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Polycarbonate and Polystyrene Nanoparticles Act as Stressors to the Innate Immune System of Fathead Minnows (*Pimephales Promelas,* Rafinesque 1820**)**

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Meiner Familie

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INDEX OF ABBREVIATIONS

ABBREVIATION	EXPLANATION
BPA	BISPHENOL A
DLS	DYNAMIC LIGHT SCATTERING
EPS	EXPANDED POLYSTYRENE
HBCD	HEXABROMCYCLODODECANE
M	METER
MT	METRIC TONNES
MMT	MILLION METRIC TONNES
NM	NANOMETRE
NP	NANOPARTICLE
PAA	POLYACRYLIC ACID
PAH	POLYCYCLIC AROMATIC HYDROCARBONS
PBDES	POLYBROMINATED DIPHENYL ETHERS
PBT	PERSISTENT BIOACCUMULATIVE AND TOXIC SUBSTANCES
PC	POLYCARBONATE
POPS	PERSISTENT ORGANIC POLLUTANTS
PS	POLYSTYRENE
SPP	SPECIES
TLR	TOLL LIKE RECEPTORS

I. INTRODUCTION

Plastic materials have high significance for our society as they are durable, cheap and easily moulded to a variety of shapes and forms. Nearly every daily routine includes contact with some kind of plastics, whether it is an early morning cup of coffee served in a styrofoam cup from the take away, sitting on a bench in a subway or using an office computer. The amount of plastics is steadily increasing since the beginning of the mass production in the 1940s. However, even highly developed waste management systems only recycle about one third of the plastic that is in use (PlasticsEurope 2015). As a consequence, plastic debris often ends up in undesired sinks in the environment such as beaches, rivers, and oceans. It is estimated that more than five trillion plastic pieces with a cumulative weight of over 250,000 metric tonnes (MT) float in the oceans (Eriksen *et al.* 2014). These plastic particles have different shapes and sizes, influencing their effects on the environment. For example, larger plastic items such as bottles, nets, or bags can lead to the entanglement and suffocation of different animals including turtles, fish, birds, and mammals (Lusher 2015).

Smaller plastic items at microplastic and nanoplastic scale may be interacting with organisms on tissue and cellular levels since their size allows them to be ingested, or pass through tissue or cell boundaries (Von Moos *et al.* 2012, Tenzer *et al.* 2013). Once inside a body, plastic particles can get in contact with organismal defence mechanisms, including cellular immune responses. The impact of internalized nanoplastics is likely of high significance for neutrophils, due to their respective phagocytic and killing role acting as one of first defence lines against potentially toxic agents. For example, it has been shown that parenteral application of TiO_2 nanoparticles in fish can modulate neutrophil

function, increase susceptibility to diseases, and decrease ability of an organism to defend itself against pathogens (Jovanovic *et al.* 2015b). Since nanoplastic concentration in the environment is expected to increase, and aquatic organisms are chronically exposed to potential nanoplastic particles, it is important to evaluate their possible adverse health effects in aquatic organisms.

We propose that evaluation of the neutrophil function can be used as a tool to assess the effects of plastic nanoparticles on innate immune response, aswell as explore interactions concerning possible interplay between toxicity of chemical compounds anddisease pathogenesis. Therefore, the overall hypothesis of this dissertation is that polystyrene and polycarbonate nanoparticles can modulate neutrophil function in fathead minnow (*Pimephales promelas*Rafinesque 1820). To test this hypothesis, the characterization of nanoplastic particles in extracellular biological fluids (plasma) or their substitutes (Hanks balanced saline solution) was performed and a battery of *in vitro* neutrophil function assays was used to determine effect of the nanoplastics.

II. LITERATURE SURVEY

1. Plastic in the aquatic environment

1.1. Amountof plastic in the ocean

The first studies of plastic debris in the ocean reported numbers for limited geographical regions that did not allow extrapolation to the global scale of aquatic plastic pollution(Carpenter *et al.* 1972, Shaw 1977, Gregory 1978). However, since 1970' the research efforts increased to overcome the shortage of information about the concentration of plastic debris in the ocean. The worldwide plastic production in 2013 reached 299 million MTs compared to 1.7 million tonnes in 1950 which accounts for an annual increase of 8.6 %(PlasticsEurope 2015)(Fig.1).

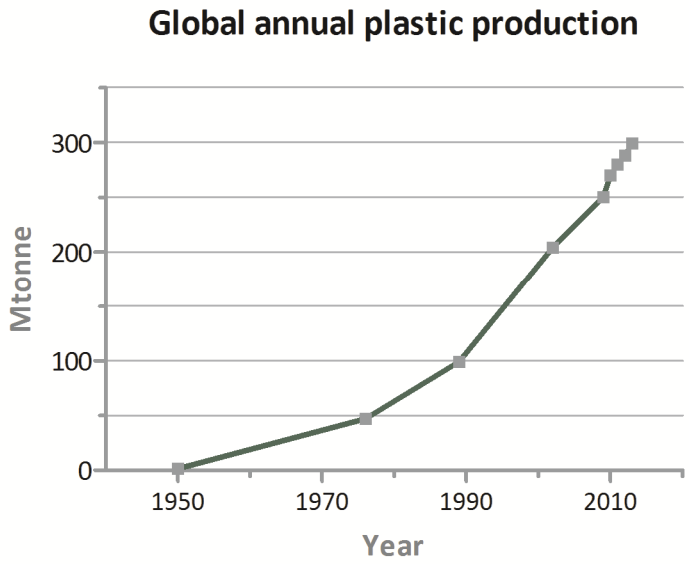

Figure 1.World plastic production in period from 1950 to 2013.Annual reported quantities include plastic materials (thermoplastics, polyurethanes), otherplastics (thermosets, adhesives, coatings and sealants) and polypropylene-fibres. (Data source PlasticsEurope 2014/2015)

Plastic in the marine habitat is increasing in comparable amounts to the

plastic production (Ryan and Moloney 1993). The techniques to collect data and calculate numbers have been constantly improving, and an increasing body of literature has recently allowed comparisons of different estimations in a meta-analysis approach(Kellen 2014).

For 2010 up to 12.7 million MT of plastic were calculated to have entered into the ocean (Jambeck *et al.* 2015). Another study by Eriksen et al. (2014) estimated a minimum of 5.25 trillion particles floating in the ocean weighing 268,940 MT (Eriksen *et al.* 2014) which does not include the amount of plastic on the seabed (Fig.2).

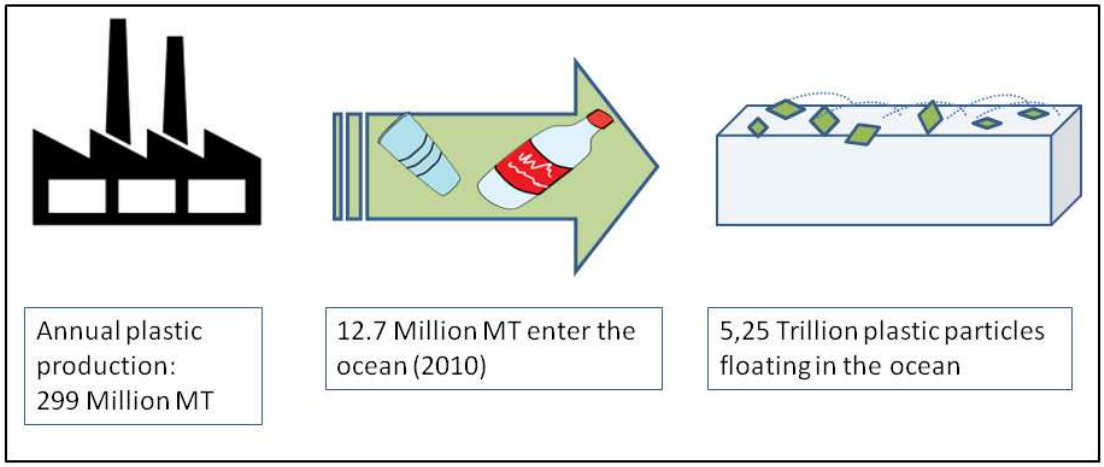

Figure 2. Relation between plastic production and pollution of the ocean.Significant shares of the annual plastic production enter into the aquatic environment which has been estimated to contain 5.25 trillion plastic particles in 2014.

The data collection originates mainly from four different methods: water sampling, beach litter collection, necropsy of dead animals and computer modelling (Kellen 2014). Combing the data of all the different collection methods with their specific uncertainties leads to comprehensive projections about the plastic concentration in the ocean (Kellen 2014). This data is of high significance

since it helps to understand the extent of the plastic pollution, specifies the different areas of the marine environment that are affected by plastic debris and highlights the ecosystems that are possibly impacted.

Plastic debris enters the environment in different ways. Some plastic is filled into landfills and gets incinerated. Some plastic is blown away by the wind and enters rivers and streams. Other plastic is negligently discarded and released into the environment. Fact is that only 26 % of the manufactured and discarded plastic are recycled (PlasticsEurope 2015) and until now no environmentally safe and sustainable system is developed to handle the ever increasing amounts of plastic debris.

Extensive research revealed several facts: the amount of marine litter keeps increasing even on remote islands. Ryan and Moloney (1993) counted 400 pieces of plastic stranded on the west side of inaccessible island in 1984 compared to more than four times the amount (1840 particles in 1990) (Ryan and Moloney 1993). Remote island areas are valuable locations to estimate the plastic debris transported from the currents in the ocean from more urbanized areas since additional input from urbanization is missing. The mass of microplastic exceeds by far the amount of macroplastic indicating the extent of macroplastic break down and the importance of a research focus on microplastics and nanoparticles (Norén 2008, Eriksen *et al.* 2014). Small particles tend to disappear from the sea surface, a phenomenon which has not been explained yet. It has been hypothesized that the removal process from the surface might be due to ingestion by organism, sinking to the sea floor by decreased buoyancy due to fouling organisms and increased weight and entrapment in settling detritus (Eriksen *et al.* 2014).

Secondly, certain areas in the ocean are more polluted due to different

currents that concentrate the plastic in the five giant plastic gyres (Fig. 3)(Barnes *et al.* 2009).On a global scale it has been observed that urbanization increases the waste production and the urbanization trend leads to a further increase of waste. Whereas wealthy societies tend to curb their waste production, east Asia counters this trend by an estimated increase of waste production from 520,550 MT per day in 2005 to 1.4 million MT per day in 2025 (Hoornweg *et al.* 2013).

Figure 3. Accumulation of plastic debris in the surface waters of the five main ocean gyres,-the North Atlantic, South Atlantic, North Pacific, South Pacific, and Indian Ocean gyres. Discarded plastics are caught in the swirling action and remain in the gyre where they gradually break down into smaller pieces. Data source: (Cózar *et al.* 2014)

1.2. Sources of plastic entering into the environment

Plastic in the ocean has four main origins: inefficient waste management, plastic pollution by the shipping industry, beach littering and natural disasters (Kellen 2014).The majority of plastic items are so called “short term plastics”, which is defined as plastic that has been in use for less than one year. Estimations calculated that of the 275 million metric tonnes (MMT) of plastic produced

worldwide in 2010 31.9 MMT were classified as mismanaged and a maximum of 12.7 MMT of plastic were estimated to end up in the ocean (Jambeck *et al.* 2015).

At the end of the product life cycle only 26 % of the plastic are recycled, 36 % are used for energy production and 38 % are placed in landfills (PlasticsEurope 2015). The main input of plastic into the ocean are land based sources, and most plastic is washed into the sea via rivers and stormwaters (Ryan *et al.* 2009). The tested freshwater bodies carry the same high concentrations of plastic as found in marine waters (Dris *et al.* 2015).The land based sources of plastic debris are as diverse as the useof plastic. Plastic debris originates from poorly managed landfills, untreated sewage and storm water discharges, street litter, agricultural plastics, industrial and manufacturing facilities with inadequate controls, recreational use of coastal areas and tourist activities (Fig. 4)(Pruter 1987, Barnes *et al.* 2009). Another entry route is washing laundry- up to 1900 synthetic fibres are released into the wastewater with a single wash (Browne *et al.* 2011).

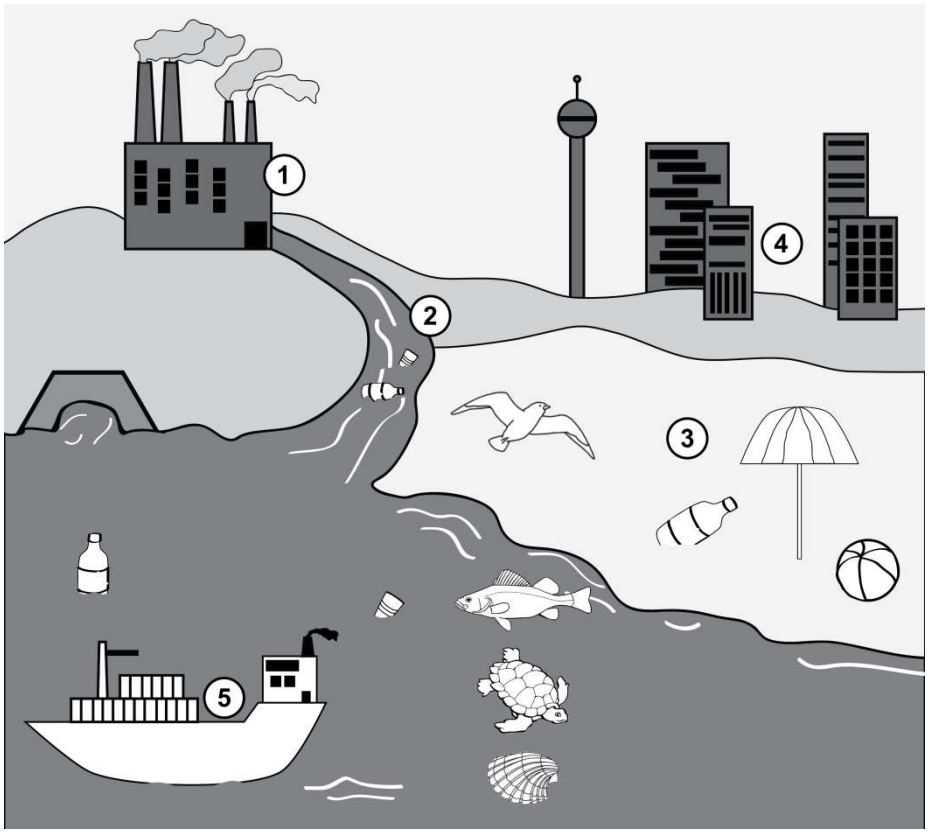

Figure 4. Schematic diagram showing the main sources for plastic in the marine environment and marine organisms affected by plastic.1. Industrial spillage, 2. Transport of plastic into the ocean via rivers carrying plastic debris; 3. Beach littering; 4. Mismanaged waste from urban areas; 5. Shipping industry.

Up to the 1960 many authorities considered the oceans as self-cleaning. Due to their vast geographic expanse it was widely accepted that they have an infinite capacity to assimilate waste of all kinds (Andrady 2003). This belief has changed over time, and after studies revealed the extent of debris in the ocean due to shipping activities, the annex V of Marpol in 1988 prohibited 'the dumping of plastic at sea'. This legislation decreased the plastic waste that could be backtracked to shipping activities, as indicated by measurements of the plastic content in stranded seabirds. The gut content changed in composition from primarily industrial plastics in the 1970s and 1980s to predominantly consumer plastics in the measurements taken from 1995 to 2004 (Van Franeker *et al.* 2011).

This was a rather surprising outcome since the implementation and enforcement of this law is often inadequate (Galgani *et al.* 2010). Furthermore potential ocean based sources include input from industrial and recreational shipping as well as oil and gas platforms and aquaculture facilities (Kershaw *et al.* 2011).

Natural disasters are another reason for plastic input into the ocean as shown in the example of the Tsunami 'Great Tohaku' that reached Japan on the 11th of March in 2011. It was estimated by the Japanese government that 5 MMT of debris were swept into the ocean of which 1.235 MMT were calculated to be plastic (Kellen 2014). This means that within a single event the tsunami released 216.79 kg / capita of the 5.7 Million inhabitants affected by the disaster. This is equivalent to the amount of almost 4 years of plastic waste generation (Kellen 2014).

1.3. Plastic production process

Plastic materials play an important role in our everyday life. Since the beginning of the mass production it is often replacing more conservative materials like paper, woods, glass and metals in increasing numbers due to low unit costs and an improved performance. Its' high value is due to its numerous characteristics as it can be used at a very wide range of temperatures, it is resistant to chemicals and light, products are strong and durable but the material can be easily molded to shape as a hot melt (Andrady and Neal 2009).

The main components of plastic materials are synthetic polymers. These polymers are mainly produced from petroleum based raw materials with 8 % of the global oil production being used in plastic synthesis (Andrady and Neal 2009).

Biodegradable plastics are made of biomass-based feedstock which results in the same chemical polymer product. Polymers can be formed in two ways; 1) by a heat driven condensation between two monomers and 2) the reaction of a molecule that has been activated and functions as a free radical. The free radical starts a chain reaction by interacting with another monomer and forms long chain polymers instantly (Bolgar *et al.* 2007).

Plastic polymers are barely used in their pure form. Additives are introduced into the production process to achieve a variety of desirable properties as for example to protect the plastic from embrittlement and colour fading. In a process called ‘Compounding’ a range of different additives are introduced to raw material (Bolgar *et al.* 2007). Plasticizers, as some of the most often used additives, help to render plastic flexible and durable. Other additives include thermal stabilizers which enable the processing at high temperatures, flame retardants, UV stabilizers to enhance the resistance to oxidation, inorganic fillers to reinforce the plastic material and other components such as colorants, opacifiers, mating agents, and lustre additives. Plastic contains additives to various amounts: from 5-15 % additives were detected in electronic waste (Schlummer *et al.* 2005)while up to 50% of additives were present in a soft PVC material (Mulder 1998).

These additives can leach from the polymers at various times during product life cycle (Sajiki and Yonekubo 2003, Lithner *et al.* 2011, Velzeboer *et al.* 2014), and their potential or established toxicity is a continuing topic of discussionamong researchers, frequently leading to controversial statements and strong opinions(Andrady and Neal 2009). It is impossible to assess the toxicity of additives in a general manner, since the variety of chemical compounds summarized under “additives” is too large forgeneral assumptions. For

example,some plasticizers such as diisononyl phthalate have a full EU health technology assessment and are considered to be safe for the use in PVC items, but other plasticizers like dibutyl phthalate require risk reduction measures (Andrady and Neal 2009). Due to their known or suspected toxicity, and to their large production volumes (11.1 million MT in 2009), the additives are presenting as considerable factors in the evaluation of the toxic impact of plastic (Leslie *et al.* 2011).

1.4. Plastic types

Plastic is chemically very diverse material made from synthetic or semisynthetic organic polymer. The production and modification of plastic polymers can be varied in numerous ways which leads to 'plastic' being a material highly heterogeneous in characteristics as well as in composition of a final product. The annual plastic production of 299 million MT includes more than 15 polymer types (PlasticsEurope 2015). Plastic polymers vary in the chemical process used in their synthesis, such as condensation, polyaddition, and cross-linking production process and also in the kind of additives that are often used to enhance certain properties like UV stabilization.

In this dissertation the focus was on two types of plastic, - polystyrene and polycarbonate, due to their large production volume, and differences in additives used in the production such as bisphenol A in polycarbonate.

1.4.1. Polystyrene

Polystyrene (PS)is a synthetic aromatic polymer made from the monomer

styrene in a polymerization process (Fig.5A). General purpose PS is a clear, hard and brittle material which can be easily colored. It is the fourth most often produced plastic type worldwide after polyethylene, polypropylene and polyvinylchloride (Abts 2014) with several billion kilograms produced per year (Maul *et al.* 2000) with 14.6 million MT PS produced worldwide in 2013 (Chemical Industry Education Centre University of York 2015a).

It is mainly used for protective packaging, containers, lids, bottles, trays, tumblers and disposable cutlery (Fig 5B). PS is licensed for food packaging and modified atmosphere food packaging helps to extend the shelf life of meat and vegetables, decreases the risk of food born infection and reduces food waste (Gerhard W. Becker 1995, Andrady and Neal 2009).

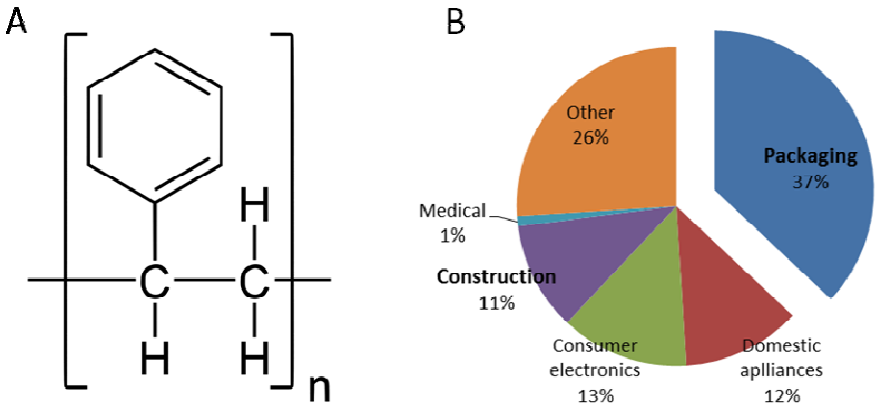

Figure 5. Polystyrene; A: structural formula, B: Purpose of use for PS. Modified from:(Chemical Industry Education Centre University of York 2015a).

Polystyrene was first described in 1893 by Eduard Simon but the industrial

production began with the polymerization in 1931 in Ludwigshafen. Soon after the start of mass production of polystyrene, a modification of expanded polystyrene (EPS) was invented and patented as Styropor. The EPS material is made by the same monomer styrene but with a different production process. EPS contains expended gas bubbles that melt together and form a low density material with good thermal insulation properties that make it very suitable for the usage in construction. The additional good damping characteristics are used in the packaging of fragile goods.PS and EPS show a high resilience and inertness which leads to a slow biodegradability especially under exclusion of UV radiation.

PS and EPS are highly flammable and most products contain flame retardants especially in the construction section. Flame-retardant additives like polybrominated diphenyl ethers and Hexabromcyclododecane (HBCD)are used to discourage ignition. Polybrominated diphenyl ethers (PBDE) are listed as Persistent Organic Pollutants (POPs)by the parties of the Stockholm Convention and the usage and production of certain PBDE such as penta brominated diphenylether was prohibited in 2009. The use of polybrominated diphenyl ethers is critically monitored due to its' bioaccumulation potential in the human body and the environment and the adverse health effects including reduced fertility in humans (Harley *et al.* 2010).

HBCDs are classified by the REACH (Registration, Evaluation, Authorisation and Restriction of Chemicals)European Union regulation asa substance of very high concern which persists, bioaccumulates and displays toxic properties. The migration of HBCD into the environment from intact expanded polystyrene is nominal (Kemmlein *et al.* 2003); however, emission can happen during fires, photolysis and recycling. During the recycling process monitoring

programs showed that PS-construction debris is often not isolated from other PS debris. This can lead to significant residual contents of flame retardant additives in sensitive recycled plastic items like packaging and flower trays (Fraunhofer-Institut für Verfahrenstechnik und Verpackung , Knauf *et al.* 2005).

With their low density of 1004 kg/m^3 PS and 16-640 kg/m^3 for EPS they are easily transported by the wind and blown wide distances. Both PS and EPS are widely found in marine litter and are reported to be mistaken as food by many animals. Several studies report that the majority of plastics observed in ocean surveys were PS or EPS (Williams *et al.* 2011, Eriksen *et al.* 2014). A study by Eriksen et al. (2014) revealed that the majority (1116 out of 4291) of macroplastics observed in over 890 surveys were EPS items (Eriksen *et al.* 2014).

1.4.1. Polycarbonate

Polycarbonate (PC) (Fig. 6) is a thermoplastic polymer mostly manufactured by condensation polymerization of bisphenol A (BPA) and phosgene. It was first invented in 1898 by Alfred Eichorn in Munich but the commercialization did not start until 1955. With a worldwide annual production of 2,38 million MT in 2012 (United Nations Statistics Division 2012) polycarbonate accounts for 1.3 % of the annual plastic production.

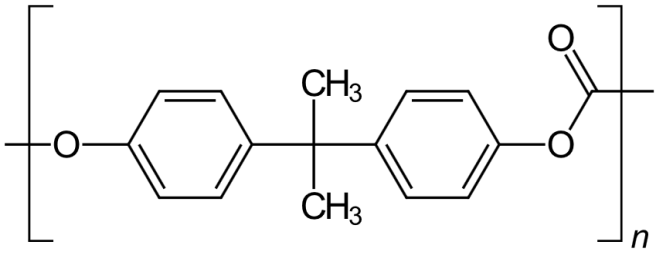

Figure 6. repeating chemical structure of polycarbonate

Polycarbonate has uniquely high impact strength and an exceptional clarity compared to other plastic types. It is heat resistant with a melting point of 155 ° C and yields flame retardant properties (Chemical Industry Education Centre University of York 2015b). With these characteristics PC is mainly used in the construction industry, for electrical components, automotive parts, compact discs (CDs, DVDs) and break resistant lenses and windows (PlasticsEurope 2015).

The application of injection molded PC in beverage containers such as drinking bottles, glasses and food containers has decreased due to the leaching of BPA from PC after hydrolysis at high temperatures. Controversial studies with concern of BPA leaching of heated polycarbonate baby bottles lead to the prohibition of the production and sale of baby bottles containing BPA by the European Union in 2011 (EU directive 2011/8/EU). The chemical BPA is an endocrine disrupting substance that can lead to cardiovascular disease, diabetes and disorders of sexual development (Umweltbundesamt 2010, CHEM Trust 2012, Angle *et al.* 2013).

Polycarbonate derived from bisphenol A is the most widely used polycarbonate. However, in order to avoid health risks and obtain slightly different material characteristics such as enhanced flame resistance, co-polymers have been developed in which substituted bisphenols are added and reacted with diphenyl carbonate (Chemical Industry Education Centre University of York 2015b).

1.5. Degradation process of plastic

The degradation time of plastic in the environment is difficult to calculate and is widely unknown, with estimates ranging from hundreds to thousands of years (Dřímal *et al.* 2006, Shah *et al.* 2008, Barnes *et al.* 2009). This uncertainty is due to varying scenarios and factors that influence the break down process of plastic materials. Four main factors are important: firstly the surrounding media (land/beach, water), secondly the temperature, thirdly the UV radiation and lastly fouling processes. All factors influence the plastic, make it brittle and eventually induce the break down into smaller pieces.

In beach or terrestrial environment the photodegradation through UV radiation (290-315 nm) plays an important role together with the temperature. Higher temperatures generally accelerate degradation process. Mechanical abrasion on the beach is mainly due to the wind and the surrounding sand. In aquatic environment, the break down process is usually slower since water temperatures are generally lower and less variable than air/ground temperatures observed on a beach, and abrasion by the waves alone is commonly less destructive than the impact of sand particles (Andrady 2011).

The influence of photodegradation on the breakdown process depends on the location of the plastic in the water column. On the surface the scattering and reflection of the UV radiation can enhance the break down process of floating plastic. When plastic objects sink down to the sea bed the break down process is slowed significantly since the UV radiation penetrating the seawater below 10 meters is considerably decreased compared to the surface water and the temperatures are lower (Dunne and Brown 1996).

Plastic debris in the ocean is prone to fouling processes which covers the surface of items with a biofilm which blocks it from radiation and enhances the density of the debris(Andrady 2000, Muthukumar *et al.* 2011). With the increase in density, plasticssuch as polyethylene and polypropylene that used to be positively buoyant,tend to sink. Other plastic types (polystyrene, polyamide, polyethylene terephthalate) that have higher densities than seawater were observed to sink to the seabed(Stefatos *et al.* 1999).

Polystyrene, which is a plastic type that is mainly used for food packaging, is taken here as an example for the estimated break down times: foamed plastic cups≤ 50 years, plastic beverage holders ≤ 400 years, and plastic bottles ≤ 450 years (Kellen 2014). However,it appears that there is more break down of plastics that take place in the oceansthan presented in several simulations/models. Studies by Morét- Ferguson et al. (2010) compared the average size of plastic particles from 1991-1995 and 2004-2007 and found a decrease by more than 50%(10.66 mm to 5.05 mm) (Morét-Ferguson *et al.* 2010).

Bioplastic or biodegradable plastics are often advertised as the environmentally friendly solution, but when it comes to degradation processes, this is not correct: bioplastics are synthetic polymers made from plant biomass and do not differ chemically from synthetic polymers made from fossil fuels raw materials such as oil. Therefore, on chemical level, the degradation process of both polymers does not differ. The difference comes in the approach of connecting polymer grains, wherebiodegradable plastics consist mostly of microplastic particles held together by starch (Russo *et al.* 2009, Raquez *et al.* 2011).So the breakdown of starch connections may be rapid and environmentally friendly, but the microplastics beads produced from biomaterials have the same degradation characteristic as oil produced polymers. The real biodegradation

requires action of specific bacterial strains to complete the oxidation process turning polymers into methane, carbon dioxide, and water(Andrady 2011). This process is not likely to be happening on a large scale in oceans, since it requires controlled environments with temperatures above 58 °C (Narayan 2009, Song *et al.* 2009). However, a possibility of biodegradation of polymers by a different microorganism in a different environment cannot be excluded (Guillet *et al.* 1988).

1.6. Plastic distribution in marine and freshwater environments

Much research has been dedicated to understand the abundance and fate of plastic pollution in the marine environment. Plastic is known to persist for 100-1000s of years, accumulate in the water surface, shoreline, seafloors, and beaches depending on their buoyancy and the surrounding environment.

According to the Convention of biological biodiversity in (2012) 633 marine species are known to be impacted by plastic debris (Secretariat of the Convention on Biological Diversity 2012). Even though rivers were identified to be important transport routes for plastic into the ocean (Lechner *et al.* 2014, Wagner *et al.* 2014, Dris *et al.* 2015, Klein *et al.* 2015) little research has focused on freshwater plastic concentrations. Studies including Lake Huron (Canada) the Laurentian great lakes (US) lake Garda (Italy) the Danube (Austria) and the River Rhine (Germany) showed very clearly that the plastic burden is comparable to marine concentrations(Avery-Gomm *et al.* 2012).

Keeping the small body of available literature in mind, it needs to be considered whether all harmful consequences of microplastic that have been

described for marine organisms like ingestion and introduction of alien species that float on plastic items to new ecosystems may operate in freshwater environments as well (Carson *et al.* 2013, Lechner *et al.* 2014, Wagner *et al.* 2014). A study conducted in the Danube River showed that the plastic abundance was higher than the average biomass and mean densities of ichthyoplankton larvae and juvenile fish. The high concentrations of harmful and unsuitable food items can pose a threat to freshwater biota comparable to marine organisms. Sanchez et al. (2014) showed that 12 % of the sampled wild freshwater fish gudgeon (*Gobio gobio*, L. 1758)had plastic particles in the intestines(Sanchez *et al.* 2014).

The sources of microplastic in rivers and lakes were found to be similar to marine sources, including sandblasting, consumer products, and input from sewage sludge (Eriksen *et al.* 2013).

1.7. The influence of size on bioavailability and toxic impacts

The size of plastic items plays an important role when it comes to their fate and effects in the environment. Plastic debris can be divided into megadebris (> 20 mm), macrodebris (20-5 mm), microdebris (< 5 mm) and nanoplastics (< 100 nm) (Table 1)

Name	Size
Megadebris	> 20 mm
Macrodebris	20-5 mm
Microdebris	< 5 mm
Nanodebris	< 100 nm

Table 1, Size definition of plastic samples

The suffocation of birds and the entanglement of cetaceans are well documented examples of the possible threats that large plastics items pose for many species (Kühn *et al.* 2015). Mega debris such as discarded fishing nets and other large items are known to affect >260 marine species by entanglement and ingestion (STAP 2011). Small plastic items can harm a broad range of species after ingestion and is specifically important due to its availability to a large range of smaller organisms like waterfleas (Daphnia spp.) and other planktonic organisms(Bhattacharya *et al.* 2010, Besseling *et al.* 2014b).

Microplastic can have different sources: primary microplastic is produced in the micro range to be used as raw material for the plastic industry, and large amounts of microplastic are released into the environment during transport and un/loading in harbours. Secondary microplastic is derived by the break down process or abrasion of macroplastic (Fig.7). This process is driven by wind, UV-B radiation and other factors, and is enhanced on shores and beaches where microplastic has stranded and is surrounded by warmer temperatures, wind, and sand.

Samplings from beaches close to Auckland (NZ) showed plastic concentrations exceeding 100,000 plastic pellets/m (Gregory 1978). Microplastic can be detected with sieves from beaches and can be filtered from water bodies with trawling nets. After the collection, the samples are quantified and qualified. Microplastic may impact the bottom of the food chain with serious consequences for larger animals and humans (Teuten *et al.* 2007).

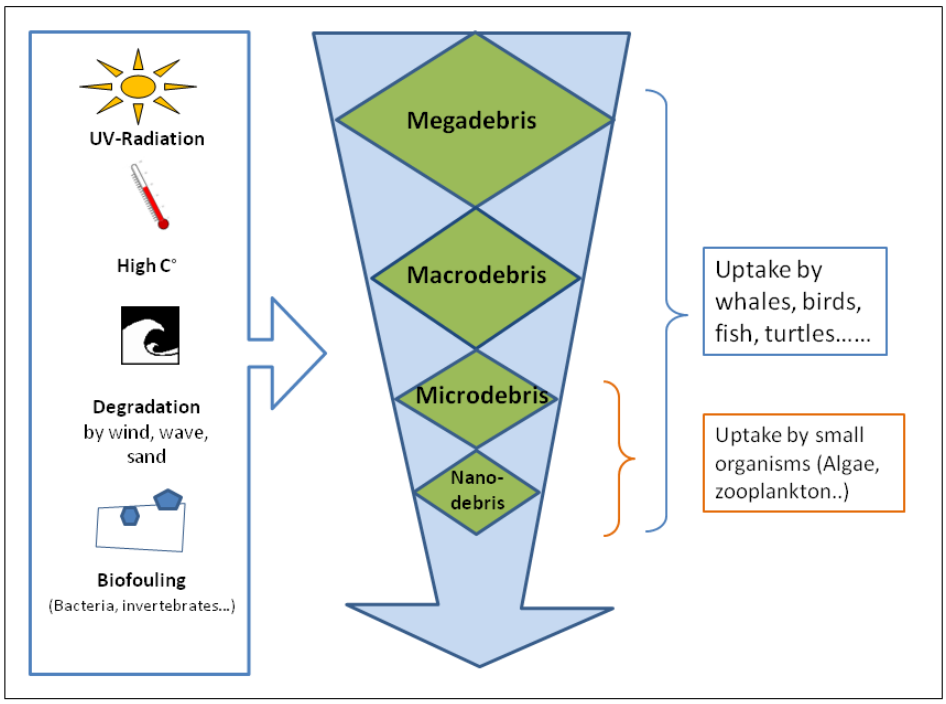

Figure 7. Breakdown process and availability of plastic debris to different species.Degradation in aquatic environments is driven by physical processes and biota. (Modified from: Stap: marine debris as a global environmental problem, p.11(STAP 2011))

An increase of microplastic over the past decades has been reported accompanied by a decrease in the average size of litter (Barnes *et al.* 2009, Cole *et al.* 2011, Thompson 2015). In highly polluted areas like the pacific ocean gyre, the plastic concentration is six time higher than the plankton biomass (Moore 2008). This leads to a high availability of potentially harmful, and unsuitable food items to consumers (Lechner *et al.* 2014).

The smaller the plastic items are, the easier they can pass cell boundaries and enter into the surrounding tissue. Studies by von Moos et al. (2012) showed the uptake of microplastic particles into digestive glands which induced granulocytomas and a decrease in lysosomal stability (Von Moos *et al.* 2012).

Nanoplastic is intrinsically linked to macro and microplastic particles because nanoplastic reaches the environment not only by direct emission of

manufactured nanoplastics, but is also likely the near-end product of degradation process starting from macroplastic, over microplastic and eventually nanoplastic particles, to disintegration of polymers(Shim 2014, Koelmans *et al.* 2015).

Nanoplastics are divided into different sources as well as microplastics. Primary nanoplastics are produced for industrial and medical applications especially for drug administration and surface coatings. Secondary nanoplastics are derived in the same way as microplastics which are mostly driven by an abrasion process that breaks down microplastics into nanoplastics. Until this time nanoplastics have not been detected in the water most likely due to detection difficulties (Koelmans *et al.* 2015).

Nanoplastics have properties and characteristics that differ from the larger plastic compounds. Their surface properties including charge and size allow them to interact differently with living cells, rendering them as a potentially valuable tool in the medical fields of disease diagnostics and treatment. But, rising concern is that the same surface properties could also display toxic effects, if they enter a body in an uncontrolled manner. Due to their specific surface characteristics and their large surface area, nanoplastic materials such as polystyrene exert proinflammatory activity and can interfere with the immune system (Brown *et al.* 2001, Bhattacharya *et al.* 2010).

In addition to the facilitated interaction of micro,- and nanoplastic with living cells, small plastic debris shows higher accumulation rates for contaminants such as persistent organic pollutants (POPs) and other chemicals due to the high surface to volume ratio (Koelmans *et al.* 2013, Velzeboer *et al.* 2014). Moreover, the size scale also impacts the exchange of organic chemicals, and it was demonstrated that nano- and micrometre size plastic has higher exchange ratesof

for example PCBs than macroplastics due to the large surface area and the short diffusion path length (Koelmans *et al.* 2013). Therefore smaller plastic items that can be ingested by a broad spectrum of organisms may also pose an increased risk of sorption and release of possibly toxic contaminants. Potential for population impacts of small scale plastic debris on aquatic organisms can therefore be similar or even higher than larger macro- or megadebris.

2. Impacts of plastic on organisms

2.1. Toxic impact of plastic on wildlife

The hazards of plastic debris on wildlife occur through three different pathways: firstly physical damage due to entanglement and blockage of the gut; secondly ingestion of plastic debris yielding high concentrations of sorbed environmental contaminants which can leach into the body after ingestion and thirdly the chemical hazards posed by the toxic monomers that can be released during production, use and the end of life cycle (Galgani *et al.* 2010).

The size of the plastic material is an important value when it comes to risk assessment. Macroplastics are known to be a threat through entanglement and ingestion. The threatof entanglement posed to organisms by ingested microplastics and smaller plastic beads is low, but the potential for direct exposure to sorbed chemicals and additives after ingestion is high because of large surface area to volume ratio (Cole *et al.* 2011). The adsorbed contaminants, and also additives used in the plastic production (see also chapter 1.5), can interfere with biologically important processes and lead to endocrine disruption, metabolic changes, carcinogenesis, and reduced fitness (Barnes *et al.* 2009, Lithner *et al.* 2011, Mattsson *et al.* 2015).

Therefore the health risks associated with plastic debris vary between polymer types and size, production process and constitutive or adsorbed chemicals.

2.1.1. Mechanical damage

Deleterious effects of plastic debris on ocean wildlife have been reported as early as 1931(Gudger and Hoffmann 1931). Mechanical damage derives from entanglement in lost fishing gear and other debris, and plastic is frequently mistaken for food leading to suffocation and death after ingestion.Plastic debris is one of many impacts that drive species to extinction(Gall and Thompson 2015). The number of species that are affected by entanglement or ingestion has risen from 267 to 557 species in less than twenty years (Kühn *et al.* 2015). Species range cover all vertebrate classes, and 17% of species reported to be affected by entanglement and ingestion of plastic debris are listed on the IUCN Red List as threatened or near threatened.

It needs to be considered that smaller sizes of plastic particles allow smaller organisms to ingest or interact with them. Microplastic and nanoplastic are reported to be ingested by various species of zooplankton such as Pacific Krill and *Daphnia magna*(Andrady 2009, Cedervall *et al.* 2012, Besseling *et al.* 2014b). Higher layers of the food web feed on zooplankton and can possibly ingest substantial amounts of plastic with their prey(Eriksson and Burton 2003, Lusher 2015).

Only a handful of studies have so far focused on health effects of microplastic and nanoplastic after ingestion and possible translocation from one organ to the surrounding tissue, blood vessels, or other organs. Blue mussels are able to translocate microplastic from the gut into the surrounding tissue which leads to the formation of granulocytomas and reduces lysosomal stability (Von Moos *et al.* 2012). Studies with polystyrene nanoplastic beads ingested by fish indicate behaviour changes and metabolic effects including a reduced feeding

activity. In addition, the brain of fish that ingested polystyrene beads showed abnormal swelling and colour change compared to the control group (Mattsson *et al.* 2015) . In broader perspective,the effects described above can lead to reduced fitness and decreased survival potential in the natural ecosystem (Mattsson *et al.* 2015).

2.1.2. Chemical ingredients of plastic and additives

Plastic polymerization requires the use of monomers, catalysts and polymerization solvents. In addition plastics are rarely used as a raw polymer but additives such as flame retardants, UV stabilizers, colours etc. are added to enhance the desired properties of the plastic material (see also chapter 1.5). The backbone structures of plastic polymers are considered to be chemically inert and due to their large molecular size they are considered as non-hazardous for the environment (Teuten *et al.* 2009, Lithner *et al.* 2011).

However, more than 50 % of the plastics in use are classified as hazardous due to their constituent monomers, additives and by-products (Lithner *et al.* 2011). It is very rare that the polymerization reactions run complete and varying amounts of residual monomers can be found in the polymeric compound (Araújo *et al.* 2004). Several of those monomers such as styrene and bisphenol A are considered to be hazardous chemicals and due to their low molecular weight the release into air, water and food is facilitated (Crompton 2007).

The plastics that were used in this study contain the monomer styrene, (needed to form polystyrene) which is listed as carcinogenic and mutagenic (Lithner *et al.* 2011). Polycarbonate is most often produced with bisphenol A

which yields endocrine disrupting effects (Oehlmann *et al.* 2009).

Chemical hazardous by-products such as polycyclic aromatic hydrocarbons (PAHs) are released during manufacturing process (in this case, polymerization of polystyrene). PAHs are listed in the United States Environmental Protection Agency regulations as carcinogenic, mutagenic and teratogenic(United States Environmental Protection Agency EPA 260-B-01-03 2001). These residuals are extremely difficult to remove after the production process and can carry over into the plastic material (Rochman 2015).

The release of hazardous substances or degradation products can happen at any time during the life cycle of plastic (Lithner *et al.* 2011). In conclusion, the toxicity of plastic materials linked to chemical ingredients and additives depends on the production process and the amount of unwanted by-products.

2.1.3. Chemical contaminants and persistent organic pollutants in plastic

Plastic debris in the ocean accumulates a complex mixture of chemical contaminants that are ubiquitously found in low levels across the oceans (Mato *et al.* 2001, Teuten *et al.* 2007, Rochman *et al.* 2013a). It has been shown that plastic has the potential to transport accumulated contaminants from the water to organisms (Teuten *et al.* 2007). The uptake of small plastic particles has been shown for many species(Lusher 2015). Since the sorbed (absorbed and adsorbed) chemicals from the plastic can become bioavailable to the organisms after ingestion there is great concern, that organism can be negatively affected by the contaminants (Endo *et al.* 2005, Hirai *et al.* 2011).

Plastic with sorbed chemicals is found everywhere in highly populated coastal areas to remote beaches and the open sea (Hirai *et al.* 2011). The persistent organic pollutants such as insecticides, pesticides and industrial chemicals derive from waste water and runoff (Wurl and Obbard 2004). The hydrophobic chemicals are prone to accumulate in plastic due to their high affinity for the water free environment found between the long chains of plastic materials (Katsnelson 2015). The concentration of contaminants in plastic particles such as dioxins, polychlorinated biphenyls (PCBs) and dichlorodiphenyltrichloroethane can be up to one million times higher thanthe concentration in seawater which highlights a cleaning effect of the ocean by the plastic (Endo *et al.* 2005, Takada 2013).

Studies have shown that the desorption of the contaminant once absorbed by the plastic is very slow (Teuten *et al.* 2007). Specific plastic materials are placed in aquatic environments as passive samplers to quantify persistent bioaccumulative and toxic substances (PBTs) (Lohmann 2011). The absorption of contaminants varies in different plastic types and is additionally influenced by the temperature of the surrounding water, the degree of crystallinity of the plastic and the non-equilibrium effects (Andrady 2011). The environmental risk of plastic related chemical contaminants is not due to the low concentrations of environmental chemicals in the water, but rathera consequence of aquatic organisms direct contact or ingestion of plastic particles with high concentrations of sorbed chemicals, that might later diffuse from digestive tract or gills into the tissues(Andrady 2011).

After ingestion, several factors influence the dose of contaminants that might be delivered to the organism; the dose depends on the ingested volume, the residence time in the organism and the kinetics of repartition of the chemicals

between the tissue medium and the plastic material (Andrady 2011). Overall, the most important question is whether the contaminants from plastic will enter into the food chain with considerable effects and if bioaccumulation occurs. These topics can be explored with the help of computer modelling, assessing the correlation between plastic ingestion and chemical body burdens and laboratory bioaccumulation tests (Rochman 2015).

Some studies explore these questions; the tissue of wild myctophid fish collected from the South Atlantic ocean showed similar congener patterns of flame retardants as those found on the plastic debris in the area (Rochman *et al.* 2014b). A study by Rochman et al. (2013) showed the transfer of hazardous chemicals from weathered plastic fragments to fish which induced hepatic stress (Rochman *et al.* 2013b). A study with lugworms (*Arenicola marina*) suggested facilitated uptake of polychlorinated biphenyls from contaminated sediment with plastic particles compared to sediment without added plastic (Besseling *et al.* 2012). In the Great Shearwaters (*Puffinus Gravis*) a positive correlation has been found between the amounts of plastic in the gut and the concentration of polychlorinated biphenyls in adult fat tissue (Ryan *et al.* 1988). PBTs have a range of chronic health effects, including endocrine disruption, mutagenicity and carcinogenicity (UNEP Year Book 2011). Raw material spherules (3 mm) spread on beaches worldwide were observed to contain organic pollutants like dioxin, pcbs and DDTs. The toxins are concentrated one million times higher than in the ocean waters (Takada 2013).

2.2. Detection of the adverse effects of plastic materials on aquatic organisms

The impacts of plastic on wildlife as described above can be difficult to detect particularly if only sublethal effects occur. Especially the effects of micro, - and nanoplastic particle impacts are challenging since the effects are less obvious than for example the entanglement in large plastic objects and resulting external wounds. Studies that use different detection methods to study the health effects of ingested and transferred particles from the intestines into the surrounding tissues and circulation are scarce.

Recent studies include the work on blue mussels (*Mytilus edilus*), that showed retention of microplastic in the digestive tract, immune response and the transfer to the lymph system (Browne *et al.* 2008, Köhler 2010, Von Moos *et al.* 2012). Decreased fecundity and mortality was observed in the copepod *Tigriopus japonicus* after ingestion of PS microbeads (Lee *et al.* 2013). The exposure of the Japanese medaka (*Oryzias latipes*) to microplastic led to hepatic stress, liver toxicity, altered gene expression, as well as decreased choriogenin regulation and vitellogenin concentrations (Rochman *et al.* 2013b, Rochman *et al.* 2014a).

Fish, being one of the largest group of animals with great importance to the ecological balance and for the food web, also serve as sensitive indicators of hazardous effects in aquatic environments (Rochman *et al.* 2013b). In addition to the histological detection of tissue samples, evaluation of altered gene expression, metabolic and behavioural effects, immune system of vertebrates can serve as a valuable tool to assess the toxicity of substances.

2.2.1. The immune system of fish

The immune system of fish defends the body against a wide range of pathogens and is comprised of the innate immune response and the acquired immune response.

Pathogens on their way into the body need to pass the surface barrier build by the skin, the gills and the gut which are covered in a protective mucus layer. Once pathogens successfully surpassed the mucus layer, specific immune cells induce the immune response. This response needs to be rapid and strong enough to eradicate the pathogens yet excessive responses need to be avoided in order to prevent harm to the host.

The acquired immune response in fish includes all fundamental features of the specific immune response including immunoglobulins, T-cell receptors, major histocompatibility products and recombination activator genes (Watts *et al.* 2001). The immune response of fish is highly temperature dependant with lower temperatures resolving in a slower proliferation of lymphocytes(Bly and Clem 1994). Fish yield fewer antibody isotypes than humans with mainly IgM, IgD and IgT antibodies reported for teleost fish. No class switching of antibodies was observed and the IgM response shows a poor affinity maturation (Mutoloki *et al.* 2014). However, IgM antibodies show a high functional heterogeneity which was suggested to be favourable for the immune response since they might be able to accommodate more epitopes (Kaattari *et al.* 1998).

Since the specific immune response in fish is slower than in humans and the anamnestic response is usually less pronounced the innate immune response in fish is especially important in fighting pathogens, and responding to other

potentially noxic agents, such as nanoparticles(Pilström and Bengtén 1996, B. Jovanovic and D. Palic 2012).

The piscine innate immune system includes both humoral and cell-mediated immunity components and is activated as soon as pathogens enter into the body. The major functions of the innate immune response include cytokine production, activation of the complement cascade and phagocytosis. Circulating immune cells like macrophages, among others identify microbes with pattern recognition receptors on their surface. In fish, 21 different Toll like receptors (TLR) have been identified which vary between different fish species (Palti 2011). Each subset of TLRsrecognizes a specific group of pathogen associated molecular patterns (PAMPs). TLR 3 and TLR 9 have been identified to detected virus associated dsRNA and dsDNA in teleosts such as common carp(Yang and Su 2010).

Since fish lack bone marrow which is the primary production site for pluripotent stem cells in mammals,the immune competent cells are produced in head kidney, trunk kidney and thymus of fish (Zapata *et al.* 2006). Macrophages and neutrophils are the most prominent immune-competent cells involved in phagocytosis in fish (Secombes and Fletcher 1992).

Neutrophils as polymorphonuclear leukocytes can perform several functions as part of the innate immune response. If one neutrophil function is impaired the disease resistance is decreased. Neutrophils can exert: rolling, adhesion, migration, polarization, degranulation of primary granules, release of cytokines, phagocytosis and release of neutrophil extracellular traps(NETs) (Lee *et al.* 2003, Kasama *et al.* 2005, D. Palic *et al.* 2005, Papayannopoulos *et al.* 2010). The NETs release is a cell death mechanism which involves the release of DNA fibres bound to specific antimicrobial proteins which have been observed to trap and at times

kill pathogens extracellularly (Fuchs *et al.* 2007).

The interactions of nanoparticles with the immune system can have different outcomes ranging from immunostimulation to immunosuppression. These effects are used in the medical field since specific treatments can benefit from an inhibited or enhanced immune response (Zolnik *et al.* 2010). In a natural environment it is essential to understand the effects of nanoparticles on the immune system, since immunomodulatory effects can severely interfere with the survival of a species.

In fish,TiO_2 nanoparticles were shown to induce respiratory burst in neutrophils as well as the release of NETs (Jovanovic *et al.* 2011b) whereas nanosized hydoxylated fullerenes supressed release of NETs and decreased oxidative burst activity and degranulation of primary granules (Jovanovic *et al.* 2011a). Differences in nanoparticle reaction with neutrophils rely on the mode of interaction.TiO_2 particles enhance the neutrophil immune response since the particles do not interfere with the ROS production after phagocytosis and the release of primary granules and the NETs release are not hindered. In contrast, hydroxylated fullerenes scavenge the ROS produced in the process of respiratory burst and the release of primary granules and NETs is decreased (Jovanovic *et al.* 2011a, Jovanovic *et al.* 2011b).

In rats poly(lactide acid) nanoparticles incubated with polymorphonuclear cells of rats lead to an increase of oxidative burst after phagocytosis (Mainardes *et al.* 2009). Gold nanorods were observed to be trapped by NETs released predominantly by neutrophils and NETs were discussed as a physical barrier for nanoparticles (Bartneck *et al.* 2009). Due to their phagocytic function and their large number neutrophils are most likely the major cells to interact with

nanoparticles. Since neutrophils can be activated by nanoparticles and die after the NETs release the number of neutrophils can be reduced and the organismal defence negatively affected (B. Jovanovic and D. Palic 2012). Due to the high importance of neutrophils in the innate immune response a decreased neutrophil function can put the normal development and survival of a species at risk (Segal 2005).

2.2.1.1. Immune response of fish to nanoparticles.

Nanoplastic particles that are likely to be present in the aquatic environments (Andrady 2011) can interact with the immune system of fish. Currently there is no information available on the possible immunotoxic effects of nanoplastic particles on aquatic organism. However, titanium dioxide (TiO_2) administered to fish has been shown to interfere with gene expression and neutrophil function as well as reduced disease resistance (Jovanovic *et al.* 2011b, Jovanovic *et al.* 2015b). In goldfish (*Carassius auratus* L.) several tested metal-oxide nanoparticles (TiO_2, CeO_2, Fe_2O_3, ZnO functionalized with polyacrylic acid (PAA) (which helps to keep particles dispersed in suspension and prevent dissolution of free metal ions from the core) negatively affected neutrophil function. PAA-TiO_2 andPAA-ZnO nanoparticles decreased neutrophil viability, PAA-CeO_2 and PAA-Fe_2O_3 increased neutrophil degranulation and in addition PAA-Fe_2O_3increased neutrophil respiratory burst. This study highlights that the toxicity of nanoparticles depends on the core material (Ortega *et al.* 2013).

Since the occurrence of microplastic is reported for all areas in marine habitats it is very likely that nanoplastics will be detected once technical issues are solved (Cózar *et al.* 2014, Koelmans *et al.* 2015). In addition the use of plastic

nanoparticles in technical and medical applications is rising and as time proceeds the input of primary nanoplastic into the environment is expected to increase (Cedervall *et al.* 2012). Since aquatic animals are chronically exposed to plastic particles in water the immunotoxicological effects are even more significant than for terrestrial animals (B. Jovanovic and D. Palic 2012). Reviews focusing on the impacts of nanoparticles on the immune system showed that the particles can suppress or enhance immune function and that surface properties are especially important (Dobrovolskaia and McNeil 2007, Dobrovolskaia *et al.* 2008).

Nanoparticles in the water get in contact with the mucus membrane layer of gills, skin and intestines as the first line of fish immune defensive mechanism against invading pathogens. Once the nanoparticles surpass the mechanical barrier of the mucus layer the innate and acquired immune response are activated. The uptake of nanoparticles through the diet appears to be a much more significant pathway than the absorption via the gills or the skin (Handy *et al.* 2008).

Nanoplastic particles enter the digestive tract with the ingestion of prey that is contaminated with nanoparticles (Cedervall *et al.* 2012, Besseling *et al.* 2014b). In the intestinal tract nanoparticles interact with the enterocytes and the cells of the diffuse gut immune system characteristic for teleost fish which include intestinal neutrophils, macrophages and leucocytes (Mestecky *et al.* 2005, Brandtzaeg *et al.* 2008). Nanoparticles can pass through enterocytes via transcytosis without disrupting the cell function (Koeneman *et al.* 2010)and can enter into the circulatory system and adsorb biomolecules from biological fluids such as plasma.

Particularly proteins bind to the surface of the nanoparticles and form a protein corona (Monopoli *et al.* 2012). The protein corona forms rapidly (< 0.5

minutes) and affects haemolysis, thrombocyte activation and nanoparticle uptake (Tenzer *et al.* 2013). The corona formation can act as a stimulator to the complement system and enhance phagocytosis of the nanoparticles leading to inflammatory reactions (Boris Jovanovic and Dusan Palic 2012).

Studies in aquatic animals showed the transport of nanoparticles from epithelium gut cells via hepatic portal system into the entire body and amassments in kidneys and liver (Moore 1990, Scown *et al.* 2009). Fish kidneys play an important role in hematopoiesis and are the main production site for neutrophils (Zapata *et al.* 2006). If nanoparticles accumulate in the kidneys the exposure of developing immune cells to nanoparticles can lead to an increased potential of immune cell function change (Jovanovic *et al.* 2011a, Jovanovic *et al.* 2011b).

Nanoparticles within the neutrophils are transported via circulatory system throughout the entire body,and in fish may preferentially concentrate in kidneys or spleen(B. Jovanovic and D. Palic 2012). This is especially critical since the immune cells cannot dissolve the nanoparticles. This leads to a potentially never ending cycle of repetitive transport process through the body and exocytosis of the nanoparticle after the apoptosis of the immune cell (Symonová 2007).

2.2.1.2. Neutrophil function assays

In this study we used a battery of neutrophil function assays to assess the impact of nanoplastic particles (PSNPs and PCNPs) on the innate immune response of fathead minnows (*Pimephales promelas*, Rafinesque 1820). In inflammatory lesions of fish the number of neutrophils is increased (Smith and

Lumsden 1983). Measuring the neutrophil function is a valuable parameter to evaluate the health status of individuals and populations (Lamas and Ellis 1994).

Neutrophilic granulocytes are activated during inflammatory processes. They can destroy pathogens through the release of cytotoxic compounds from granules and the production of reactive oxygen species (ROS)(Epstein and Weiss 1989) as well as entrap the pathogens in DNA nets (NETs release(Palic *et al.* 2007b)). The neutrophil function in fish is measured on the basis of the degranulation of primary granules (MPO assay), the oxidative burst activity (HDFFDA assay) and the release of neutrophil extracellular traps (NETs) (Hermann *et al.* 2004, D. Palic *et al.* 2005, Palic *et al.* 2007a).

Neutrophil function assays in fish have been applied before to study the impacts of immunomodulating substances (ß-glucan), to measure toxic impacts of titanium dioxide to zebrafish and to evaluate the impact of hydroxylated fullerenes on fathead minnows(Jovanovic *et al.* 2011a, Brogden *et al.* 2014, Jovanovic *et al.* 2015b). Therefore these well-established assays have been chosen to study the effect of PSNPs and PCNPs on the innate immune response of fathead minnows.

2.2.2. The effects of nanoparticles in plasma

Nanoparticles interact with biomolecules in biological systems and form a protein corona which has a large impact on the interaction, uptake and capacity to interfere with biological processes. The parameters affecting the composition and evolution of the protein corona include nanoparticle size, surface characteristics and plasma exposure time (Tenzer *et al.* 2013).

Common surface modifications of PSNPs include nanoparticles displaying carboxylated groups (COOH) (anionic) and unsaturated amines (NH2) (cationic) (Casado *et al.* 2013). Cationic PSNPs were reported to induce oxidative stress, mitochondrial damage and toxic oxidative stress in a phagocytic cell line, whereas anionic PSNPs showed fewer reactions (Xia *et al.* 2006).

Plastic nanoparticles can accumulate in the lysosome (cell organelles that break down cellular debris and waste material) where they affect their functionality by releasing the lysosomal content in the cytosol and inducing apoptosis (Wang *et al.* 2013). Several studies suggest that the nanoparticle toxicity strongly depends on their size, surface characteristics and their interaction with the surrounding medium. It has been hypothesized, that positively charged NPs preferentially interact with the negatively charged cell membrane to result in facilitated cellular uptake. Positively charged PSNPs with a protein corona showed an increased attachment and cellular uptake in endothelial cells compared to negatively charged protein coated PSNPs (Tenzer *et al.* 2013).

Nanoplastic particles were able to transfer from the gut of the mussel (*Mytilus edilus*) into the circulatory system (Ward and Kach 2009). In biological fluids such as plasma the surface of nanoparticles has a strong influence on the formation of the protein corona. The analysis of the protein corona of PSNPs in plasma included proteins involved in the lipid metabolism, the complement system and the blood coagulation among others (Gessner *et al.* 2003, Lundqvist *et al.* 2008).

It has been shown that PSNPs have an impact on the balance between pro and anti-coagulants and therefore are able to influence the blood coagulation. More specifically, positively charged amine modified PSNPs decreased the

amount of thrombin formed and lead to a prolonged clotting time which could lead to bleeding. In contrast, negatively charged carboxyl-modified PSNPs increased blood coagulation by activating the intrinsic pathway and could lead to a thrombotic state (Oslakovic *et al.* 2012). This is in accordance with the findings of Smyth et al. (2014) who observed the ability of PSNPs to induce and enhance platelet aggregation which can lead to thrombotic events such as myocardial infarction (Smyth *et al.* 2014).

In our study we tested the agglomeration behaviour of PSNPs and PCNPs in fish plasma which to our knowledge has not been tested before. The positive surface charge of both particle types and the size detected for PSNPs in plasma (155 nm) is in a similar size range as the positively charged PSNPs that promoted blood coagulation in the study of Oslakovic et al. (2011) and might therefore be able to influence the blood coagulation as well. The finding that the particles did not agglomerate enables neutrophils and other phagocytic cells to ingest the particles; however, since nanoparticles cannot be eradicated from the system the uptake could lead to the depletion of the phagocytic system and could interfere with the natural immune response.

2.3. Effects of nanoplastic on marine organisms

The application of nanoplastics is rising and more nanoplastic is released into the environment. Therefore it is important to assess the possible environmental hazards deriving from the nano-specific properties which differ from polymers in the bulk form (Klaine *et al.* 2012). Commonly produced nanoplastics are used in the medical field for drug delivery purposes where

theirsurface properties are able to control and delay the drug release (Guterres *et al.* 2007). In addition, the formation of nanoplastic has been observed as a by-product in the cutting process of expended polystyrene and in the 3D printing process (Zhang *et al.* 2012, Stephens *et al.* 2013).

The weathering of macroplastic into plastic on the microscale is a well described process. The breakdown process of microplastic into nanoplastic (< 100 nm) on the other hand is a less studied area of growing interest, especially since calculations of plastic masses in the oceans reveal a so far unexplained loss of plastic at the lower end of the expected size scale that could be explained with the fragmentation into nanoplastics (Cózar *et al.* 2014). Up to this point no nano sized plastic has been detected in aquatic environments; however, the fragmentation of expanded PS to micro and nanoscale plastics has been observed in abrasion experiments over one month (Shim 2014).

Another possible decrease in size could be linked to a degradation process due to photo oxidation (Sivan 2011). Yet degradation of a 1 mm sized plastic particle into a nano scale particle (< 100 nm) has been calculated to take more than 300 years. It has been concluded that nanofragmentation might be driven mainly by the surface fragmentation of macro and microplastics and to a lesser extent by degradation (Koelmans *et al.* 2015).

Few studies are available concerning the fate of nanoplasticparticles in organisms. These studies revealed several nanospecific outcomes such as an easy permeation of lipid membranes by polystyrene nanoparticles. The biological membranes are essential for the cellular function and control a large number of membrane protein activities. Inside the cells PSNPs were observed to alter the

membrane structure and decreases the molecular diffusion while softening it at the same time. These changes in the membrane properties were hypothesized to severely affect cellular function such as membrane protein sorting and functioning (Killian 1998, Rossi *et al.* 2013).

A reduction of population growth and reduced chlorophyll concentrations in algae (*Scenedesmus obliquus*) were observed after the administration of PSNPs. In *Daphnia magna* PSNPs decreased the number of neonates and lead to a decreased body size whereas the percentage of malformations in the offspring increased (Besseling *et al.* 2014b). The role of the surface charge was shown in a study with sea urchin embryos. Positive (NH_2 PSNPs) and negatively (COOH PSNPs) charged polystyrene particles accumulated in sea urchin embryos (*Paracentrotus lividus*). The anionic COOH PSNPs showed no embryotoxicity whereas cationic NH_2 PSNPs caused developmental defects. The positive charge of NH_2 PSNPs was assumed to be the reason for the observed cell death by apoptosis mechanisms in sea urchin embryos. (Della Torre *et al.* 2014). In addition cationic polystyrene nanoparticles showed various immunomodulatory effects on the marine mussel *Mytilus gallo provincialis.* High concentrations (50 µg/ml) were cyctotoxic and induced apoptotic processes. Lower concentrations (1/5 µg/ml) decreased the phagocytic activity and increased the extracellular ROS (Canesi *et al.*).

In algae (*Chlorella and Scenedesmus*) the presence of PSNPs increased the ROS production which is a signal for antimicrobial defence and is an indicator for environmental stress. Furthermore the photosynthesis rate was decreased which is indicated by a reduced depletion of CO_2. This effect may be due to a decreased light penetration or the CO_2 gas flow and the nutrient uptake pathways are blocked (Bhattacharya *et al.* 2010).

In fish PSNPs absorbed from ambient water accumulated in medaka eggs. PSNPs were found in various organs and were also found in the brain after penetrating the blood-brain barrier (Kashiwada 2006). Other studies in fish showed the influence of PSNPs transported through the food chain in an altered fat metabolism and negatively affected feeding behaviour of crucian carp (*Carassius carassius*). Fish hunted more slowly, exhibited stronger shoaling behaviour, showed weight loss and lipid metabolism changes after the uptake of nanoparticles. These changes might have potential effects on the function of these fish in the ecosystem. (Cedervall *et al.* 2012, Mattsson *et al.* 2015)

Comparable to the assumption that macroplastic might lead to starvation since it creates a false feeling of filled stomachs in fish, PSNPs affect the feeding behaviour of the blue mussel. *Mytilus edilus* produced pseudofeces which indicates that PSNPs are recognized as low nutritional food. Yet the opening of the valve was decreased which results in a reduced filtering activity (Wegner *et al.* 2012).

Since there are no actual numbers for the concentration of nanoplastics in aquatic environments available the experimental outcomes need to be considered with caution. The lowest observed effect concentration of 0.54 mg/ml (Casado *et al.* 2013) is comparable to the highest concentration of microplastic in marine waters (0.51 mg/ml) (Lopez Lozano and Mouat 2009, Besseling *et al.* 2014a) .

No research is available to assess the specific hazards of nanoplastic to transport and transfer chemical contaminants to the organism after incorporation. As described before (chapter 2.1.3) plastic is able to accumulate contaminants from the surrounding water. Nanoplastic with its high surface area could exert exceptionally high sorption affinities (Velzeboer *et al.* 2014). The transfer of contaminants sorbed to microplastic was observed to show different effects

depending on the concentration of the contaminants and the contamination of the organism at the beginning of the study. For nanoplastics the high surface to volume ratio combined with the ease of nanoparticles to permeate membranes, pass cell walls and reside in tissues for prolonged times might enhance the health hazards for the organism (Koelmans *et al.* 2015).

2.4. Plasticparticles in the food chain

Microplastic is abundant in every habitat of the ocean including surface waters, beaches, the water column, sediments and the deep sea floor. The widespread distribution and accumulation of microplastic is an important aspect when it comes to the assessment of possible entry of plastic into the food chain. In contrast to macroplastics, micro, and nanoplastic particles are bioavailable to a wide range of biota in benthic and pelagic ecosystems (Lusher 2015). The ingestion of microplastic is the most likely route of uptake for a variety of aquatic organisms. Either the particle uptake is indirect since some species cannot distinguish between prey and debris due to specific feeding mechanism or a direct uptake occurs if plastic particles are mistaken for food (Moore *et al.* 2001, Moore 2008).

Microplastic ingestion is reported for various species including invertebrates, birds, fish, turtles and mammals (Lusher 2015). The uptake of microplastic can interfere with the feeding and digestion of organisms (GESAMP 2010).

The uptake of microplastic at the vary base of the food web is highlighted by the absorption of PSNPs by the marine algae (*Scenedesmus*) which caused a reduced

photosynthesis rate and produced signs of oxidative stress (Bhattacharya *et al.* 2010). In addition zooplankton was observed to be negatively affected after the ingestion of PSNPs and nauplius larvae of the copepod (*Tigriopus japonicas*) showed increased mortality rates in a two-generation chronic toxicity test (Cole *et al.* 2013, Lee *et al.* 2013). Invertebrates such as lugworms (*Arenicola marina*), amphipods (*Orchestria gammarellus*) and blue mussels (*Mytilus edilus*) directly feed on microplastic with various consequences (Thompson *et al.* 2004, Wegner *et al.* 2012). Particles were observed to be rejected before digestion, excreted after digestion, and pseudofaeces production occurred which requires additional energy reserves. Weight loss, reduced feeding activity and decreased energy reserves were detected in lugworms (Besseling *et al.* 2012, Wright *et al.* 2013).

Microplastic that is introduced at one trophic level into the food web may transfer to other, higher trophic level organisms. (Farrell and Nelson 2013). In laboratory conditions food chain transfer of PS microspheres (10 mm) from mesozooplankton (*Marenzelleria spp.*) and copepods to macrozooplankton pelagic mysid shrimps (*Mysis relicta*) occurred after 3 h incubation (Setälä *et al.* 2014). Zebrafish (*Danio rerio*) fed with naupli of artemia species containing polyethylene microparticles (1-20 µm) showed a transfer of particles with adherence to intestinal vili which may lead to an uptake by endothelial cell (Batel 2015).

Baleen whales filter water to feed on the extracted planktonic organisms and trapped fish (Nemoto 1970). Microplastic uptake could occur directly during the filtering process or via trophic transfer from plankton and fish. Erikson et al. (2003) showed the microplastic transfer from lanternfish (myctophids) to seal (*Arctocephalusspp.*) scat which serves as an example for active transfer of plastic in the food chain (Eriksson and Burton 2003). A possible pathway of microplastic

uptake in turtles might occur via a trophic transfer since they feed on crustaceans, bivalves and sea cucumbers which ingest microplastic (Bjorndal *et al.* 1997, Graham and Thompson 2009). At this point research data is not sufficient to assess the impacts of microplastic debris on the food chain. However, since sea food consumed by humans in whole (with gut content) such as cod, whiting, haddock bivalves and brown shrimp contain microplastic, the trophic transfer is not limited to the lower end of the food web but possibly accumulates and affects organisms from invertebrates to humans.

2.5. Possible effects of plastic on humans

The impacts of plastic on humans are numerous. They can be divided into economic impacts in the tourism, fishing and navigation sector, and possible adverse health effects. The economic impacts in tourism are due to possible residue in tourism if recreational areas are covered with unsightly debris. The cleaning of beaches and the prevention of further pollution is costly. The fishing industry reports losses in the standing stock due to unwanted bycatch in plastic debris and with decreased numbers of fish the long term sustainability due to reduced reproductive abilities is negatively affected. In addition fisheries can be affected by damaged fishing gear and vessels (EPA 2013). The collision of mariners with plastic debris can be a navigational hazard and can endanger the life of people (Gold *et al.* 2014).

The health effects of plastic are due to the fact that we are surrounded by plastic in almost every aspect of our life. Especially in the food sector plastic is widely used to serve as containers and wrapping material which alone accounts for 14.5 million MT of plastic per year (Galloway 2015). On the one hand, plastic

food packaging prevents infections and decreases food waste, yet microplastic has been found as a contaminant in beer and honey and the migration of contaminants associated with plastic from food packaging into food is considered a major exposure route for humans (Grob *et al.* 2006, Hanning *et al.* 2009, Liebezeit and Liebezeit 2014, Liebezeit and Liebezeit 2015).

Human biomonitoring revealed that chemicals used in the plastic production are present in the human population (Galloway 2015). Plastic polymers are considered to be chemically inert and health risks for humans are associated to the plastic additives that may leach from the plastic compound (Crompton 2007) and to the release of constituent monomers such as BPA.

A possible pathway for human exposure to plastic particles is the ingestion of seafood containing plastic. Former studies have shown that 90 % of human POP exposure originates from food (Safe 1998). Recent studies by Van Cauwenbergh et al. (2014) identify the possible risks of microplastic in bivalves that were cultured for human consumption in the North Sea and the Atlantic Ocean. It was observed that depending on the annual dietary exposure European shellfish consumers can ingest up to 11 000 particles per year and once inside the human digestive tract, intestinal uptake of the particles might occur (Van Cauwenberghe and Janssen 2014).

The uptake of particle through the gut depends on different factors such as size, surface charge and hydrophilicity (Awaad *et al.* 2012) and the uptake of smaller particles exceeds the uptake of larger particles (Jani *et al.* 1992, Florence and Hussain 2001). The consumption of fish and seafood (even though it is only a small part of the diet at about 10% is one of the major routes of persistent organic pollutants into the human body (Alcock *et al.* 1998). In that sense the

accumulation of plastic particles and chemical contaminants in the food chain may pose a serious threat to human health. Nanoplastic with its various surface properties and its high ability to interact with cell membranes could pose an even higher threat than microsized particles.

A possible risk from plastic debris might be due to the microbial colonization of plastic particles in the ocean. The hydrophobicity of plastic stimulates the colonization and enhances biofilm formation. Zettler et al. (2013) described ‘plasticspheres’ asa term relating to the microbial community found on plastic debris in the north Atlantic (Zettler *et al.* 2013). Due to its durability, it lasts much longer than natural floating debris like feathers and bark and can act as an artificial reef (Law *et al.* 2010). Plastic has been described as a vector of transportation for harmful algae species (Masó *et al.* 2003) and members of the genus *Vibrio* were found to be dominating microbe in a plastic biofilm sample (Zettler *et al.* 2013). Several members of the vibrio genus are pathogens and cause gastroenteritis as for example *Vibrio cholera*e.

Municipal sewagetreatment plants in Germanywere observed to annually release 93 million to 8.3 billion microplastic particles with the effluent water into the environment(Alfred-Wegener-Institut *et al.* 2014). Therefore microplastics that serve as a habitat for waste water microorganisms can enter into freshwater bodies and interact with the ecosystem(Habib *et al.* 1998, Leslie *et al.* 2011).

Furthermore plastic is able to travel large distances and can introduce alien species into foreign ecosystems (Law *et al.* 2010). If animals ingest plastic containing microbes, plastic could serve as a vector for infectious diseases. Fish were observed to be carriers of human pathogenic Vibrio strains (Senderovich *et al.* 2010) and plastic as a possible vector for human pathogenic microbes should be considered.

III. MATERIALS AND METHODS

1. Nanoparticles

1.1. Nanoplastic synthesis

Plastic nanoparticles were synthesized at the Max Planck Institute for Polymer Research (MPI) Mainz, Germany.

1.1.1. Polystyrene nanoparticles

The Polystyrene nanoparticleswere labeled with Bodipy fluorophores in order to allow fluorescent microscope imaging. For the synthesis sodium dodecyl sulfate (30 mg) was dissolved in 10.5 mL water followed by the addition of distilled styrene (1.24 mL, 10 mmol), divinylbenzene (0.16 g, 1.22 mmol) and 3 mg Bodipy-styrene (3 mg, 0,0657 mmol) and stirred for 30 min at 600 rpm. The dispersion was degassed with argon and heated at 70°C for one hour. $K_2S_2O_8$ (10 mg, 0.037 mmol) was dissolved in 0.7 mL H_2O and added to the stirred emulsion. After 6 h, the emulsion was cooled to room temperature (20 °C) and dialyzed for 3 days against water (MWCO =25 kDa). The dialyzed emulsion was freeze dried for 24 h to obtain solid polystyrene nanoparticles. A working suspension of PS was prepared in Hank's balanced salt solution with Ca, Mg, no phenol red (HBSS+) (HyClone Laboratories, Inc, USA).

1.1.2. Polycarbonate nanoparticles

For the PolycarbonatePoly(isoprene-*block*-polymethylmethacrylate) (200 mg) were used to serve as emulsifier. It was placed in a flask, dried under vacuum

overnight and degassed with argon. Dry cyclohexane (8.3 mL) was added, and the mixture stirred for 3 hours at room temperature, followed by addition of BPA (609.5 mg,2.67 mmol in 1ml of acetonitrile). Anhydrous pyridine (0.29 mL, 3.59 mmol), was added and the dispersion was sonicated for 10 minutes. Triphosgene (291.5 mg, 0.98 mmol) in 0.71 mL acetonitrile) was sonicated for 20 min and added dropwise (5 mL h^{-1}) to the stirred emulsion. The emulsion was stirred at room temperature for 24 h under inert atmosphere. An aliquot was removed to analyze the particle size and morphology via Dynamic Light scattering (DLS) and Scanning electron microscope (SEM). The particles of the remaining emulsion were precipitated in methanol and separated by centrifugation to form a white solid. The emulsifier was washed out by repeated dispersion in fresh cyclohexane and centrifugation. In order to remove cyclohexane the suspension was washed with methanol and afterwards the solid was dried under vacuum for 24 h. Finally, the working suspension of PC was prepared in Hank's balanced salt solution with Ca, Mg, no phenol red (HBSS+) (HyClone Laboratories, Inc, USA).

1.2. Nanoplastic particles characterization:

To obtain the size distribution and the zeta potential of the PC and PS particles dynamic light scattering measurements were performed with a Malvern Zetasizer 3000HSA. For the measurements particles were suspended in HBSS+ which is used as a cell medium in the neutrophil function assays. (Table 2, Fig. 8A).

Particle type	Size (nm) in H_2O (PS), Cyclohexane (PC)	Hydrodynamic diameter(nm) in HBSS+	Z-potential in HBSS+	Fluorophore
PS	41 ± 0.2	683.1 ± 454.7	2.1 ± 0.4 mV	Bodipy
PC	158.7 ± 41	1710.47 ± 425.6	2.0 ± 0.5 mV	None

Table 2, Size and zeta-potential of polystyrene and polycarbonate nanoparticles

1.3. Scanning electron microscopy images of the particles:

Scanning electron microscopy (SEM) images of PC and PS particles were taken using a Zeiss Gemini 912 microscope with samples of PC and PS nanoparticles dispersed in cyclohexane (PC) and water (PS) and drop casted on a silica wafer (Fig.8 B,C).

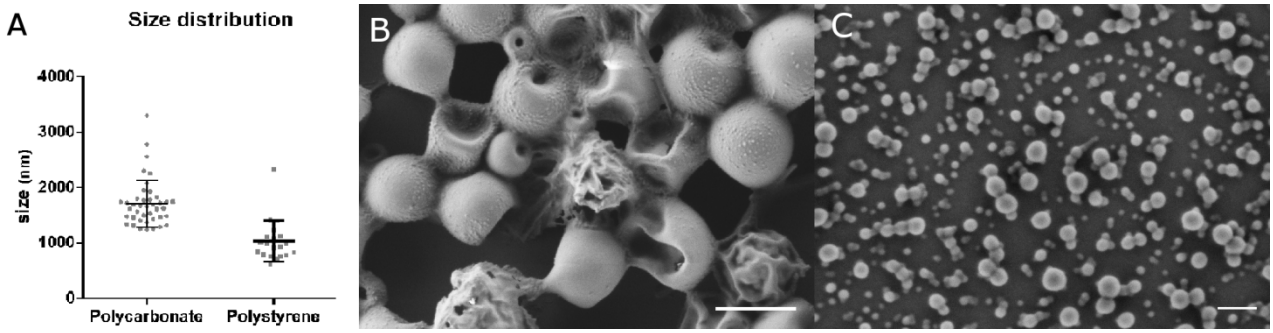

Figure 8.Characterization of polystyrene and polycarbonate nanoparticles.
A: Hydrodynamic size distribution graph of PSNPs and PCNPs in HBSS+.Mean size PS: 1710.47 ± 425.6 nm, mean size PC: 683.1 ± 454.7 nm. Dynamic light scattering measurements were performed with Malvern Zetasizer 3000 HAS. B and C:Scanning electron microscopy (SEM) images of PCNP (B; cyclohexane, scale bar = 1 µm and PSNP (C; water, scale bar = 100 nm).

2. Dynamic light scattering in fathead minnow plasma:

Characterization of fathead minnow plasma and nanoparticle mixtures was performed with a dispersion of 100 µg of polystyrene particles in 1 ml fathead minnow plasma. Due to macroscopic precipitation in the polycarbonate dispersion (c = 0.1 µg/ml) the exact concentration could not be determined. However, the precipitate was separated via filtration. The remaining solution was measured by DLS and added to fathead minnow plasma. All solutions were filtered through Millex SV filters with a pore size of 5 µm (Merck Millipore, Billerica, USA) into dust-free quartz light scattering cuvettes (inner diameter 18 mm, Hellma, Müllheim), which were cleaned before with acetone in a Thurmont-apparatus. Light scattering experiments were performed with an ALV-CGS 8 F SLS/DLS 5022F goniometer equipped with eight simultaneously working ALV 7004 correlators and eight QEAPD Avalanche photodiode detectors (ALV, Langen, Germany). A HeNe laser (632.8 nm, 25 mW output power) was utilized as the light source.

3. Neutrophil phagocytosis of plastic nanoparticles:

Fluorescent microscopy was used to visualize the phagocytosis of the fluorescently labeled polystyrene (PS) particles (Max Planck Institute for Polymer Research (MPI) Mainz, Germany) by fathead minnow neutrophils. Cells were incubated with a PS suspension for one hour followed by cytospin slide preparation. Air dried cell preparations were examined immediately with an

Olympus BX 63 microscope equipped with an Olympus DP80 digital camera using a Texas Red filter (Em 583 nm) and total magnification of 100 X (under oil), and images captured by Olympus Imaging software cellsSens Dimension and further processed in Photoshop CS6. Due to the occurrence of strong auto fluorescence of the neutrophils after fixation, native (not fixed or stained) preparations were used.

4. In vitro neutrophil assays:

4.1. Animal care:

Adult fathead minnows (average weight 4.5 g) were maintained at the Chair for Fish Diseases and Fisheries Biology at the faculty of Veterinary Medicine, Ludwig-Maximilian University, Munich, Germany. Fish were kept in a water recirculation system supplied with 300 L of filtered tap water and fed dried flake food (2:3 w/w mixture of Tropical® Breeder Mix/D-Vital plus, and Tropical Heimtierbedarf Deutschland GmbH) twice daily. Fathead minnows were cared for in accordance with the German guideline for laboratory animal care (§11 TierSchG).

4.2. Cell preperation:

Fathead minnows were euthanized with an overdose of MS-222 (Pharmaq, U.K.). Kidneys from eight fish were pooled in a sample and standard neutrophil suspensions (2×10^7 cell mL^{-1}) were prepared as described previously (Dušan Palic *et al.* 2005). Cells were exposed to standard stimulants (Phorbol 12-Myristate 13-Acetate, PMA 1 µg mL^{-1}, Fisher Scientific, US, and calcium

ionophore A23187, CaI, 5 µg mL^{-1}, Fisher Bioreagents, Switzerland); and also to Polystyrene Nanoparticles (PSNP) and Polycarbonate Nanoparticles (PCNP) at 0.025, 0.05, 0.1, 0.2 µg $µL^{-1}$ MPI Mainz), or to Hank's balanced salt solution with Ca, Mg, no phenol red (HBSS+) as negative control. PS and PC were suspended in HBSS+, sonicated (Elmasonic S40H, 340 W) for 2 h, and filtered through a 70 µm sterile cell strainer (Fischerbrand®) to remove aggregated NPs prior to loading wells.

4.3. Neutrophil assays:

Degranulation of primary granules, respiratory burst, and neutrophil extracellular traps (NETs) release assays were performed according to established protocols with minor modifications (D. Palic *et al.* 2005, Jovanovic *et al.* 2011b).

4.3.1. Degranulation of primary granules

The degranulation of primary granules assay was modified by using ready-made liquid 3,3′,5,5′-Tetramethylbenzidine (TMB) working solution (2.5 mM in H_2O_2, Sigma-Aldrich, Germany) instead of powdered TMB. Neutrophil granulocytes release myeloperoxidase (MPO) from primary granules into phagosoms or to the extracellular space during degranulation. MPO is the most abundant proinflammatory lysosomal protein and it plays an important role in the regulation and termination of inflammatory reactions that occur after the stimulation of neutrophils. The enzyme catalyses the oxidation of chloride anion (Cl-) and hydrogen peroxide (H_2O_2). From those products cytotoxic hypochlorus acid (HOCl) can be build which kills bacteria and other pathogens.

MPO catalyses the reaction: $H_2O_2 + Cl^- \rightarrow H_2O + ClO^-$,

ClO^- can further react to hypochlorus acid HClO: $ClO^- + H^+ \rightarrow HClO$

4.3.2. Respiratory burst assay

Polymorphonuclear neutrophils generate reactive oxygen species (ROS) such as hydrogen peroxide (H_2O_2)during phagocytosis of pathogens such as bacteria, fungi or toxic substances. This process is referred to as 'oxidative burst' or 'respiratory burst' and is a major component in the host defense. The ROS production helps to degrade internalized pathogens (Chen and Junger 2012). The intensity of the immune response representing the health status of an organism can be measured by the amount of ROS produced (Hermann *et al.* 2004). The degranulation of primary granules releases an enzyme that catalyses the chemical reaction of chloride to hypochlorus acid. These close interactions between the chemical process and products show the complexity in the neutrophil host defence. The oxidative burst assay is based on the diffusion of 5(6)-Carboxy-2′,7′-dichlorofluorescein diacetate (HDFFDA) into the cell where it oxidates and reacts to H_2O_2. This oxidative product can be quantified with a spectrofluorometer.

4.3.3. Neutrophil extracellular trap release asssay

The neutrophil extracellular trap release is based on the ability of neutrophils to release decondensed chromatin from their nucleus after activation through reactive oxygen species. These chromatin threads form a net to trap and

kill pathogens extracellularly. This process is called 'neutrophil extracellular traps' formation (NETs) (Papayannopoulos *et al.* 2010). These NETs contain antimicrobial factors including neutrophil elastase and MPO (Brinkmann *et al.* 2004).

The NET assay was performed as previously described (Jovanovic *et al.* 2011c) with minor modifications. Briefly, 10 µL of standard neutrophil cell suspension was seeded into 96 well plates and stimulated with 90 µL of nanoparticle suspensions of different concentrations in HBSS+. For the positive control 10 µL of standard cell suspension was seeded into 96 well plates prefilled with 40 µL HBSS+ and 50 µL of the standard stimulant, (PMA - positive control) of 1 $\mu g\, mL^{-1}$. For the negative control 10 µL of standard cell suspension were seeded into 96 well plates preloaded with 90 µL HBSS+. All samples were incubated for 2 h at room temperature. After incubation, NETs generated by stimulated neutrophils were digested with 500 mU mL^{-1} (One µmolar unit = approx. 85 A_{260} units, where an A_{260} unit is equivalent to a ΔA_{260} of 1.0 in 30 min at pH 8.8 at 37 °C in a 3.55 mL reaction volume) of Micrococcal Nuclease (MNase) for 20 min at 37 °C. The MNase was inactivated with 5 mM EDTA, plates were centrifuged on 4 °C for 5 min at 400 g, supernatants collected for DNA quantification and stained with Picogreen (Quant-iT^{TM,} Thermo Fisher Scientific, US) dsDNA fluorescent dye according to manufacturer's instructions.

4.3.4. Photometric measurements and calculations

Plates for all assays were read in a plate reader (Spectra Max M5, Molecular Devices, SOFTmax Pro 6.2.2.). All assays were also tested without

cells to exclude spectrophotometric interference of the compounds and chemicals themselves. None of the tested chemicals (including NPs) interfered with photometric readings.

The NET and respiratory burst stimulation index were calculated using the following formula:

((Average PS (PC) value)/ (average of HBSS))

The percentage of degranulation (MPO) was calculated based on the following formula:

((average PS (PC) value)-(average of HBSS))/ ((average of CTAB value)-(average of HBSS)).

An index > 1 means that the compound is a stimulant; while an index <1 means that the compound is a suppressor. In order to statistically compare the differences between experimental groups and controls, values were divided by the average HBSS value for each experiment. Thus, although the average stimulation index of the control is 1, a background variability in the response of the control population is calculated and statistically compared to the variability of the treatments.

4.4. Statistics

Statistical testing for the neutrophil function assay data was done using Student's t-test for independent samples. Dunnett's procedure for post hoc comparison of mean between single control and multiple experimental groups was used to analyze the PS and PC concentration gradient data for significance. Results with a *P*-value equal or below 0.05 were considered as statistically significant. The significance is indicated as * $p<0.05$; # $p< 0.005$; § $p< 0.001$ and $ $p< 0.0001$.

IV. RESULTS

1. Nanoplastic particle characterization:

Dynamic light scattering of nanoparticles (NP) indicated aggregates in HBSS+ with an average hydrodynamic diameter of 1006.9 ± 380.8 nm for polycarbonate (PC) and 1710.47 ± 425.6 nm for polystyrene (PS), and zeta potential of PSNPs and PCNPs dissolved in HBSS+ was 2.1 ± 0.4 mV (PS) and 2.0 ± 0.5 mV, respectively (Table 2).The multimodal distribution of PSNPs and PCNPs hydrodynamic diameter was observed (Fig. 8a). The scanning electron microscopy of polycarbonate revealed aggregates with round shaped spherules in the µm range (Fig.8b). Polystyrene particle aggregates were below 100 nm scale and with a higher level of dispersion compared to PCNP (Fig 8a, c).

2. Polystyrene and polycarbonate nanoparticles in plasma

The Dynamic Light Scattering (DLS) measurements of polystyrene and polycarbonate nanoparticles were analyzed by adapting the method of Rausch *et al*. (Rausch *et al.* 2010). The autocorrelation function (ACF) of the untreated fathead minnow plasma could be perfectly described by a sum of three exponentials (equation 1), similar to a human serum, giving an averaged hydrodynamic radius of Rh = 14 nm.

$$g_{1,P}(t) = a_{1,P} \exp\left(-\frac{t}{\tau_{1,P}}\right) + a_{2,P} \exp\left(-\frac{t}{\tau_{2,P}}\right) + a_{3,P} \exp\left(-\frac{t}{\tau_{3,P}}\right) \qquad (1)$$

with the amplitudes a_i and the decay times $\tau_i = \frac{1}{q^2 D_i}$ while q is the absolute

scattering vector ($q = \frac{4\pi n}{\lambda_0} \sin\left(\frac{\theta}{2}\right)$) and $D_i$the Brownian diffusion coefficient of component i. Data obtained from light scattering analysis of fathead minnow plasma and the respective particles are provided in figure 9a.

The ACFs for the polystyrene and polycarbonate particles alone were successfully fitted by a sum of two exponentials (equation 2) when given a size of Rh = 155 nm for the PS particle and a size of Rh = 500 nm for the PC particle in PBS (phosphate buffered saline) buffer solution.

$$g_{1,C}(t) = a_{1,C} \exp\left(-\frac{t}{\tau_{1,C}}\right) + a_{2,P} \exp\left(-\frac{t}{\tau_{2,C}}\right) \quad (2)$$

Knowing the ACF of fathead minnow plasma and the respective particles, the correlation function of the plasma particles mixtures could be analyzed. If no aggregation occurs, the resulting ACF of the plasma particle mixture correlates to the so-called force fit. In the force fit, the sum of the individual correlation functions with the known parameters of the two components (plasma/particle) is kept fixed and the intensity contributions for plasma f_P and particle f_C are the only fit parameters (equation 3).

$$g_{1,m}(t) = f_P g_{1,P}(t) + f_C g_{1,C}(t) \quad (3)$$

For none of the investigated systems the formation of aggregates with sizes larger than the largest size of the original components (particle/fathead minnow plasma) was indicated by the fittings.

Figure 9b shows the results of the mixture of PS with undiluted fathead minnow plasma.

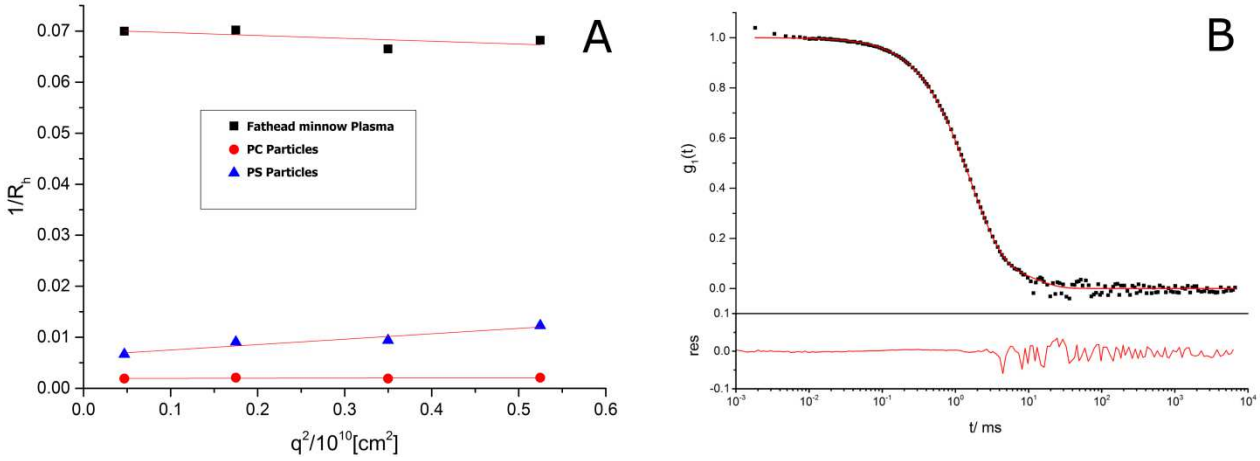

Figure 9.Dynamic light scattering measurements of the aggregation behaviour of PC and PS nanoparticles in fathead minnow plasma.A: angular dependency of the reciprocal hydrodynamic radius of fathead minnow plasma (black ■), PS-Particles (blue triangle) and PC particles (red o), T = 310K. B: Auto correlation function of the PS-particles in fathead minnow plasma. Red line, fit with eq.3 and the resulting residue. Data points of the ACF. Scattering angle 60°, T = 310 K.

3. Neutrophil phagocytosis of plastic nanoparticles

Neutrophils phagocytized larger PSNP aggregates, and observed cytoplasmic fluorescence also indicates presence of smaller diameter nanoparticles in the observed cells (Fig 10. a-c). Control images show no neutrophil auto fluorescence in native preparations (Fig 10. d-e).

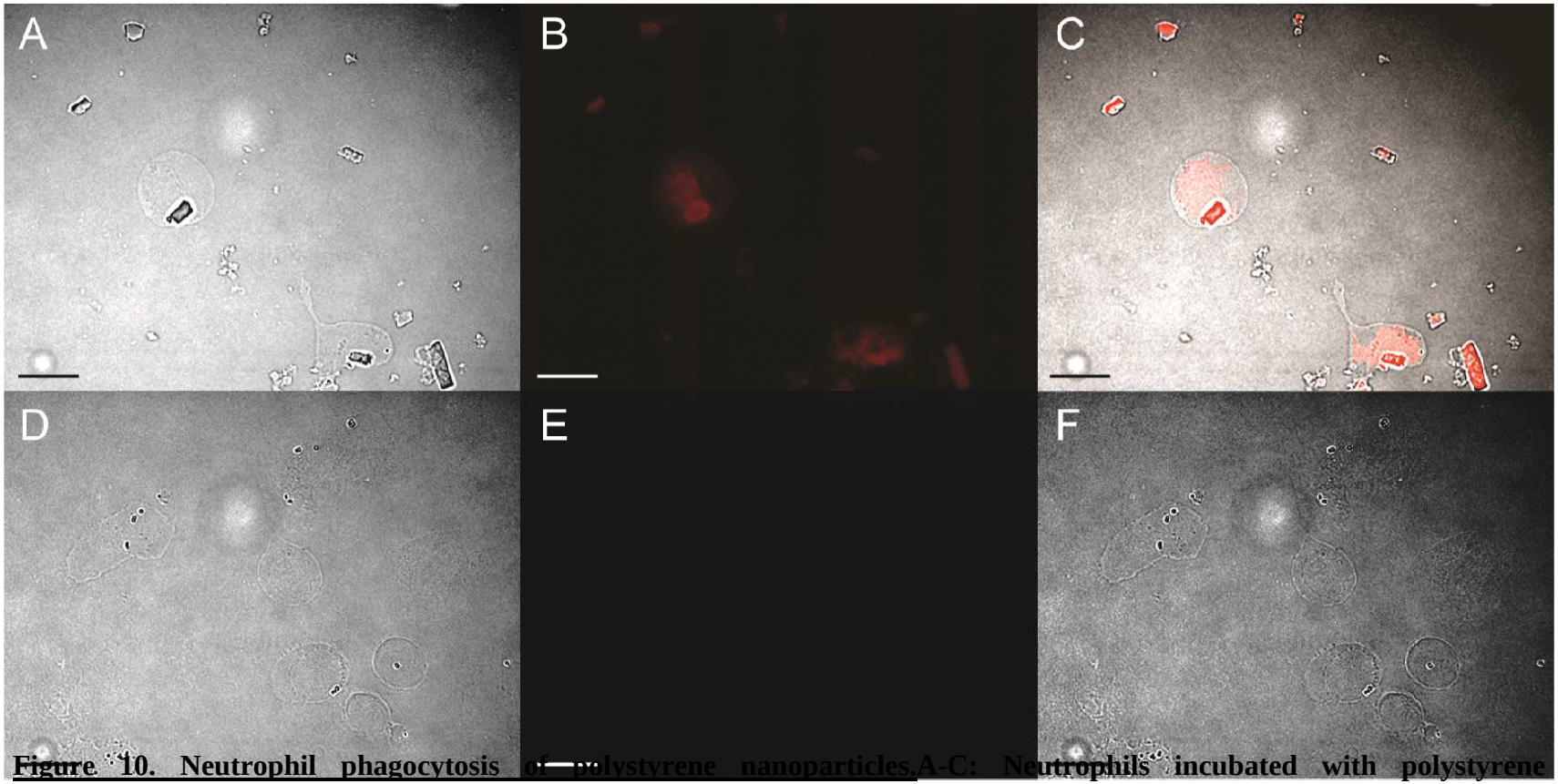

Figure 10. Neutrophil phagocytosis of polystyrene nanoparticles.A-C: Neutrophils incubated with polystyrene nanoparticles. A: Bright field microscopy of native cells and nanoparticles, B: Fluorescent microscopy of native cells and nanoparticles, with Texas Red filter (em 583, exposure time 178 ms), C: Overlay of image A and B showing phagocytized nanoparticles and aggregates in the cytoplasm of neutrophils. D-F. Control images, no PS added, with identical microscope and imaging settings. Scale Bar = 10 µM.

4. Neutrophil function assays

Neutrophil function was assessed with degranulation of primary granules, oxidative burst, and NETs release, after the exposure to PSNP and PCNP *in vitro*. PS and PC nanoparticles caused a significant degranulation increase *in vitro* compared to negative control (HBSS+) at a dose of 0.1 µg µL^{-1} (t-test, $p < 0.0001$,Fig.11). A significant induction of NETs release was observed *in vitro* for both particles types at a concentration of 0.1 µg µL^{-1} ($p < 0.0001$, Fig.11). PC caused a significant increase of respiratory burst at a concentration of 0.1 µg µL^{-1} ($p < 0.001$, Fig.11). The concentration-response curve for the PCNPs and PSNPs was tested with four different NP concentrations: 0.025; 0.05; 0.1; 0.2 µg µL^{-1}.

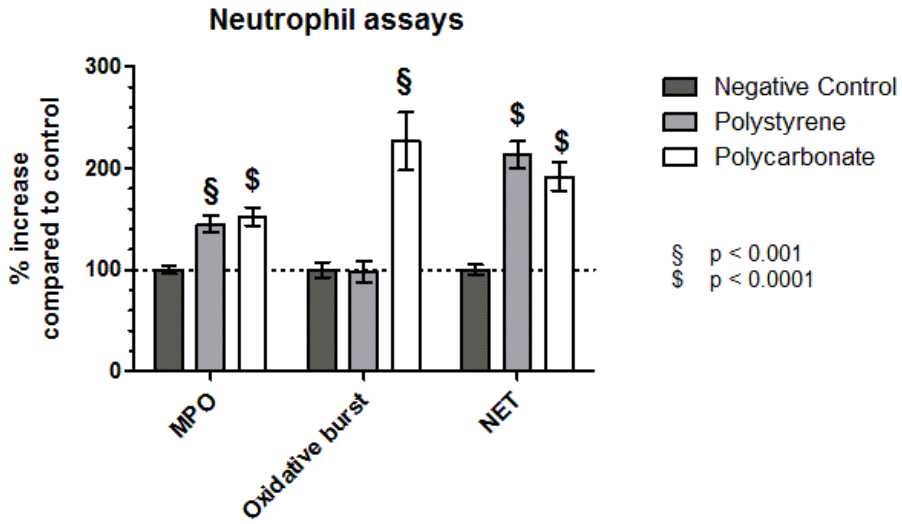

Figure 11.Polystyrene and polycarbonate nanoparticles increase response of fathead minnow neutrophils in vitro.PSNP (grey) and PCNP (white) bars indicate neutrophil function compared to non-treated control (HBSS+). MPO: Degranulation of neutrophil primary granules measured as MPO exocytosis compared to control (HBSS+, n = 18). Oxidative burst: cumulative production of oxygen radicals compared to control (HBSS+, n = 17). NET: Neutrophil extracellular traps release from fathead minnow neutrophils compared to the negative control (HBSS+, n = 23). Data are shown as mean ± SEM, and level of significance is indicated with § (p <0.001) or $ (p <0.0001).

The PCNP (Fig. 12a) initiated maximum neutrophil function response at 0.1 µg µL^{-1} in all three assays. All PSNP concentrations caused a significant increase of the degranulation of neutrophil primary granules in a dose dependent manner. (Fig.12b). The PSNP induced NETs release at all concentrations (Fig. 12b) and the oxidative burst activity significantly increased at 0.1 µg µL^{-1} and 0.2 µg µL^{-1} of PSNP compared to the negative control (HBSS+).

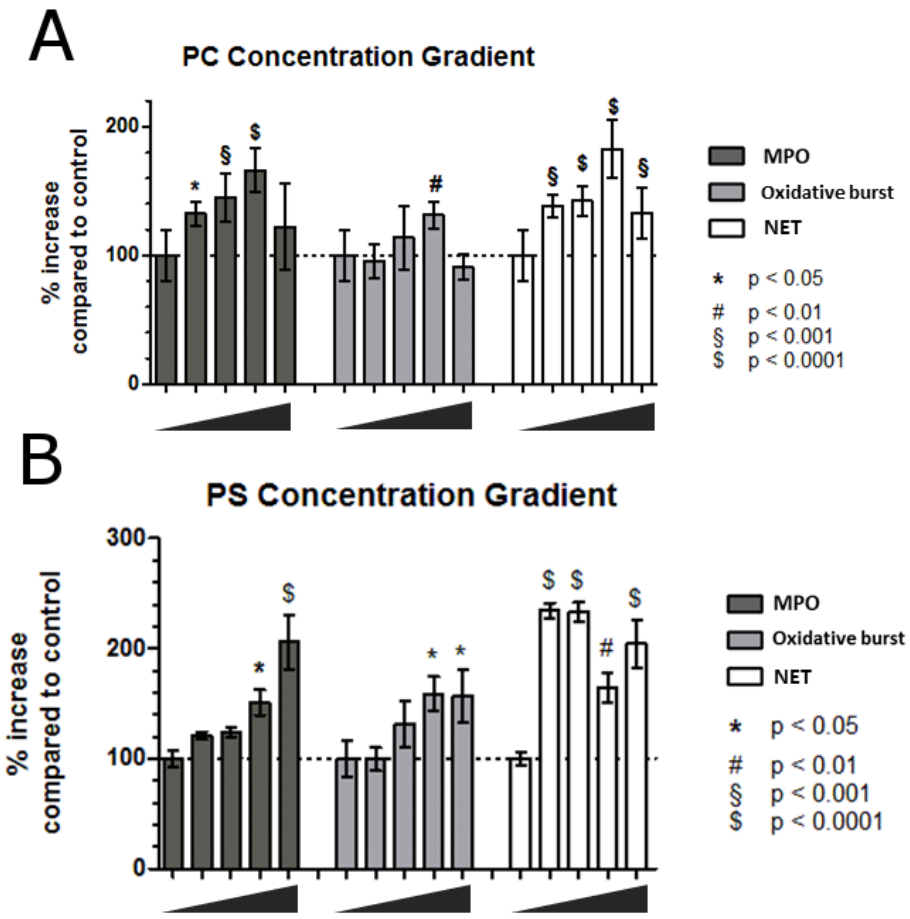

Figure 12.Different concentrations of Polycarbonate and Polystyrene nanoparticles increase neutrophil function responses.PCNP (A) and PSNP (B) significantly increased MPO, oxidative burst, and NETs release compared to control (HBSS+, n = 6). Concentrations used in this study were 0.025 µg µL^{-1}, 0.05 µg µL^{-1}, 0.1 µg µL^{-1}, 0.2 µg µL^{-1}for both PCNP and PSNP. Data are shown as mean ± SEM and level of statistical significance is indicated with * (p < 0.05), # (p < 0.01), § (p < 0.001), $ (p < 0.0001).

V. DISCUSSION

Polystyrene and Polycarbonate nanoplastic particles were observed to increase the production of primary granules, enhance the neutrophil extracellular traps release and trigger the oxidative burst response. In addition the size measurement of the particles in plasma as a biological fluid showed no increase in size which potentially allows the nanoplastics to exert their characteristic interaction with the surrounding cells and tissues.

These results are important in the context that the small body of available literature concerning the possible toxic effects of nanoplastic on wildlife suggests that the small size, the chemical contamination and the physical effects of plastic can be a threat to animal populations.

The immune system is important in protecting the body against invading pathogens and toxic compounds. A well-functioning immune system is essential to ensure the survival and well-being of an organism. By examining the reaction of neutrophils as the first line of the innate immune response against invading pathogens in fish we identified the potential of polystyrene and polycarbonate nanoparticles to act as stressors to the immune system. The particles utilized in this study suspended in plasma did not agglomerate to an extent that would prevent the phagocytosis of the particles by neutrophils. This is important since neutrophil function can also be hindered by excessive attempts to delete particles which are too big for phagocytosis (Jovanovic *et al.* 2015a). In addition the microscope images of the fluorescently labeled particles show strong fluorescence in the cytoplasma which is a solid indicator for the phagocytosis of the particles.

It can be difficult to detect the impact of nanoplastics in the environment

on the health of an organism since many other factors can influence the body. Studies focusing on the impact of TiO_2 on aquatic organisms used neutrophil function assays and observed comparable changes in neutrophil function (Jovanovic *et al.* 2011b) as we did in our study. These authors tested the disease resistance and found an increased mortality in fish (Jovanovic *et al.* 2015b).This indicates that neutrophil function assays can be a helpful tool to assess the toxic health effects.

The immune system is challenged every day by physical, biological and chemical impacts. The encounter with an increasing amount of nanoplastic particles could be an additional stressor to the immune system. It has been observed that PSNPs fed to daphnia magna negatively influenced the reproduction with an increase of malformations in the offspring and a decreased body size of neonates (Besseling *et al.* 2014b). Alterations in the reproduction can be due to a decreased immune function since the health of an organism significantly influences the ability of an organism to reproduce (Leatherland and Woo 2010).

Our findings that nanoplastics induce oxidative stress in living organisms is in accordance with the findings of Bhattacharya et al. (2010) who observed the production of reactive oxygen species in algae (Bhattacharya *et al.* 2010). Both different taxa use ROS production as an ancient defense mechanism against pathogens. Even though fish show a higher complexity in biological functions both organism shows similar signs of oxidative stress after the encounter with nanoplastics. Algae as well as the fish species fathead minnows are important parts of the base of the food web and changes in their quantity and quality can negatively influence the balance of the food chain.

In crussian carp the ingestion of nanoplastics over the food chain induced

severe effects in the metabolism and behavior of the fish which was concluded to lead to a decreased fitness. Similar to an impacted immune system this can negatively affect their survival in a natural ecosystem (Mattsson *et al.* 2015).

The positive surface charge of the particles utilized in this study might have influenced the corona formation and the impact on the neutrophil function. The corona formation plays an important role in the stimulation of the complement system which leads to the priming of the nanoparticles for phagocytosis and increases the inflammatory reaction (B. Jovanovic and D. Palic 2012). In sea urchin embryos the positive surface charge of amine polystyrene nanoparticles caused embryotoxicity which was not observed for negatively charged carboxylated polystyrene nanoparticles highlighting the importance of the surface characteristics (Della Torre *et al.* 2014).

In this study we used nanoparticles specifically synthesized with a positive surface charge and without additives. No weathering of the particles (for example in seawater, or in the sun) occurred that could have led to the absorption of chemical contamination from the environment. This design of the particle was chosen to enable the evaluation of the toxicity of the particle in its purest form in order to gain a basic understanding of the nanoplastic impact on the immune system. In natural conditions of aquatic environments these pure particles barely exist since they may aggregate, are prone to biofouling and absorb chemical compounds. Especially polystyrene has been shown to be a source and sink for polycyclic aromatic hydrocarbons (PAHs) in the marine environment and was observed to absorbs PAHs up to 200 times more than other common plastic polymers as for example polypropylene and polyethylene terephthalate (PET) (Rochman *et al.* 2013c). PAHs are considered as pollutants of concern since PAHs have been identified to be mutagenic, carcinogenic and teratogenic (Luch2005).

Since the detection of nanoparticles in natural aquatic environments is not possible at the moment due to technical detection limitations we chose a concentration similar to the lowest concentration found in the literature that showed adverse health effects in aquatic organisms (Cedervall *et al.* 2012, Koelmans *et al.* 2015). The chosen concentration of 0.1 g/L nanoplastics used in this study is about 8 to 12 magnitudes higher than the microplastic concentration of 0.4-34 ng/L calculated for European and American freshwater environments (Eriksen *et al.* 2013, Besseling *et al.* 2014a). However, it is several magnitudes lower than the highest concentration (0.5 mg/ml) of microplastics measured in marine waters (Lopez Lozano and Mouat 2009, Besseling *et al.* 2014a). Therefore the concentrations used in this study lie well within the detected concentration ranges of microplastic in the natural environment.

Several studies that measure the plastic concentration in the ocean highlight a loss of particles at the lower end of the scale which is possible due to the degradation of small microplastics into nanoplastics (Eriksen *et al.* 2014). Since the microplastic concentration is increasing, the nanoplastic concentration is likely to increase as well which is why we decided to test the toxic potential of a rather high concentration (Andrady and Neal 2009, Thompson 2015).

In vitro studies can help to identify possible reactive mechanisms; however, these results are of limited validity in the extrapolation of the effects of nanoplastics on the immune system *in vivo*. The *in vitro* results indicate that an impaired immune function could result in living organisms after the uptake of nanoplastics. Therefore future *in vivo* studies could conduct a bacterial challenge after the dietary uptake of nanoplastics to assess if the immune function of the organism is impaired.

VI. ZUSAMMENFASSUNG

Die Ansammlung von Plastikmüll in der Umwelt ist ein Problem von internationaler Tragweite. Kalkulationen gehen von einem Minimum von 5,25 Milliarden Kunststoffteilchen aus, die auf der Oberfläche der Ozeane schwimmen. Dieser große Eintrag in die Umwelt ist auf die geringe Recyclingrate (37%) und das unzureichende Abfallmanagement zurückzuführen. Tiere die große Plastikpartikel aufnehmen sterben häufig durch die negativen Auswirkungen auf das Verdauungssystems oder ersticken, nachdem sie sich in Netzen verfangen haben. Kleine Kunststoffpartikel, wie Mikro (<5 mm) und Nanoplastik (< 100 nm), können Organismen auf zellulärer Ebene schaden, da ihre geringe Größe es ihnen ermöglicht, Zellgrenzen zu passieren und so in das umgebende Gewebe einzudringen.

Durch die wachsende Plastikproduktionsrate und dem Zersetzungsprozess, dem das Plastik in der Umwelt ausgesetzt ist, steigt auch die Masse an Mikro,- und möglicherweise Nanoplastik in der Umwelt an. Mikroplastik ist in der Lage aus dem Verdauungssystem von Muscheln in den Blutkreislauf und in das umgebende Gewebe überzutreten. Im Gewebe induziert das Plastik, da es ein Fremdkörper ist, Entzündungsreaktionen und begünstigt die Entstehung von Granulozytomen. Nanopartikel können aufgrund ihrer geringen Größe und den spezifischen Oberflächeneigenschaften leichter in Zellen eintreten und mit den Immunzellen interagieren, als größere Partikel.

Aufgrund der genannten Erkenntnisse ist es notwendig, eine mögliche Gesundheitsgefährdung von aquatischen Lebewesen, die durch ihre Lebensweise dauerhaft den Partikeln ausgesetzt sind, zu untersuchen. In dieser Arbeit wird die

Hypothese überprüft, dass Nanopartikel aus Polycarbonat (158,7 nm) und Polystyrol (41,0 nm) Auswirkungen auf das Immunsystems von Dickkopfelritze (*Pimephales promelas*, Rafinesque 1820) haben. Dickkopfelritzen sind Süßwasserfische, die häufig als Modellorganismen in toxikologischen Untersuchungen eingesetzt werden. Im Rahmen der Untersuchung wurde die Größe der Teilchen im Plasma gemessen und die Wirkung von Polystyrol und Polykarbonat-Nanopartikeln (0,1 µg / µl) auf das angeborene Immunsystem von Dickkopfelritze mit Hilfe von *in vitro* Neutrophilen- Funktions Assays untersucht.

Die Plastiknanopartikel führten zu einer signifikante Erhöhung der oxidativen Burst-Aktivität, einer erhöhten Freisetzung von primären Granula und einer gesteigerten Freisetzung von extrazellulären Netzen im Vergleich zu der unbehandelten Kontrollgruppe. Die Ergebnisse zeigen, dass das angeborene Immunsystem von Fischen durch Polystyrol und Polycarbonat Nanopartikel gestresst wird und weisen auf das Potential von Nanoplastik hin, die Krankheitsresistenz von Fischpopulationen negativ zu beeinträchtigen.

VII. SUMMARY

The accumulation of plastic debris in the environment has been recognized as a matter of international concern. A minimum of 5.25trillion plastic particles were estimated to float on the surface of the ocean which is due to a low amount of recycling (37 %) and mismanaged waste that enters into the environment. Large plastic debris can lead to suffocation and death after entanglement or ingestion. Small plastic items like microplastic (< 5 mm) and nanoplastics (< 100 nm) can harm organisms on the cellular level since their small size enables them to pass cell boundaries and enter surrounding tissues.

The amount of microplastic, and potentially nanoplastic, is increasing due to their release to the environment and the breakdown of larger plastic items in aquatic ecosystems. Microplastic has been observed to enter into the circulatory system of mussels inducing inflammatory reactions and granulocytoma formations. A facilitated uptake of nanoparticles into the cells and an interaction with the immune system is enhanced due to their small size and their specific surface characteristics. Therefore, the health hazards of nanoplastics on aquatic organisms that are potentially chronically exposed to these compounds were assessed.

The hypothesis tested here is that that polycarbonate (158.7 nm) and polystyrene (41.0 nm) nanoparticles impact the immune system of the freshwater fish fathead minnow (*Pimephales promelas*, Rafinesque 1820) which is as a model fish organism frequently used for toxicology tests. The size of the particles was measured in plasma and *in vitro* neutrophil function assays were used to assess the effect of polystyrene and polycarbonate nanoparticles (0.1 µg/µl) on the innate immune system of fathead minnows. The exposure of neutrophils to the

particles revealed a significant increase in oxidative burst activity, release of primary granules and release of extracellular nets release (NETs) compared to a non-treated control. The results underline the stress response of the fish innate immune system to polystyrene and polycarbonate nanoparticles and highlight their potential to interfere with the disease resistance in fish populations.

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IX. APPENDIX

1. List of tables

2. List of figures

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