highest PsNP (100 µg/mL) had a significant cytotoxic effect compared to all other mixture combinations (fig. 6).

Lysosomal activity

In the Neutral Red assay, the relative cell viability (lysosomal activity) was lower in cells exposed to 80% WAF mixtures compared to 10% and 50% WAF mixtures. There was no clear dose-response trend caused by WAF in the 10% and 50% WAF mixtures, which showed a similar pattern and percentage of viable cells. However, the addition of the highest concentration of PsNPs (100 µg/mL) significantly decreased the lysosomal activity in 50% WAF (95.12±3.26, $p<0.05$) and in 80% WAF (7.90±2.03, $p<0.001$) mixtures compared to 10 µg/mL in 50% WAF (104.62±2.86) and in 80% WAF (67.12±4.27) mixtures. Same tendency was seen in cells exposed to 10% WAF mixtures with 100 µg/mL PsNPs (92.10±4.98) compared to 50 µg/mL (99.73±3.39, $p=0.347$) and to 10 µg/mL (100.00±2.79, $p=0.282$) mixtures, although it was not significant (fig. 6). The combination of the highest WAF (80%) and highest PsNPs (100 µg/mL) concentration had a significant cytotoxic effect compared to all other mixture combinations, which was observed also in the alamarBlue and CFDA-AM assay.

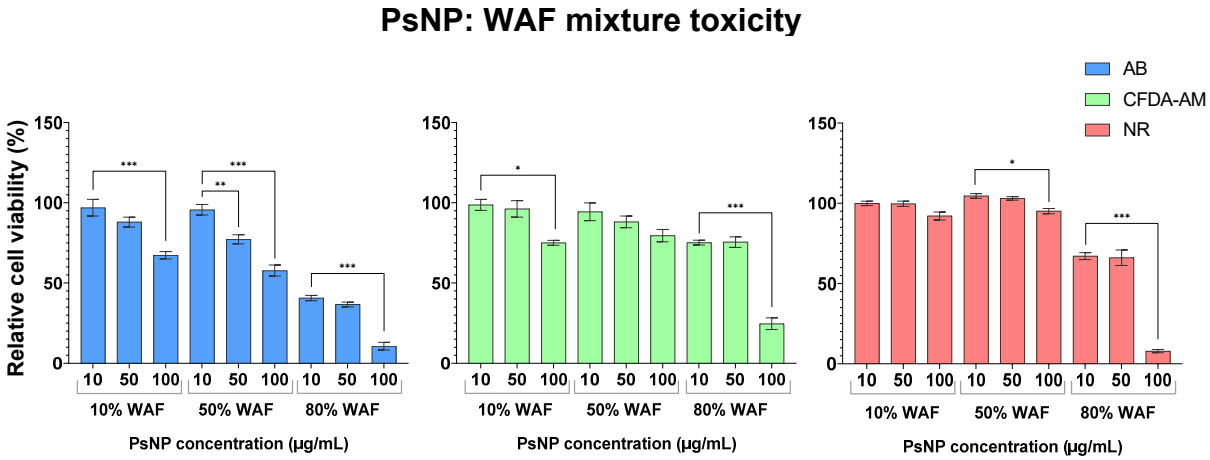

Fig. 6. Relative cell viability in rainbow trout gill (RTgill-W1) cells after 24 h mixture exposures of polystyrene nanoparticles (PsNPs, 10, 50, 100 µg/mL) and water-accommodated fractions (WAF, 10, 50, 80%) of North Sea marine oil, measured with alamarBlue (metabolic activity), CFDA-AM (cell membrane integrity) and Neutral Red (lysosomal function) cell viability indicator dyes. Cells were cultured in L-15 medium (+5% FBS) prior to exposure and cell viability is expressed as fluorescence units relative to the negative control (cells treated with L-15 medium). Each bar shows the mean value of five biological replicates ± SEM. Significance level *$p<0.05$, **$p<0.01$, ***$p<0.001$.

Measurement of oxidative stress (generation of reactive oxygen species) in rainbow trout gill cell line

Generation of ROS in response to PsNPs and WAF single exposures

Generation of intracellular reactive oxygen species (ROS) was measured with the fluorescence based DCFH-DA assay in RTgill-W1 cells exposed to a range of PsNPs (1-100 µg/mL) or WAF (10-80%) concentrations for 24 h. Three biological replicates with different passage numbers were used for both the PsNPs- and WAF experiments. Data is represented as average ROS generation (fluorescence units) for all plates (n=3) ± SEM (fig. 7), and not as relative to the negative control (as in the cytotoxicity assays) since we had high ROS generation in cells exposed to L-15-medium. This needs to be taken into consideration when interpreting the results, since the cells themselves do naturally produce ROS. A time dependent increase in ROS generation was observed after the exposure of all concentrations of both PsNPs and WAF (fig. 7). The generation of intracellular production of ROS after 5 and 6 h was not statistically significant different in cells exposed to the different concentrations of PsNPs. However, the ROS generation was significantly lower (p=0.031) in cells exposed to 80% WAF (239.41±38.41) after 6 h compared to cells exposed to 10% WAF (460.88±93.96) after 6 h (fig. 7). The cells exposed to 70% WAF (303.69±44.98) also had a tendency (p=0.108) to generate slightly lower amount of ROS compared to cells exposed to 10 % WAF (see appendices).

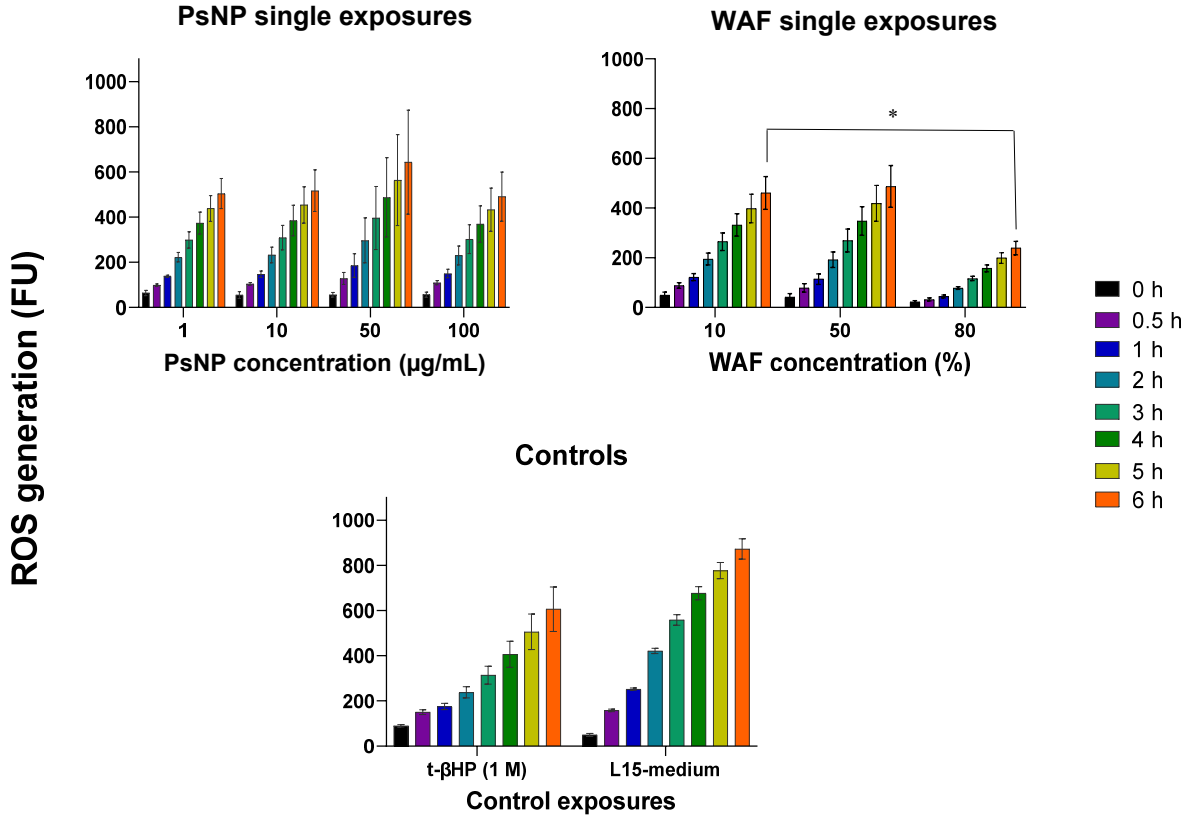

Fig. 7. Generation of reactive oxygen species in rainbow trout gill (RTgill-W1) cell line after exposure to polystyrene nanoparticle (PsNP) or water-accommodated fraction (WAF) single exposures, measured over a 6 h period. Each bar shows the mean value of three biological replicates ± SEM. Significance level *p<0.05, **p<0.01, ***p<0.001.

Generation of ROS in response to PsNPs and WAF mixture exposures

The generation of intracellular ROS in RTgill-W1 cells exposed to mixtures of PsNPs and WAF increased in a time-dependent manner over a 6 h period (fig. 8), as seen also in the single exposure experiments (fig. 7). Three biological replicates were used, data is represented as ROS generation (fluorescence units) ± SEM. No statistically significant differences in ROS production were seen between cells exposed to 10, 50 or 100 µg/mL PsNP concentrations in any of the mixtures after 5 and 6 h. Nevertheless, there was a slight increase of ROS generation with lower PsNP concentration. This trend was especially observed in the 10% WAF mixtures where the ROS generation was 221.14±78.42 in 10 µg/mL PsNP mixture after 6 h compared to 130.90±51.01 in 100 µg/mL mixture, but also in the 80% WAF mixtures where the ROS production was 113.56±23.63 in 10 µg/mL PsNP mixture after 6 h, compared to 87.95±35.70 in 100 µg/mL PsNP mixture. The ROS generation was similar between the different mixtures in the 50% WAF mixture, even if it was slightly higher in 10 µg/mL (124.13±26.04) PsNP mixture compared to 100 µg/mL (114.85±29.56) PsNP mixture. The ROS production was generally lower in the mixtures (fig. 8) compared to single exposures (fig. 7) of both PsNP and WAF, this also applies to cells exposed to L15-medium in the different experiments (see discussion). Cells in the negative control (L15-medium) and positive control (t-BHP) produced lower respectively higher intracellular ROS compared to the exposures, which was expected. After 5 h, the ROS generation in 80% WAF single exposure (fig. 7) differed significantly from mixtures with 80% WAF and both 10 µg/mL ($p<0.05$), 50 µg/mL ($p<0.05$) and 100 µg/mL PsNPs ($p<0.01$) (fig. 8). After 6 h, a significant difference in ROS generation between 80% WAF single exposure and 80% mixture with 100 µg/mL PsNP ($p<0.05$) was seen, but not with 10 or 50 µg/mL PsNPs. No significant difference was observed when comparing 50% single exposures to mixture with 50% WAF and PsNPs after 5 and 6 h, or when comparing 10% WAF single exposure to mixture with 10% WAF and 10, 50 or 100 µg/mL PsNPs.

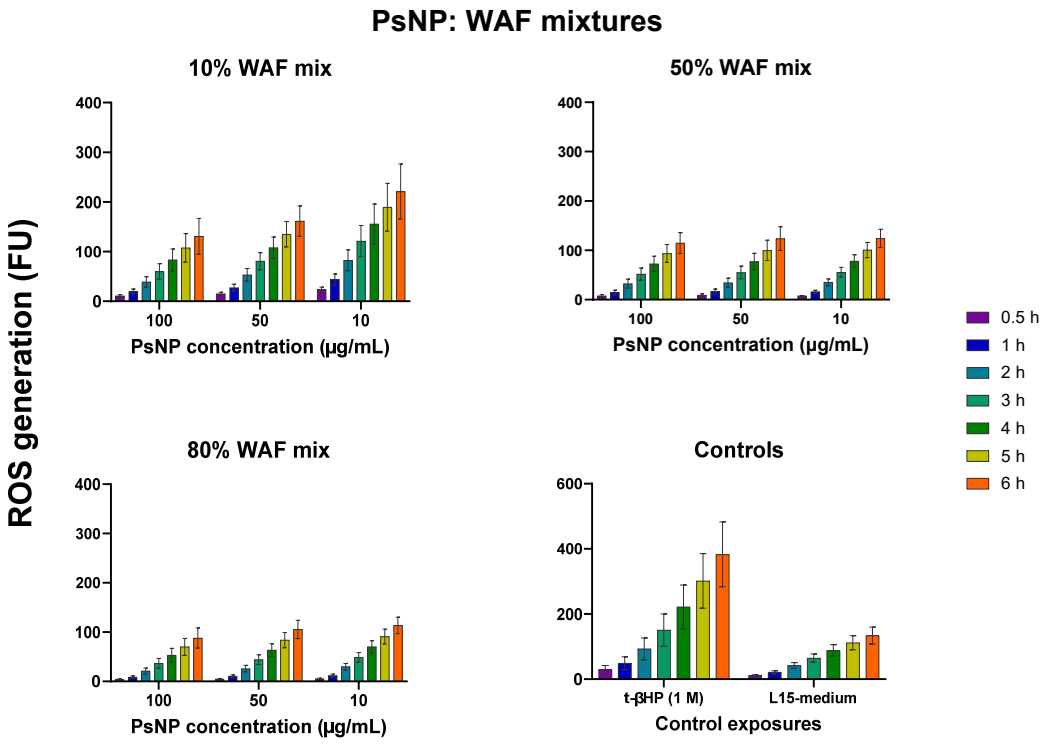

Fig. 8. Generation of reactive oxygen species in rainbow trout gill (RTgill-W1) cell line after exposure to polystyrene nanoparticle (PsNPs) and water-accommodated fraction (WAF) mixtures, measured over a 6 h period with DCFH-DA assay. Note that a different y-axis was used for the controls. Each bar shows the mean value of three biological replicates ± SEM. Significance level *$p<0.05$, **$p<0.01$, ***$p<0.001$.

Measurement of CYP1A dependent EROD-activity in rainbow trout liver cell line

EROD-activity in response to PsNPs and WAF single exposures

The CYP1A dependent EROD-activity (amount of produced resorufin) in RTL-W1 cells increased in a dose dependent manner from 0.77±0.56 ρmol/min to 2.67±1.56 ρmol/min after 24 h exposure to 20-50% WAF. The difference in EROD-activity was closer to significantly different when comparing cells exposed to 10% WAF and 60% WAF single exposures (p=0.062) (due to less variation between replicates) than when comparing cells exposed to 10% WAF and 50% WAF single exposures (p=0.250), although the mean EROD-activity was higher in cells exposed to 50% WAF. No produced resorufin could be measured in cells exposed to 10% or 80% WAF or to any of the PsNP concentrations (1-100 µg/mL) (fig. 9). Four biological replicates with different passage numbers were used to include biological variation, all data is expressed as average produced resorufin for all plates (n=4) ± SEM. No statistically significant differences were seen between the 20-80% WAF concentrations and the lowest (10%) concentration of WAF, when using a Games-Howell post hoc test (which was used since homogeneity of variances could not be assumed).

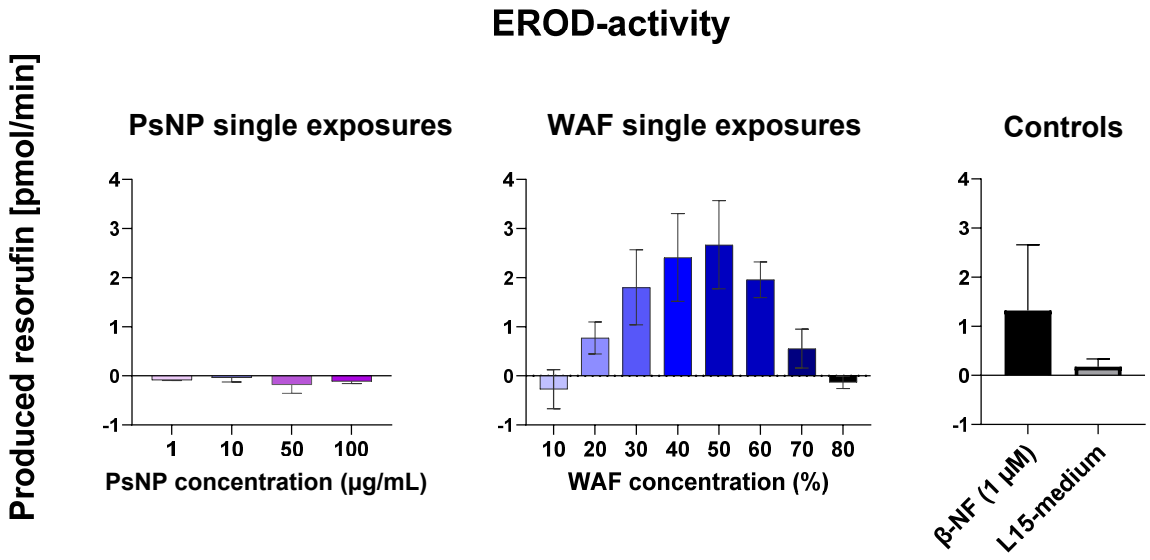

Fig. 9. Induction of CYP1A dependent EROD-activity in rainbow trout liver (RTL-W1) cell line after 24 h exposure to polystyrene nanoparticles (PsNPs) or water-accommodated fraction (WAF) single exposures. Data is expressed as mean produced resorufin (ρmol/min) for four biological replicates ± SEM. Significance level *p<0.05, **p<0.01, ***p<0.001.

EROD-activity in response to PsNPs and WAF mixture exposures

The total substrate (ethoxyresorufin) turnover [ρmol/min] increased in a dose dependent manner in RTL-W1 cells exposed to PsNPs/WAF mixtures with WAF concentrations between 10% and 50% (fig. 10), which was also observed in the single exposures of WAF (fig. 9). No statistically significant differences in EROD-activity were seen between cells exposed to mixtures with different concentrations of PsNPs (10, 50, 100 µg/mL) combined with the same concentration of WAF (10, 50 or 80%). However, there was a trend of increasing amount of produced resorufin in cells exposed to the highest concentration of PsNPs (100 µg/mL) in the mixtures compared to cells exposed to mixtures with lower concentrations (10-50 µg/mL) of PsNPs in both 10% and 50% WAF mixtures. 10% WAF mixtures induced a higher EROD-activity (produced resorufin) both with 10 µg/mL (0.56±0.19), 50 µg/mL (0.46±0.14) and in 100 µg/mL (1.48±0.43) PsNPs concentrations (fig. 10) compared to EROD-activity in cells

exposed to 10% WAF single exposure (-0.27±0.69) (fig. 9). The negative values equal decreased EROD-activity (since amount of resorufin produced at time 10 is subtracted by the amount of resorufin at time 0). On the contrary, cells exposed to 50% WAF mixed with 10 µg/mL (1.38±0.50), 50 µg/mL (1.59±1.02) and 100 µg/mL (2.07±0.55) PsNPs produced less resorufin compared to cells exposed to corresponding (50%) WAF single exposure (2.67±1.56). Cells exposed to 80% WAF mixtures did not induce EROD-activity regardless of PsNP concentration (fig. 10). Three biological replicates with different passage numbers were used to include biological variation, all data expressed as average produced resorufin for all plates (n=3) ± SEM.

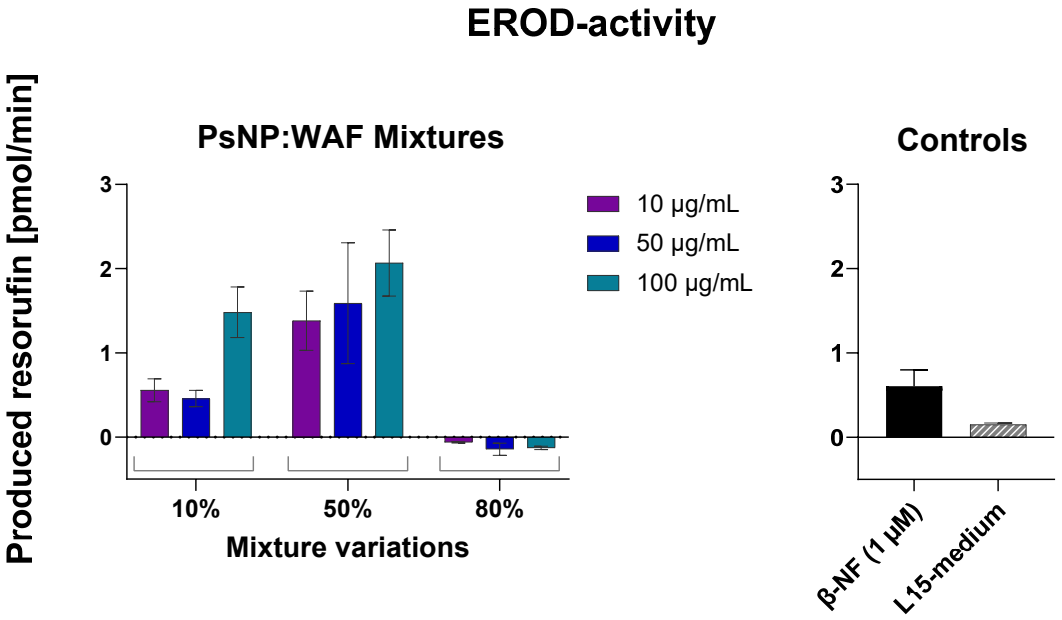

Fig. 10. Induction of CYP1A dependent EROD-activity in rainbow trout liver (RTL-W1) cell line after 24 h exposure to polystyrene nanoparticles (µg/mL PsNPs) in or water-accommodated fraction (% WAF) mixture exposures. Data expressed as mean produced resorufin (*ρ*mol/min) for four biological replicates ± SEM**.** Significance level *p<0.05, **p<0.01, ***p<0.001.

Acute toxicity in zebrafish embryos

Zebrafish embryo toxicity in response to PsNPs and WAF single exposures

A pilot study of zebrafish embryo toxicity test was performed to reflect a different level of organization that includes the complexity of a whole organism. Newly fertilized zebrafish eggs were collected and exposed to 50 or 100 µg/mL PsNPs, or to 10, 50 or 80% WAF single exposures, both diluted in zebrafish embryo medium. Lethal and sublethal effects were observed with a microscope after 96 h of exposure. Observations that were recorded as lethal effects included: coagulation of fertilized eggs, unhatched, non-viable embryos. Sublethal effects included: scoliosis, oedema, lack of inflated swim bladder (fig. 12), modified protocol from test No. 236 from OECD (2013), see also Huang et al. (2010). Data is expressed as percentage (%) of total, by dividing the number of embryos with no (healthy), lethal or sublethal effects by the total number of embryos in each exposure concentration (fig. 11). Three experiments (different batches) were performed with the single exposures with 30-40 eggs/exposure concentration. The zebrafish egg quality in these batches varied, which was observed by eye and seen as varying percentage of lethal effects in the negative control. To avoid that the effects seen in the exposed embryos were caused by bad egg quality rather than the exposure itself, the presented results will focus on the experiments with good egg quality

(this also applies for the mixture exposures below). All numbers in percentage for single exposures are therefor based on one experiment (instead of an average of the three experiments) where 40 eggs were used per exposure concentration, illustrated in figure 11. Effects observed in zebrafish embryos exposed to 80% WAF single exposure: 60% of the zebrafish embryos had sublethal effects, 12.5% had lethal effects and 27.5% were healthy. Effects observed in zebrafish embryos exposed to 10% WAF: 0% had sublethal effects, 12.5% had lethal effect, 87.5% were healthy. Effects observed in zebrafish embryos exposed to 50 µg/mL PsNP single exposure: 5% had sublethal effects, 10% had lethal effects and 85% were healthy. This should be compared to the negative control (only zebrafish embryo medium) where 0% of the zebrafish embryos had sublethal effects, 3% lethal effects and 97% were healthy (fig. 11). 50% WAF had similar effects as the 80% WAF with a large percentage of embryos with sublethal effects compared to the negative control (not illustrated). No clear differences were observed between 50 and 100 µg/mL PsNPs single exposures.

Zebrafish embryo toxicity in response to PsNPs and WAF mixture exposures

Zebrafish embryos were exposed to four different mixtures of WAF and PsNPs consisting of 10% WAF and 50 µg/mL PsNPs (C1a), 10% WAF and 100 µg/mL (C2a), 50% WAF and 50 µg/mL PsNPs (C3a) and 50% WAF and 100 µg/mL PsNPs (C4a). All numbers in percentage for the mixture exposures are based on one experiment where 30 eggs/exposure concentration were used (fig. 11). Zebrafish embryos exposed to 100 µg/mL PsNP mixture had high percentage of lethal effects both in combination with 10% and 50% WAF, compared to 50 µg/mL PsNPs mixed with the same WAF concentration. Embryos exposed to higher WAF concentration (50%) in combination with 50 µg/mL had larger percentage of sublethal effects compared to lower WAF concentration (10%) with the same PsNP concentration. 10% WAF: 50 µg/mL PsNP mixture: 17% of the embryos had sublethal effects, 20% had lethal effects and 63% were healthy. 10% WAF: 100 µg/mL PsNPs: 23% sublethal effects, 77% lethal effects and 0% healthy. 50% WAF: 50 µg/mL PsNPs: 77% sublethal effects, 10% lethal effects and 13% healthy. 50% WAF: 100 µg/mL PsNPs: 17% sublethal effects, 83% lethal effects and 0% were healthy. Negative control (zebrafish embryo medium): 3% sublethal effects, 7% lethal effects and 90% were healthy.

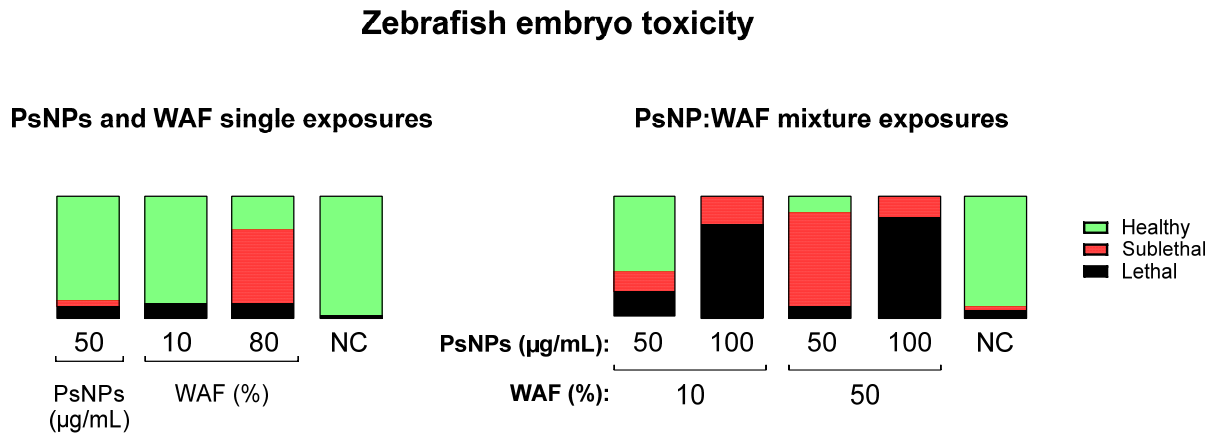

Fig. 11. Percentage of healthy (green), sublethal (red) and lethal (black) effects observed in zebrafish embryos after 96 h of either single exposure of polystyrene nanoparticles (PsNPs) or water-accommodated fraction (WAF) or a mixture of them both. 40 eggs/exposure concentration were used or single exposures and 30 eggs/concentration for the mixtures.

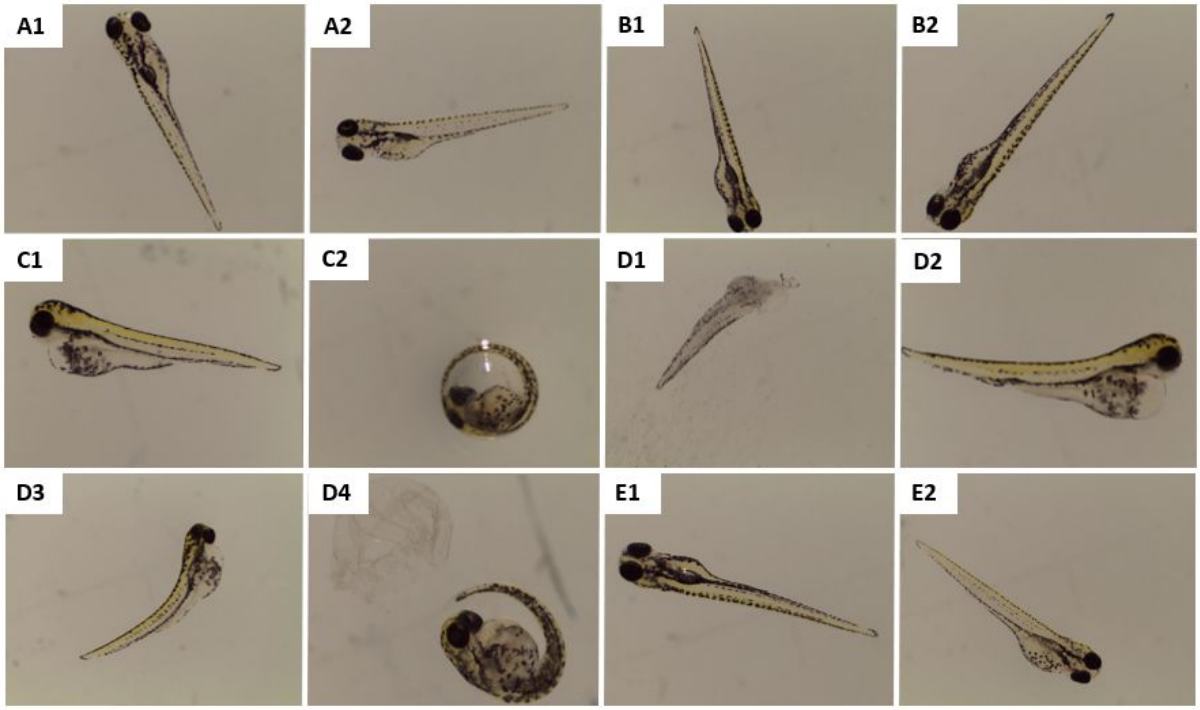

Fig. 12. A1 and A2: embryos exposed to 50 µg/mL polystyrene nano particles (PsNPs) where A1 are healthy and A2 lacked inflated swim bladder. B1 and B2: embryos exposed to 10% WAF where B1 was healthy and B2 lacked inflated swim bladder, respectively. C1 and C2: embryos exposed to 50% WAF, where C1 had oedema and lacked inflated swim bladder and C2 was non hatched. D1-D4: embryos exposed to the highest 80% WAF, where D1 was non-viable, D2 had oedema and lacked inflated swim bladder, D3 had scoliosis, oedema and lacked inflated swim bladder and D4 was non hatched, eggshell seen to the left of the embryo. E1 and E2: embryos exposed to zebrafish embryo medium (negative control), where E1 was healthy and E2 lacked inflated swim bladder.

Discussion

The biological effects of Mps and Nps on aquatic organisms are well documented (Banerjee and Shelver, 2021) and several studies have reported that Mps have strong capacity to adsorb different organic micropollutants (Yu et al., 2021). However, the studies that examine Nps capacity to adsorb organic micropollutants are limited (Razanajatovo et al., 2018), and it is still a matter of debate whether this adsorption is posing multiple health risks to organisms (Mei et al., 2020). This project aimed to examine the possible toxic effects associated with single and combined exposure of PsNPs and WAF of North Sea marine oil. This implied measurements of acute toxicity in two *in vitro* fish models where standardized *in vitro* methods were used, and more sensitive *in vitro* assays were explored.

Why fish *in vitro* models?

In vitro experiments with fish cells are valuable 3R (replace, reduce, refine) tools that allow researchers to study the effects of a chemical inside a cell or on cell surface (Dayeh et al., 2013), and have been a useful tool in several previous studies (Bols and Lee, 1991). One advantage with fish cell lines (used in this study) is that they can be permanent in comparison to primary cultures which are temporary (Bols and Lee, 1991). Cell lines are a reproducible source of cells since they can be cryopreserved indefinitely (Wolf, 1979) and become homogeneous during early passaging. This can be advantageous in contrast to primary cell cultures that often are more heterogenous which can contribute to difficulties in obtaining an identical primary culture at another date (Bols and Lee, 1991). Although, the choice of cell type or if it is more suitable to use cell lines or primary cell cultures can vary among different studies depending on their

goals (Bols and Lee, 1991). Experiments with zebrafish embryos (also considered a 3R tool up to <120 h post fertilization) add an additional level of biological organization that includes the complexity of a whole organism. These two *in vitro* models together with the several assessed endpoints of toxicity in this study provide an overall picture of the toxicity of PsNPs, WAF and the mixture of them both on a cellular and embryonic level. Fish are important model systems in environmental biology and embryology, such when studying the effects of eco-toxicants of concern both to aquatic organisms and to human health, since these are often released into aquatic environments (Bols et al., 2005). Therefore, the models used in this study are relevant tools when evaluating ecotoxicological effects in the aquatic environment induced by nanoplastics and other environmental pollutants, such as organic contaminants found in crude oil (e.g., PAHs). However, if (or to what extent) these effects observed *in vitro* under controlled laboratory conditions apply to aquatic organisms in the wild is yet to be determined. Thus, there is a need of both further toxicological and ecotoxicological studies to examine the effects of toxicants on both individual organisms and on ecosystems.

Cytotoxicity of PsNPs and WAF single and combined exposure in RTgill-W1 cells

Cell viability was measured in RTgill-W1 cells with alamarBlue, CFDA-AM and Neutral Red fluorometric dyes that monitor metabolic activity, cell membrane stability and lysosomal activity, respectively. These protocols have been used in several previous studies in different formats (Dayeh et al., 2003; Dayeh et al., 2005; Dayeh et al., 2013; Bols et al., 2005). They are useful, cost-effective, simple and rapid tools when studying environmental toxicity at the cellular level and provides a broad overview of the sensitivity of cells to chemical contaminants (Dayeh et al., 2013). RTgill-W1 and RTL-W1 are two examples on fish cell lines previously used to study cytotoxicity of PAHs (Schirmer et al., 1998; Schirmer et al., 2000).

Cytotoxicity in response *to PsNP single exposures*

PsNP single exposures (1-100 µg/mL) were generally not cytotoxic and did not have a statistically significant effect on metabolic activity, lysosomal activity, or membrane stability in RTgill-W1 cell lines (fig. 5). However, there was a slight decrease in relative cell viability (%) in cells exposed to the highest concentration of PsNP (100 µg/mL) especially in the alamarBlue but also in the CFDA-AM assay, indicating that high concentrations of PsNPs may have a slight effect on metabolic activity and membrane stability/enzymatic activity, and that the potential toxic effects in that case are targeting these endpoints before affecting the lysosomal activity, since there was no trend of decreased cell viability in the Neutral Red assay (fig. 5).

Cytotoxicity in response to WAF single exposures

Cytotoxicity in RTgill-W1 cells exposed to WAF single exposures (10-90%) increased with higher WAF concentrations in a dose-dependent manner especially in the alamarBlue but also in the Neutral Red assay (fig. 5). The relative cell viability was significantly decreased in cells exposed to the highest concentrations (80-90%) of WAF (compared to the lowest concentration of 10% WAF) in alamarBlue, CFDA-AM and Neutral Red assay, indicating that high WAF concentrations affect both metabolic activity, membrane stability and lysosomal activity. However, in the alamarBlue assay, which revealed the most dose-dependent pattern, a significant cytotoxic effect was observed already at 40% WAF concentrations, indicating that the toxic compounds found in WAF mainly affect the metabolic activity in lower WAF concentrations and that this may be a more sensitive endpoint to study. The results also indicate that the lysosomal activity is affected after mid-high WAF exposure, since the dose-dependent

pattern was mainly observed in cells exposed to 40-90% WAF, with a significant decrease in cell viability after 70-90% WAF exposure. No significant decrease in cell viability was observed in cell exposed to low to mid WAF concentrations in the CFDA-AM assay, indicating that the membrane stability/enzymatic activity is affected first at high WAF exposures, and that this endpoint is not as sensitive as the metabolic activity when observing the cytotoxic effects of WAF single exposures.

Cytotoxicity in response to PsNP and WAF mixtures

A decrease in relative cell viability in a more distinct dose-dependent manner was seen in RTgill-W1 cells exposed to the mixtures of PsNPs and WAF when using the alamarBlue assay compared to the CFDA-AM and Neutral Red assay (fig. 6). This is similar to what was observed in cells exposed to WAF single exposures (fig. 5) and indicating that the mode of action of the mixture is to target the metabolic activity rather than lysosomal activity or membrane stability/enzymatic activity. However, in all three assays a lower relative cell viability was observed in cells exposed to the highest concentration (100 µg/mL) of PsNPs in mixture with WAF, especially in the highest WAF concentration (80%), indicating an additive effect on all three endpoints (lysosomal activity, membrane stability and metabolic activity). No clear difference in cytotoxicity was observed in cells exposed to mixtures with 10% and 50% WAF in any of the cell viability assays, in contrast to the measurements of generation of ROS and EROD-activity (see below).

Interpretation of the cell viability assays

Enzymes with both mitochondrial and cytoplasmic locations, such as diaphorases, are today thought to be responsible for the reduction of alamarBlue (Bols et al., 2005), compared to the original thought that only mitochondrial enzymes reduced the dye (de Fries and Mitsuhashi, 1995). Thus, impairment of cellular metabolism is indicated by a decline in alamarBlue reduction, rather than specific mitochondrial dysfunction (Bols et al., 2005).

The explanations about what CFDA-AM assay is monitoring can be more complex than only “impairment to plasma membranes” due to subtle differences in how the toxicant acts or how the test is performed. Bols et al. (2005) discuss that fluorescent readings in the CFDA-AM assay measures changes of the total esterase activity in the 96-well plate cell culture, since the dye that are applied to the exposed cells is not removed before the fluorescent reading and CF both inside and outside of the cells are measured. Treatment with toxicants (like organic pollutants from crude oil and/or nanoplastics like in this project) may decrease the esterase activity by interference with either plasma membranes or cellular esterases (Bols et al., 2005). Partial or complete lysis of the cells are one possible explanation of how the esterase activity can be decreased by plasma membrane integrity loss. The esterases are lost when they are released into the medium after toxicant exposure and the medium is removed and replaced with CFDA-AM solution (Bols et al., 2005). A change in plasma membrane integrity could be another explanation of the reduction in esterase activity, where the esterases remain within the cells that are attached to the microplate surface, even if the “cytoplasmic constitutes are lost to the medium” (Bols et al., 2005). A third explanation to a decline in esterase activity could be that the toxicant exposures could interfere with cellular esterases specifically, while the membrane integrity is left unimpaired. For example, a toxicant can inhibit the catalytic activity of the esterases or interfere with the CFDA-AM substrate uptake across the plasma membrane (Bol et al., 2005).

The endpoints measured in the neutral red assay is either accumulation or retention of the dye, which depends on how the exposure of toxicants affect the plasma membrane integrity or specifically the lysosomes. Although, both a functioning lysosome, an intact plasma membrane and adequate energy metabolism is affecting the retention of the neutral red (Bols et al., 2005).

Shirmer et al. (1998) detected "hints of specific lysosomal damage" to the RTgill-W1 cell line after xenobiotic exposure when using the neutral red which was not observed with other indicator dyes, indicating that lysosomal impairment in some cases can occur before decrease in cell viability. However, lysosomal activity was not observed to be a more sensitive endpoint compared to metabolic activity (alamarBlue assay) after PsNP or WAF exposure in this project, it may be a more sensitive endpoint compared to the membrane stability/enzymatic activity (CFDA-AM assay).

CYP1A dependent EROD-activity in RTL-W1 cells after single and combined exposure of PsNPs and WAF

Fish cell lines are useful *in vitro* models when examining xenobiotic metabolizing enzymes, where CYP1A or P4501A is one of the most studied (Bols et al., 2005). Measurements of CYP1A catalytic activity as EROD-activities is one way to demonstrate the induction of CYP1A (Lee et al., 2003). Dioxin-like compounds act as ligands for the aryl hydrocarbon receptor (AhR), which has been identified in for example RTL-W1 cell lines (Billard et al., 2002). They have been shown to cause strong induction of CYP1A in rainbow trout liver cells like RTL-W1 (Lee et al., 2003), whereas little or no induction was seen in other liver cell lines from rainbow trout, like RTH-149 and R1 (Lorenzen and Okey, 1990; Schirmer et al., 2000). Thus, we found the RTL-W1 cell line suitable for the measurements of EROD-activities in this study.

EROD-activity in response to PsNP and WAF single exposures

No increase in resorufin production was observed in RTL-W1 cell line after PsNP single exposures (1-100 µg/mL), indicating that PsNPs do not induce CYP1A dependent EROD-activity. No statistically significant increase in EROD-activity was observed in the RTL-W1 cells exposed to WAF single exposures. However, there was a dose-dependent increase in EROD-activity in the cells exposed to 10-50% WAF, where the 10% WAF exposed cells had no increase in resorufin production, and the cells exposed to 50% WAF produced the highest amount of resorufin (fig. 9). There was a pattern of decreasing amount of produced resorufin with higher WAF exposure in cells exposed to 60-80% WAF. This may be due to high cytotoxicity, which was observed in cells exposed to high WAF concentrations in the cytotoxicity assays (fig. 5), and this observation is also in accordance with the lower ROS-generation in cells exposed to the highest (80%) WAF single exposure (fig. 7). This indicates that the resorufin production at 50% WAF exposure is the "limit" of what the cells can produce before the concentration is "too toxic" to measure EROD-activity, which is a more sensitive endpoint. These results also indicates that the North Sea marine oil contains compounds that act as ligands for the AhR and contributes to CYP1A induction.

As mentioned in the introduction, induction of liver CYP1A caused by compound mixtures from crude oil has been shown in many fish species such as Atlantic salmon (Gagnon & Holdway, 1999), rainbow trout (Ramachandran et al., 2004) and others (Danion et al., 2014; Frantzen et al., 2015; Jung et al., 2009). Since compounds of PAHs and heavy metals are often found in crude oil (Nwaichi et al., 2014), we hypothesized that the WAF contains PAHs and that this would affect the cells. One indication that we have PAHs in our WAF is the induction of EROD-activity in the WAF single exposed cells, since they have significantly induced CYP1A dependent EROD-activity in previous studies (Pacheco and Santos, 1997). Loss of cell viability is one type of cytotoxic response previously observed when the PAH benzo[a]pyrene has been used as eco-toxicant to fish cell lines to study the role of xenobiotic metabolism. Bols et al. (2005) write that there is a correlation between the susceptibility to benzo[a]pyrene of piscine cell lines and the expression of inducible CYP1A, therefore "this cytotoxicity can be

attributed to the generation of toxic metabolites through a pathway(s) in which an essential step(s) is mediated by induced CYP1A".

EROD-activity in response to PsNP and WAF mixture exposures

No statistically significant increase in EROD-activity was observed in RTL-W1 cells exposed to the mixtures of PsNPs and WAF. However, a trend of increasing amount of resorufin was observed in cells exposed to the 10% WAF mixtures (fig. 10) compared to cells exposed to 10% WAF single exposure (and to cells exposed to PsNP single exposures), in which no resorufin was produced (fig. 9). This indicates that addition of PsNP stimulates a higher EROD-activity in the cells when combined with WAF and is contributing to an additive effect, which was also observed in the cytotoxicity assays (fig. 6). The highest concentration (100 µg/mL) of PsNPs contributed to a higher increase in EROD-activity compared to lower concentrations (10-50 µg/mL) of PsNPs in both 10% and 50% WAF mixtures (fig. 10), which aligns to the hypothesis that PsNPs have an additive effect in combination with organic pollutants found in the WAF. In contrast to the 10% WAF mixtures, the 50% WAF mixtures produced lower amount of resorufin compared to cells exposed to 50% WAF single exposure, which could be interpreted as a protective effect. However, it may be more likely that the cells are already stressed at 50% WAF (as seen in the cells exposed to 50% WAF single exposures in figure 9) and that the addition of PsNPs rather is having a cytotoxic effect contributing to less viable cells that are able to convert ethoxyresorufin to resorufin. This aligns with that no induction of CYP1A dependent EROD-activity was observed in cells exposed to the 80% WAF mixtures (fig. 10) or to the higher concentrations of WAF single exposures (fig. 9) and with the cytotoxicity measurements with WAF single exposed cells. The difference in response between cells exposed to 10% and 50% WAF mixtures was more distinct compared to the effects measured in the cytotoxicity assays, indicating that the EROD-activity is a more sensitive endpoint than metabolic activity, lysosomal activity and membrane stability.

One explanation for the additive effect seen in cells exposed to the mixtures with PsNPs and WAF in the cytotoxicity and EROD-activity assays may be that higher concentration of PsNPs, which equals more available nanoparticles in the exposure solution surrounding the cells, increases the possibility that they are affecting the cell membranes or are taken up by the cells with compounds from the oil adsorbed on the particle surface. As mentioned in the introduction, earlier studies have examined how the interaction between nano-sized plastics or carbon nanomaterials and co-occurring contaminants can affect the toxicity by acting as a vector and by affecting the bioavailability (Sanchís et al., 2016). The type of contaminant can determine the combined toxicity (Baun et al., 2008). MWCNT are able to either increase toxicity (Schwab, 2013) or enhance the bioavailability (Su, 2013) of co-occurring contaminants while single walled carbon nanotubes have been shown to decrease the bioaccumulation of pyrene (Petersen, 2009). When Sanchís et al. (2016) exposed *Daphnia magna* to binary mixtures of fullerene soot and malathion, the malathion was unexpectedly released inside living organisms and nanomaterials aggregates acted as a vector with an increase in toxicity proportional to the size of the carbon nanomaterials aggregates. If organic pollutants adsorbed on the PsNP surface is released inside living organisms or cells needs to be studied further, but if so, this could be one of the factors driving the combined toxicity of PsNPs and North Sea marine oil (and possibly other pollutants in the marine environment), where the PsNPs are increasing the toxicity of the organic pollutants by acting as a vector and enhancing the bioavailability. One hypothesis is that the nanoplastics may have a higher concentration of pollutants like PAHs than the surrounding environment, since the concentration of PCBs held by microplastics can be one-million-fold higher that surrounding water (Betts, 2008). Thereby the PsNPs may increase the direct exposure of pollutants like PAHs to organisms since they might be absorbed by organisms after ingestion and/or penetrate their membranes. Earlier studies have also shown that nanoparticles can accumulate in tissues in aquatic organisms

(Waring et al., 2018), this may be true also for the PsNPs with adsorbed pollutants, which possibly could enhance the toxicity, even if this also need to be examined further in future studies.

Generation of reactive oxygen species in RTgill-W1 cells in response to single and combined exposure of PsNPs and WAF

ROS generation in response to PsNP and WAF single exposures

No significant difference or trend was observed in ROS generation in RTgill-W1 cells exposed to different concentrations (1-100 μg/mL) of PsNP single exposures after 5 or 6 h (fig. 7), indicating that PsNPs themselves do not induce ROS generation in cells. In contrast to a previous study where polypropylene has been shown to cause oxidative stress in human neuronal cell lines (Waring et al., 2018). RTgill-W1 cells exposed to 50% WAF single exposures did not significantly differ in intracellular ROS generation compared to cells exposed to 10% WAF. However, the generation of ROS was significantly lower ($p=0.031$) in cells exposed to the highest concentration (80%) of WAF (fig. 7). This may be caused by high cytotoxicity in these cells, which was observed in cells exposed to 80% WAF single exposures in the cytotoxicity measurements (fig. 5), this is also in accordance with the trend seen in the EROD-activity measurements (fig. 9).

ROS generation in response to PsNP and WAF mixture exposures

No significant differences in ROS generation were seen after 5 and 6 h exposure of the mixtures (fig. 8). However, in the 10% WAF mixtures the cells exposed to lower PsNP concentration (10 μg/mL) had higher ROS generation compared to 10% WAF mixed with higher PsNP concentration, which may indicate a slightly protective effect of the addition of PsNPs. The ROS generation in the mixture (fig. 8) was also lower in general compared to ROS produced in cells that were exposed to the same percent of WAF single exposure (fig. 7), this may also indicate a protective effect of the PsNPs, even if the fact that DMSO used in the single exposures could have affected the data (higher values), so it can't be excluded that the decreased ROS is due to the use of the probe solved in ethanol in the mixtures.

As observed in the EROD-activity assay (fig. 10), a clearer difference was seen between 10% and 50% WAF mixture in the DCFH-DA assay (fig. 8) measurements compared to the alamarBlue, CFDA-AM and Neutral Red measurements (fig. 6), this indicates that also ROS generation is a more sensitive endpoint to measure than cytotoxicity.

Zebrafish embryo toxicity in response to PsNP and WAF single and combined exposures

Sublethal and lethal effects in zebrafish embryos tend to increase with higher concentration of WAF alone, and with higher PsNPs concentrations in the mixtures (fig. 11). Majority of the Zebrafish embryos exposed to 50-80% WAF had lethal effects, meaning they were non-viable or non-hatched, or had sublethal effect as oedema, scoliosis, or lacked inflated swim bladder (or a combination of these effects, all that had oedema lacked swim bladder) (fig. 12). This suggests that these concentrations, for example after an oil spill in the ocean, may be highly toxic to the aquatic organisms. Sublethal effects seen in embryos exposed to 10% WAF or 50 μg/mL PsNPs was mostly that they lacked inflated swim bladder (fig. 12), indicating that this "quality" seems to be more sensitive for exposure of lower concentration of PsNPs or WAF. However, the sublethal effects seen in the negative control was also lack of inflated swim bladder and lethal effects was due to coagulated embryos, which may be caused by bad egg quality. Therefore, it can't be excluded that the effects seen in 10% WAF and 50 μg/mL is due to the egg quality and not the exposures. The increased toxicity seen in the mixtures with higher PsNP concentration (100 μg/mL) compared to lower (50 μg/mL) indicates that PsNPs have an

additive effect in combination with WAF, as observed in the cytotoxicity and EROD-activity measurements.

Environmental pollutants like PAHs are posing a potential risk to fish populations and both laboratory and field suggests that exposure of PAHs during early life stages can cause embryotoxic effects in fish in a similar way as the most potent planar halogenated hydrocarbons (PHHs) (more specific 2,3,7,8-tetrachlorodibenzo-p-dioxin), which toxic effects is mediated by AhR (Billard et al, 2002). This is in accordance with the sublethal- and lethal toxic effects seen in the zebrafish embryo toxicity test in this project and to the increased resorufin production in cells exposed to WAF single exposures, and PsNP/WAF mixture exposures in the EROD-activity assay and in accordance with our hypothesis that the WAF contains organic pollutants for example PAHs. The CYP1A induction potency and AhR-binding affinity is used to predict the potency of PHHs. Billard et al. (2002) examined the ”binding affinity of a test set of PAHs that had been previously ranked for their potency for inducing teleost CYP1A.”. Their results suggest that the PAH potency for CYP1A induction is predicted by the binding ability of PAH to teleost AhR. Previous studies have shown that persistent organic pollutants like PCBs or PAHs adsorbed to plastic particles (nurdles) can affect both the environment and human health negatively, and which in the long-range transport of toxic pollutants can play an essential role (Karlsson et al., 2021).

Ecotoxicological risk assessment of nanoparticles

To assess Nps ecotoxicological risk, there is a need for consideration of their surface area (Yang, 2010), size (Jiang et al., 2009), aggregation/agglomeration properties in suspension (Ashraf et al., 2018), shape (Brown et al., 2007), functionalization, their diffusion capacity, and the possible interaction with other contaminants. It is therefore of interest to further study how these factors or other experimental conditions like pH or the presence of organic matter may influence the combined toxicity of PsNPs and organic pollutants (e.g., from crude oil). There is also a need for assessment of the uptake of nanoplastics with adsorbed organic pollutants from the water column by primary producer organisms and their possible transfer to food chains in the aquatic ecosystems. In this study we did in not observe a significant increased cytotoxicity, EROD-activity or ROS generation after PsNP exposure (alone) in rainbow trout cell lines, or increased toxicity in zebrafish embryos. However, the results obtained in the present study indicates an additive effect when adding PsNPs to the mixtures, the nanoplastics is surrounded by a mixture of pollutants in the aquatic environment and the results from a previous study (Cedervall et al., 2012) showed that commercially manufactured PsNPs affect lipid metabolism and feeding behavior of the top consumer when transported through an aquatic food chain from algae, through zooplankton to fish. Altogether this makes it interesting to study endpoints like feeding behavior and lipid metabolism of the top consumer also after exposure to mixtures with nanoplastics and chemical pollutants.

Book et al. (2019) investigated which properties are relevant for the ecotoxicological hazard profile of manufactured silica nanomaterials (MSNM) and reported great variety in toxicity when comparing species with different physiology, trophic levels, and morphology. MSNM were not toxic to the water flea Daphnia magna, the algae *Raphidocelis subcapitata* or bacterium *Pseudomonas putida* at least up to concentrations at several hundred mg/L, whereas most of the silica particles were cytotoxic to fish cell line (RTgill-W1) from Rainbow trout. One may discuss that it is possible that this variation applies also for the PsNPs combined with WAF, and that RTgill-W1 in that case is a suitable cell line to use during risk evaluation due to higher sensitivity, but that there is a need to have in mind that the effects observed can vary in different organisms which may be true also for the toxicity seen in this study. Disturbance of membrane function and/or structure was suggested by Book et al. (2019) to be one of the modes

of toxic action of hydrophilic MSNM, which can explain why algae and bacteria, organisms with a cell wall, were less sensitive compared to fish cell line. Whereas our results from the cytotoxicity studies with the mixture exposures indicate that the metabolic activity is the main target, although cell membrane integrity also is affected in higher exposure concentrations. Henceforth, Book et al. (2019) showed that the toxicity was clearly affected by surface modifications, and when calculating the toxicity to fish cell lines in relation to mass (mg/L), particles numbers and surface area they found that the toxicity-determining factor for the uncoated colloidal silica particles is the total surface area. This may indicate that the toxic effects obtained in the present study also are affected by the total surface area where a smaller size (larger surface to volume ratio) of the PsNPs is correlated with higher toxicity.

Limitations section

There is a need for more zebrafish embryo experiments. Due to the varying zebrafish egg quality, we did not get enough data to calculate an EC50-value and could only use the data from two of our results. It was noticed that the egg quality depended on which aquarium the eggs were collected from.

As mentioned in the results section the ROS data is presented as raw data subtracted by the emitted background light (and not relative to the negative control) which needs to be taken into consideration when interpreting the results, since the cells themselves do naturally produce ROS. The reason why we did not divide the data by the negative control is that we obtained high fluorescence values in the negative control when the DCFH-DA probe was solved in DMSO instead of ethanol, therefore the negative control is represented separately. Unfortunately, this was noticed first when three measurements had been performed with the single exposures, and therefore the probe was solved in DMSO for the single exposures and ethanol for the mixtures. The generally higher fluorescence values when using the probe solved in DMSO compared to ethanol need to be taken into consideration when comparing the results from the single and mixture exposures to each other.

The calculated substrate turnover per unit time in the EROD-assay was not standardized based on protein content of each well (measured with the fluorescamine assay) due to reading problem. Instead, the EROD data was normalized against cells per wells. However, the pipetting technique was practiced before the start of the experiments and it was controlled by measuring cytotoxicity in cells exposed to only L-15 medium, which revealed similar fluorescence values in the whole 96-well plate (where cell in all wells were exposed to only L-15 medium). Pipetting error in general, either when preparing the concentrations or when seeding/washing the cells cannot be excluded, for example do the RTL-W1 easier detach from the bottom of the well (but this was checked for in the microscope).

Due to high variance in the EROD-assay it would be beneficial to have more time to perform more EROD-activity measurements to get statistically significant results.

Some emitted fluorescence spill over between the wells cannot be excluded since we used transparent plates. However, when comparing black and transparent 96-well plate no significant difference was observed.

Future research

Future research comparing toxic effects of PsNPs of different sizes in combination with WAF would be beneficial. A follow up on the zebrafish embryo toxicity studies could be to perform a zebrafish locomotion assay looking at abnormal spontaneous movements, and to collect enough data to calculate a 96-h-LC50-value. Additionally, it would be interesting to optimize the DCFH-DA probe to zebrafish embryos. Analyzing the WAF content and also the

concentration of organic pollutants (e.g., PAHs) adsorbed to PsNPs together with analyzing the gene expression in the rainbow trout gill/liver cells after the exposures would be beneficial to get an overall picture of the single and combined toxicity of PsNPs and WAF. It would also be interesting to examine how longer exposure times affect the endpoints and how the presence of for example naturally occurring particles can affect the combined toxicity. Some advantages with *in vitro* studies like the ones used in this study is that they are following the 3R principle and also that researchers can have more control on the factors that may induce toxicity. However, this kind of studies is often less representative of what is occurring in the environment. Therefore, an interesting complement would be to study the toxicity of single and combined exposure of PsNPs and WAF in more complex laboratory systems. In addition, it would be interesting to study if the nanoplastics with adsorbed organic pollutants is enhancing the toxicity by accumulating in the tissues of aquatic organisms, are transported through aquatic food chains or are affecting for example the lipid metabolism or feeding behavior of the top consumer as seen in previous studies, since this needs to be taken into consideration when doing ecotoxicological risk assessments.

Conclusions

Toxic effects associated with single- and combined exposures of polystyrene nanoparticles (PsNP) and water-accommodated fractions (WAF) of North Sea marine oil was examined by measuring acute toxicity and by exploring more sensitive *in vitro* assays on two *in vitro* fish models. This included cytotoxicity studies where metabolic activity, membrane stability/permeability and lysosomal activity were measured in RTgill-W1 cell lines and also their potential to generate ROS. Additionally, studies of the induction of CYP1A dependent EROD-activity in RTL-W1 cell line and toxicity studies in zebrafish embryos were accomplished.

PsNPs alone was generally not found to be toxic and did not have a statistically significant effect on metabolic activity, lysosomal activity, or membrane stability. However, the metabolic activity was slightly decreased in cells exposed to the highest concentration of PsNPs. The PsNPs did not significantly affect the induction of CYP1A dependent EROD-activity, ROS generation nor the lethal- and sublethal effects in zebrafish embryos.

WAF single exposures were shown to be significantly cytotoxic, mainly affecting the metabolic activity but also the lysosomal activity in a dose-dependent manner. Membrane stability was affected first after exposure to the highest concentrations of WAF, indicating that this is not the main target of the organic pollutants found in WAF. There was a dose-dependent increase in resorufin production (although not statistically significant) in cells exposed to low-mid WAF concentration, and a dose-dependent decrease in cells exposed to mid-high WAF. These results indicate that organic pollutants found in the WAF induce CYP1A dependent EROD-activity, but that the mid WAF concentration is the "limit" before the concentration is too cytotoxic which aligns with the results in the cytotoxicity- and ROS generation measurements, and with the hypothesis that the WAF may contain PAHs. Sublethal (oedema, scoliosis, lacked inflated swim bladder) and lethal (non-viable, non-hatched) effects in zebrafish embryos increased with higher concentration of WAF.

The results from the mixture experiments indicate that PsNPs had an additive effect in combination with WAF both on cytotoxicity (metabolic activity, membrane stability and lysosomal activity), EROD-activity and on zebrafish embryo toxicity. The results indicate that the cytotoxic mode of action of the mixtures is to target the metabolic activity rather than lysosomal activity or membrane stability/enzymatic activity, which is similar to what was observed in the WAF single exposure experiments. They also suggest that PsNPs may increase the toxicity of the organic pollutants found in crude oil by acting as a vector and enhancing the bioavailability. Measurements of ROS generation and EROD-activity was shown to me more

sensitive *in vitro* assays since differences in the endpoints measured could be observed at lower exposure concentrations.

Altogether, PsNPs alone was generally not found to be toxic, WAF single exposures induced toxicity both when measuring cytotoxicity (mainly affecting metabolic activity but also lysosomal activity and membrane stability), CYP1A dependent EROD-activity and sublethal- and lethal effects in zebrafish embryos. The obtained results indicated an additive toxic effect when adding PsNPs to the mixtures both when looking at cytotoxicity, EROD-activity and toxicity in zebrafish embryos. These results contribute to an increased understanding of the interactions of PsNPs with other environmental pollutants in the marine environment, which is useful for environmental risk assessment and management, and to provide regulatory constraints. Although, more research is needed to get an overall picture of these effects and to evaluate how relevant these effects are in the ecosystem, which is a much more complex system and where the plastic nanoparticles are a minority.

Acknowledgements

I would like to thank my supervisors Joachim Sturve and Marianne Brookman-Amissah for both allowing me to perform this project and for the help in the laboratory and by answering questions. I would also like to thank Jari Parkkonen for helping me with the risk assessment in KLARA, Britt Wassmur for guiding me in the cell lab and all other personnel at the department of biological and environmental sciences that have helped me during my project. I also want to thank my examiner, Kristina Sundell.

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Popular scientific summary

Nanoplastic- and crude oil pollution: A toxic mixture?

Scientists say nanoparticles and compounds found in crude oil can be toxic to water living organisms. What about the mixture of them both?

The marine environment is polluted by a mixture of pollutants, where plastic debris and crude oil are two sources of concern. Plastic pollution are widespread and rapidly increasing in ecosystems on land (terrestrial) and in water (aquatic) due to increased consumption, irresponsible waste handling and since they stay in nature for a long time. But did you know that tiny plastic particles, that cannot be visualized by the human eye, can be found in the environment? They are called nano plastics since they are between 1-100 nm, where one nanometer is a millionth of a millimeter!

Nanoparticles in general, can be intentionally produced to give materials or products specific characteristics and are then called engineered or manufactured nanoparticles. Some examples on nanoparticles you may use in your daily life is mineral nanoparticles of titanium oxide found in sunscreen that contribute to its UV-protection, antimicrobial nanoparticles found in sport equipment to prevent bacterial growth or carbon nanotubes making your baseball bat lighter.

Nano plastics can be either engineered particles or be created by degradation of larger plastic items. They can be taken up by organisms and reach the blood stream, since their small make it possible for them to cross biological barriers such as cell membranes. They can therefor affect marine organisms and potentially be toxic to cells, by causing membrane damage, oxidative stress, immune responses or by being genotoxic, which means damage genetic material (DNA) in a cell and causing mutations, which may lead to cancer.

Several studies shows that small plastic particles can hold organic pollutants on its surface (adsorb), like compounds called poly aromatic hydrocarbons (PAHs), which can be found in crude oil. Interactions between nano plastics and surrounding contaminants may decrease or increase the toxicity of that contaminant, by changing how much of it is taken up by an organism. However, the knowledge about interactions between micro- and nano sized plastics and marine pollutants such as compounds from crude oil is limited, and it is still a matter of debate whether this adsorption is posing multiple health risks to organisms.

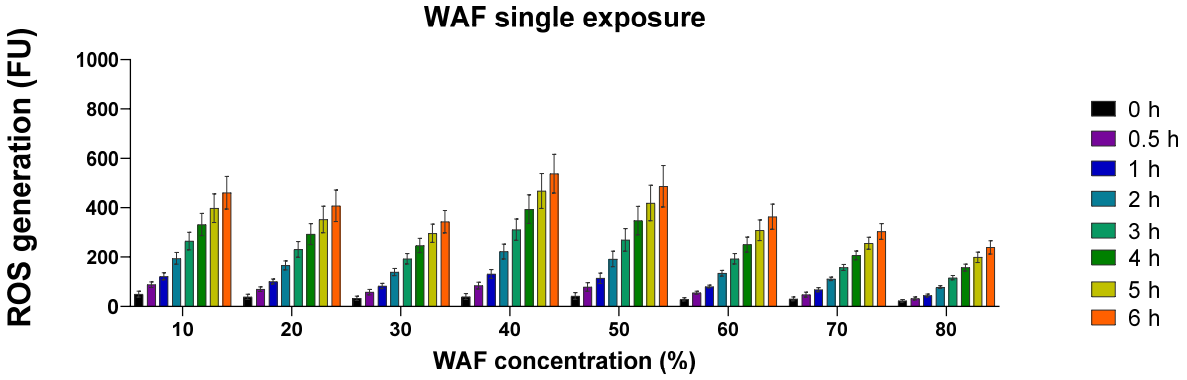
The aim of this study was to gain knowledge on the possible toxic effects associated with single exposure of nanoparticles of polystyrene (PsNPs, one of the most common plastic polymers) and Water accommodated fraction (WAF) of North Sea marine oil (compounds from the oil mixed in water), compared to a mixture of both. Two fish models were used, rainbow trout cell lines and Zebra fish embryos, to reflect different levels of biological organization (cells, individuals). Additionally, we aimed to explore more sensitive assays for the assessment of nanoparticle and WAF toxicity, meaning investigate which assays are suitable for looking at sub lethal effects where the cells or organism is affected but not killed.

Mechanisms of toxicity were studied by exposing Rainbow trout gill cells or zebrafish embryos to a range of concentrations of PsNPs (25 nm) alone, WAF or to a combination of them both for 24 h. The percentage of living or healthy cells (cell viability) was measured by using the fluorescent probes Alamar Blue, CFDA-AM and Neutral red. An assay called DCFH-DA was conducted to observe changes in generation of intracellular reactive oxygen species (ROS) up to 6h after exposure. Induction of CYP1A-dependent EROD activity was measured in Rainbow trout liver cells. Toxic effects in zebrafish embryos were observed with microscope.

PsNP alone was generally not found to be toxic for rainbow trout cells or zebrafish embryos. WAF was toxic to cells both when measuring cell viability and EROD-activity but did not

increase ROS generation. The results from the mixture of PsNP and WAF indicated that PsNP increased the toxicity of organic pollutants found in crude oil both when measuring cell EROD-activity, cell viability and zebrafish embryo toxicity.

Appendices


WAF single exposure
ROS generation (FU)
1000
800
600
400
200
0
10
20
30
40
50
60
70
80
WAF concentration (%)
0 h
0.5 h
1 h
2 h
3 h
4 h
5 h
6 h