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Environmental Toxicology and Chemistry, Vol. 35, No. 12, pp. 3093–3100, 2016

Printed in the USA

POLYCARBONATE AND POLYSTYRENE NANOPLASTIC PARTICLES ACT AS STRESSORS TO THE INNATE IMMUNE SYSTEM OF FATHEAD MINNOW (*PIMEPHALES PROMELAS*)

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(Submitted 11 December 2015; Returned for Revision 1 March 2016; Accepted 16 May 2016)

Abstract: Water pollution with large-scale and small-scale plastic litter is an area of growing concern. Macro-plastic litter is a well-known threat to aquatic wildlife; however, the effects of micro-sized and nano-sized plastic particles on the health of organisms are not well understood. Small-scale plastic particles can easily be ingested by various aquatic organisms and potentially interfere with their immune system; therefore, the authors used a freshwater fish species as a model organism for nanoplastic exposure. Characterization of polystyrene (41.0 nm) and polycarbonate (158.7 nm) nanoplastic particles (PSNPs and PCNPs, respectively) in plasma was performed, and the effects of PSNPs and PCNPs on the innate immune system of fathead minnow were investigated. In vitro effects of PSNPs and PCNPs on neutrophil function were determined using a battery of neutrophil function assays. Exposure of neutrophils to PSNPs or PCNPs caused significant increases in degranulation of primary granules and neutrophil extracellular trap release compared to a nontreated control, whereas oxidative burst was less affected. The present study outlines the stress response of the cellular component of fish innate immune system to polystyrene and polycarbonate nanoparticles/aggregates and indicates their potential to interfere with disease resistance in fish populations. *Environ Toxicol Chem* 2016;35:3093–3100. © 2016 SETAC

Keywords: Nanotoxicology Immunotoxicity Microplastic Aquatic toxicology Fish innate immunity Polystyrene nanoplastic Polycarbonate nanoplastic Neutrophil function

INTRODUCTION

Plastic debris in aquatic ecosystems is recognized as one of the most pressing emerging issues for the global environment by the European Commission [1]. Global plastic production continuously increased from 1.7 million metric tons in 1950 to 299 million metric tons in 2013, an annual average increase of 8.6% [2]. However, the recycling rate is at a low percentage (26%) [2], and plastic contributes about 10% of the municipal waste generated worldwide every year. Nearly 10% of the plastic produced each year ends up in the oceans, where it persists and accumulates; and plastic materials are the most common compound in all marine litter (60–80%) [3]. Plastic contamination is not limited to marine ecosystems as freshwater ecosystems also contain plastic particles because the majority of plastic debris is introduced to oceans by streams and rivers [4]. Most of the plastic litter is not biodegradable and is continuously weathered by physical impacts such as sunlight, rain, wind, and ocean waves. These processes break down the plastic litter into smaller particles referred to as "macroplastics" (>5 mm) or even smaller "microplastics" (<5 mm) [5]. Adverse health effects of plastic pollution in the ocean have been reported for more than 600 marine species [6]. Even though all harmful consequences of plastic described for marine environments may be relevant for freshwater environments as well, limited data are available to estimate ecological effects in rivers and lakes [4]. In the present study, the fathead minnow (*Pimephales promelas*, Rafinesque 1820) was selected as an extensively used model organism for environmental and aquatic toxicology and immunology studies [7]. The fathead minnow is an ecologically important North American forage fish species [8]. In addition, its omnivorous feeding behavior, similar to other cyprinid species, allows study of the interaction of plastic particles and a freshwater fish species [9].

Polystyrene (no. 4) and polycarbonate (no. 11) rank among the 11 highest volume–producing plastics [2] and are used for food packaging, surface coatings, baby bottles, and other household items. While polystyrene is considered a low toxic material [10], use of polycarbonate has become a controversial issue because of the manufacturing process that involves the reaction of bisphenol A (BPA) with phosgene. Bisphenol A is a known endocrine-disrupting substance, may potentially be involved in carcinogenesis, and has been detected as an environmental contaminant in human blood and urine samples [11,12]. The volume of world BPA production is predicted to surpass the 5.4 million metric tons mark by 2015 [13], and BPA is commonly present in the water because of its release during chemical manufacture and transport and the natural breakdown of plastic in the environment [14].

Microplastic particles may further fragment into "nanoplastics," a term that has not been defined uniformly in the literature [15] and may refer to nanoparticles (<100 nm), plastic particles with diameter <20 μm, as well as plastic particles which can pass through zooplankton nets with a mesh size of 330 μm. In the present study, we use the term "nanoplastics" to refer to particles with at least 1 dimension <100 nm, similar to the definition used for nonpolymer materials [15]. Detection methods for nanoplastic are in an early stage of development, and so far no one has detected nanoplastic (as defined above) in the aquatic environment [15]. However, abrasion of microplastics into particles in the nanoscale is likely to happen especially in beach environments because of mechanical

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Published online 21 May 2016 in Wiley Online Library (wileyonlinelibrary.com).

DOI: 10.1002/etc.3501

abrasion by the sand and weathering by ultraviolet radiation. Based on prediction analyses, 320 yr are needed to reduce a 1-mm plastic particle to a nanoplastic [16], a time frame which surpasses the current plastic production era. When abrasion of polystyrene in beach and river conditions is mimicked, the release of microplastic and nanoplastic particles can be observed [17]. The concentration of microplastic extracted from seawater was higher if a smaller net mesh size (80 μm) was chosen compared with a larger net mesh size (450 μm) [18], indicating that smaller plastic particles outnumber larger plastic particles.

Different interactions between plastic microparticles and aquatic organisms have been reported, and recently several studies have addressed nanoplastic material effects on different organisms and their health status, suggesting that nanoplastics can enter different organisms and could interact with the immune system [9,10,19,20]. Cellular innate immunity effectors act as 1 of the first organismal defenses against various agents, making it a likely target for interaction with nanoplastic particles. Activation of neutrophils is critical for host defense, and their function is a valuable tool to assess the health status of individuals and animal populations [21]. If neutrophils are impaired in their ability to phagocytize and kill microorganisms, the normal development and survival of animal populations are at risk [22]. Fish neutrophils can extravasate, migrate chemotactically, degranulate, release neutrophil extracellular traps, and phagocytize particulate matter such as bacteria [23]; however, information about interactions and effects of polystyrene nanoparticles (PSNPs) and polycarbonate nanoparticles (PCNPs) on fish neutrophils is not available.

Therefore, the present aim was to characterize PSNPs and PCNPs in contact with extracellular fluids emulated by fish plasma and Hank's balanced saline solution, as well as determine their potential to change neutrophil function measured as oxidative burst, degranulation, and release of neutrophil extracellular traps in vitro.

MATERIALS AND METHODS

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; Tropical Heimtierbedarf Deutschland) twice daily. Fathead minnows were cared for in accordance with the German guideline for laboratory animal care (§11 TierSchG).

Nanoplastic synthesis

Plastic nanoparticles were synthesized at the Max Planck Institute for Polymer Research, Mainz, Germany. The PSNPs labeled with Bodipy fluorophores were prepared as follows. Sodium dodecyl sulfate (30 mg) was dissolved in 10.5 mL water, followed by 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 stirring for 30 min at 600 rpm. The dispersion was degassed with argon and heated at 70 °C for 1 h. Potassium persulfate (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 d against water (molecular weight cutoff = 25 kDa). The dialyzed emulsion was freeze-dried for 24 h to obtain solid PSNPs. A working suspension of polystyrene was prepared in Hank's balanced salt solution with calcium (Ca), magnesium (Mg), no phenol red (HBSS+; HyClone Laboratories).

The PCNPs were prepared as follows. Polyisoprene-*block*-polymethylmethacrylate, 200 mg, to serve as emulsifier, was placed in a flask, dried under a vacuum overnight, and degassed with argon. Dry cyclohexane (8.3 mL) was added, and the mixture was stirred for 3 h at room temperature, followed by addition of BPA (609.5 mg, 2.67 mmol in 1 mL of acetonitrile). Anhydrous pyridine (0.29 mL, 3.59 mmol) was added, and the dispersion was sonicated for 10 min. 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 and scanning electron microscopy (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. To remove cyclohexane, the suspension was washed with methanol and afterward the solid was dried under vacuum for 24 h. Finally, the working suspension of polycarbonate was prepared in HBSS+.

Nanoplastic particle characterization

Dynamic light scattering measurements were performed with a Malvern Zetasizer 3000HSA. Size distribution and zeta-potential were determined for polycarbonate and polystyrene particles suspended in HBSS+ (Table 1 and Figure 1C). The SEM images of polycarbonate and polystyrene particles were taken using a Zeiss Gemini 912 microscope with samples of PCNPs and PSNPs dispersed in cyclohexane (polycarbonate) and water (polystyrene) and drop-casted on a silica wafer.

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. Because of 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 dynamic light

Table 1. Size and zeta-potential of polystyrene and polycarbonate nanoparticles

Particle type	Hydrodynamic diameter mean size ± standard deviation (nm) in H_2O (polystyrene), cyclohexane (polycarbonate)	Hydrodynamic diameter mean size ± standard deviation (nm) in HBSS+	Z-potential in HBSS+	Fluorophore
Polystyrene	41 ± 0.2	1006.9 ± 380.8	2.1 ± 0.4 mV	Bodipy
Polycarbonate	158.7 ± 41	1710.47 ± 425.6	2.0 ± 0.5 mV	None

HBSS = Hanks' balanced salt solution.

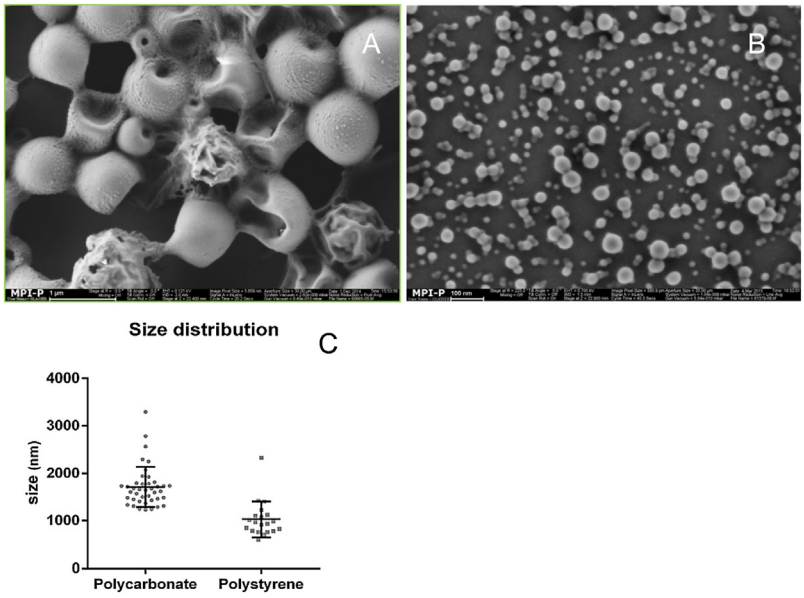

Figure 1. Characterization of polystyrene and polycarbonate nanoparticles. (**A** and **B**) Scanning electron microscopy (SEM) images of PCNP (**A**, cyclohexane) and PSNP (**B**, water). (**C**) Hydrodynamic diameter size distribution graph of PSNP and PCNPs in HBSS+. Mean size ± standard deviation.

scattering and added to fathead minnow plasma. All solutions were filtered through Millex SV filters with a pore size of 5 μm (Merck Millipore) into dust-free quartz light scattering cuvettes (inner diameter 18 mm; Hellma), which were cleaned before with acetone in a Thurmont apparatus. Light scattering experiments were performed with an ALV-CGS 8 F SLS/dynamic light scattering 5022F goniometer equipped with 8 simultaneously working ALV 7004 correlators and 8 QEAPD Avalanche photodiode detectors (ALV). A helium–neon laser (632.8 nm, 25 mW output power) was utilized as the light source.

Neutrophil phagocytosis of plastic nanoparticles

Fluorescent microscopy was used to visualize the phagocytosis of the fluorescently labeled polystyrene particles (Max Planck Institute) by fathead minnow neutrophils. Cells were incubated with a polystyrene suspension for 1 h, 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 (emission 583 nm) and total magnification of ×100 (under oil), and images were captured by Olympus Imaging software cellsSens Dimension and further processed in Photoshop CS6. Because of the occurrence of strong autofluorescence of the neutrophils after fixation, native (not fixed or stained) preparations were used.

In vitro neutrophil assays

Fathead minnows were euthanized with an overdose of MS-222 (Pharmaq). Kidneys from 8 fish were pooled in a sample, and standard neutrophil suspensions (2×10^7 cell mL^{-1}) were prepared as described [23]. Cells were exposed to standard stimulants (phorbol 12-myristate 13-acetate, 1 μg mL^{-1} [Fisher Scientific] and Ca ionophore A23187, 5 μg mL^{-1} [Fisher Bioreagents]) and to PSNPs and PCNPs at 0.025 μg μL^{-1}, 0.05 μg μL^{-1}, 0.1 μg μL^{-1}, and 0.2 μg μL^{-1}; Max Planck Institute) or to HBSS+ as negative control. Prior to loading wells, polystyrene and polycarbonate were suspended in HBSS+, sonicated in bath sonicator (Elmasonic S40H, 340 W) for 2 h, and filtered through a 70-μm sterile cell strainer (Fischerbrand®).

Degranulation of primary granules, respiratory burst, and neutrophil extracellular trap release assays were performed according to established protocols with minor modifications [24,25]. The degranulation of primary granules assay was modified using ready-made liquid 3,3′,5,5′-tetramethylbenzidine (TMB) working solution (2.5 mM in H_2O_2; Sigma-Aldrich) instead of powdered TMB. The respiratory burst assay was based on detection of reactive oxygen species with 5-(and-6)-carboxy-2′,7′-difluorodihydrofluoresceindiacetate reagent. The neutrophil extracellular trap assay was performed as described [26] 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 (phorbol 12-myristate 13-acetate–positive control) of 1 μg mL^{-1}. For the negative control, 10 μL of standard cell suspension was seeded into 96-well plates preloaded with 90 μL HBSS+. All samples were incubated for 2 h at room temperature. After incubation, neutrophil extracellular traps generated by stimulated neutrophils were digested with 500 mU mL^{-1} (1 μmol unit = ∼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 for 20 min at 37 °C. The micrococcal nuclease was inactivated with 5 mM ethylenediamine tetraacetic acid, plates were centrifuged at 4 °C for 5 min at 400 *g*, and supernatants were collected for deoxyribonucleic acid (DNA) quantification and stained with Picogreen (Quant-iT™; Thermo Fisher Scientific) double-stranded DNA fluorescent dye according to the manufacturer's instructions. Plates for all assays were read in a plate reader (Spectra Max M5, SOFTmax Pro 6.2.2; Molecular Devices). All assays were also tested without cells to exclude spectrophotometric interference of the compounds and chemicals themselves. None of the tested chemicals (including nanoparticles) had readings that were different from a background values (empty well of a 96-well plate).

The neutrophil extracellular trap and respiratory burst stimulation index were calculated using the following formula:

Average polystyrene (polycarbonate) value/average of HBSS

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

(average polystyrene [polycarbonate] value – average of HBSS)/(average of *N*-cetyl-*N,N,N*-trimethyl-ammonium bromide value – average of HBSS)

An index >1 means that the compound is a stimulant, whereas an index <1 means that the compound is a suppressor. 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 with the variability of the treatments.

Statistics

Dunnett's procedure for post hoc comparison of means between single control and multiple experimental groups was used to analyze the polystyrene and polycarbonate data for significance. Results with $p \leq 0.05$ were considered statistically significant.

RESULTS

Nanoplastic particle characterization

The SEM of synthesized nanoplastic revealed formation of aggregates with round spherules in the case of PCNPs (Figure 1A). Synthesized PSNPs had a much higher level of dispersion compared to PCNPs (Figure 1B), with occasional formation of smaller aggregates. However, after dispersion in HBSS+, both nanoplastic materials immediately formed aggregates. Dynamic light scattering indicated aggregates in HBSS+ with a mean hydrodynamic diameter ± standard deviation of 1007 ± 381 nm for polystyrene and 1710 ± 426 nm for polycarbonate, and the zeta-potential of PSNPs and PCNPs dissolved in HBSS+ was 2.1 ± 0.4 mV (polystyrene) and 2.0 ± 0.5 mV (polycarbonate; Table 1). Therefore, the cells did not interact with individual nanoplastic particles but were effectively exposed to such aggregates. A multimodal distribution of PSNP and PCNP hydrodynamic diameters was observed (Figure 1C).

PSNP and PCNP size in plasma

The dynamic light scattering measurements of PSNPs and PCNPs in fish plasma were analyzed by adapting the method of Rausch et al. [27]. The autocorrelation function of the untreated fathead minnow plasma could be perfectly described by a sum of 3 exponentials (Equation 1), similar to human serum, giving an averaged hydrodynamic radius of Rh = 14 nm.

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

with the amplitudes a_i and the decay times $\tau_i = \frac{1}{q^2 D_i}$ where q is the absolute scattering vector $\left(q = \frac{4\pi n}{\lambda_0}\sin\left(\frac{\theta}{2}\right)\right)$ and D_i the Brownian diffusion coefficient of component. Data obtained from light scattering analysis of fathead minnow plasma and the respective particles are provided in Figure 2A.

The autocorrelation functions for the polystyrene and polycarbonate particles alone were successfully fitted by a sum of 2 exponentials (Equation 2) when given a size of Rh = 155 nm for the polystyrene particle and a size of Rh = 500 nm for the polycarbonate particle in 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 autocorrelation function of fathead minnow plasma and the respective particles, the correlation function of the plasma particle mixtures could be analyzed. If no aggregation occurs, the resulting autocorrelation function of the

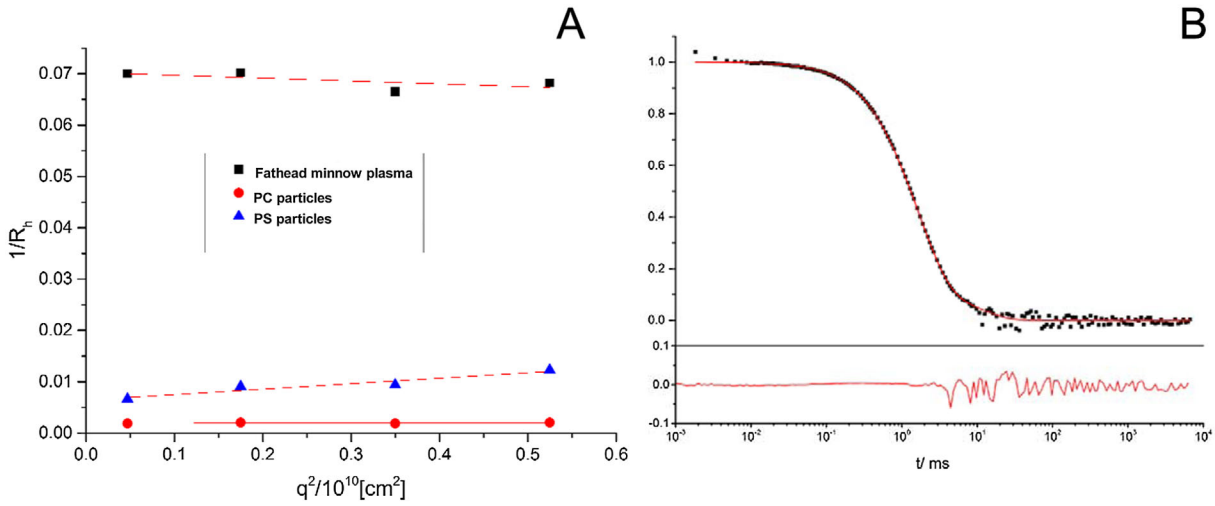

Figure 2. Dynamic light scattering measurements of the aggregation behavior of polycarbonate and polystyrene nanoparticles in fathead minnow plasma. (**A**) Angular dependency of the reciprocal hydrodynamic radius of fathead minnow plasma (black squares), polystyrene particles (blue triangles), and polycarbonate particles (red circles), temperature = 310 K. (**B**) Autocorrelation function of polystyrene particles in fathead minnow plasma. Red line, fit with Equation 3 and the resulting residue. Data points of the autocorrelation function. Scattering angle 60°, temperature = 310 K. PC = polycarbonate; PS = polystyrene.

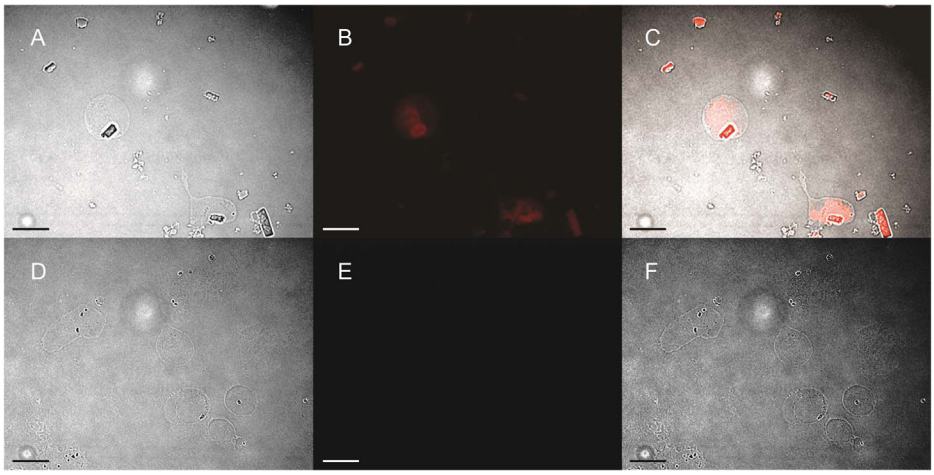

Figure 3. Neutrophil phagocytosis of polystyrene nanoparticles. (**A–C**) Neutrophils incubated with polystyrene nanoparticles. (**A**) Bright field microscopy of native cells and nanoparticles. (**B**) Fluorescent microscopy of native cells and nanoparticles, with Texas Red filter (emission 583, exposure time 178 ms). (**C**) Overlay of images **A** and **B** showing phagocytized nanoparticles and aggregates in the cytoplasm of neutrophils. (**D–F**) Control images, no polystyrene added, with identical microscope and imaging settings. Scale bar = 10 μM.

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 2 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 was the formation of aggregates with sizes larger than the largest size of the original components (particle/fathead minnow plasma) indicated by the fittings. Figure 2B shows the results of the mixture of polystyrene with undiluted fathead minnow plasma.

Neutrophil phagocytosis of plastic nanoparticles

Neutrophils phagocytized larger PSNP aggregates, and the observed cytoplasmic fluorescence also indicates the presence of smaller-diameter nanoparticles in the cells (Figure 3A–C). Control images show no neutrophil autofluorescence in native preparations (Figure 3D–E).

Neutrophil function assays

Neutrophil function was assessed with degranulation of primary granules, oxidative burst, and neutrophil extracellular traps release after the exposure to PSNPs and PCNPs in vitro. Both polystyrene and polycarbonate caused a significant degranulation increase in vitro compared to negative control (HBSS+) at a dose of 0.1 μg μL^{-1} ($p < 0.0001$; Figure 4). A significant induction of neutrophil extracellular trap release was observed in vitro for both particle types at a concentration of 0.1 μg μL^{-1} ($p < 0.0001$; Figure 4). Polycarbonate caused a significant increase of respiratory burst at a concentration of 0.1 μg μL^{-1} ($p < 0.001$; Figure 4), whereas polystyrene had no effect. The concentration–response curve for PCNPs and PSNPs was tested with 4 different nanoparticle concentrations: 0.025 μg μL^{-1}, 0.05 μg μL^{-1}, 0.1 μg μL^{-1}, and 0.2 μg μL^{-1}. The PCNPs (Figure 5A) initiated maximum neutrophil function response at 0.1 μg μL^{-1} in all 3 assays. All PSNP concentrations caused a significant increase of the degranulation of neutrophil primary granules in a dose-dependent manner (Figure 5B). At all concentrations PSNPs induced neutrophil extracellular trap release (Figure 5B). Oxidative burst activity was, however, significantly increased only at 0.1 μg μL^{-1} and 0.2 μg μL^{-1} of PSNPs compared to the negative control (HBSS+). The PCNPs induced only a significant response in respiratory burst at a single concentration of 0.1 μg μL^{-1}, which was this time much smaller than the PCNP response observed previously for the same concentration in Figure 4. A higher concentration of PCNP did not increase further respiratory burst response.

DISCUSSION

The results of the present study confirm the hypothesis that PSNPs and PCNPs can impact the immune system of fish by stimulating degranulation of primary granules, oxidative burst activity, and neutrophil extracellular trap release. This shows the potential of PCNPs and PSNPs to act as stressors for

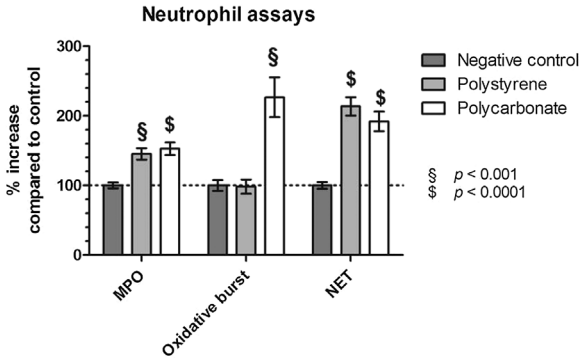

Figure 4. Polystyrene and polycarbonate nanoparticles increase response of fathead minnow neutrophils in vitro. Nanoparticles of polystyrene (gray) and polycarbonate (white) indicate neutrophil function compared to nontreated control (Hank's balanced salt solution with calcium, magnesium, no phenol red [HBSS+]). MPO = degranulation of neutrophil primary granules measured as myeloperoxidase exocytosis compared to control (HBSS+, $n = 18$; Oxidative burst = cumulative production of oxygen radicals compared to control (HBSS+, $n = 17$); NET = neutrophil extracellular trap release from fathead minnow neutrophils compared to the negative control (HBSS+, $n = 23$). Data are shown as mean ± standard error of the mean, and the level of significance is indicated: $^{\S}p < 0.001$, $^{\$}p < 0.0001$.

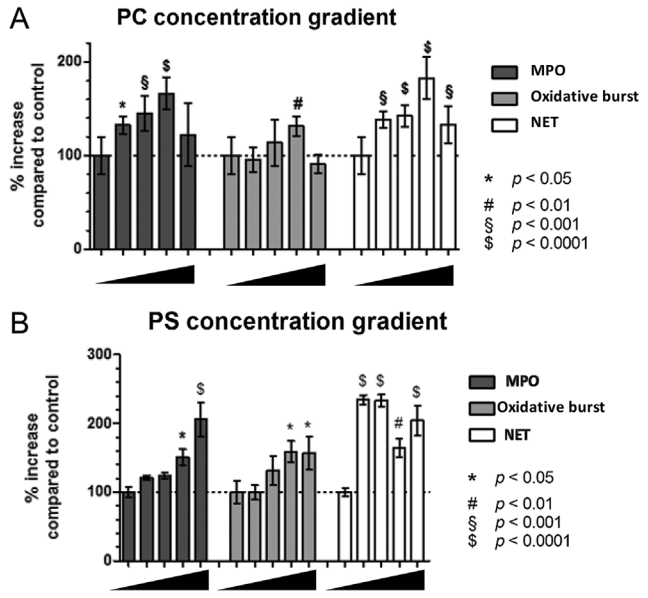

Figure 5. Different concentrations of polycarbonate and polystyrene nanoparticles increase neutrophil function responses. Nanoparticles of polycarbonate (**A**) and polystyrene (**B**) significantly increased myeloperoxidase, oxidative burst, and neutrophil extracellular trap release compared to control (Hank's balanced salt solution with calcium, magnesium, no phenol red [HBSS+], $n = 6$). Concentrations used in the present study were 0.025 μg μL^{-1}, 0.05 μg μL^{-1}, 0.1 μg μL^{-1}, and 0.2 μg μL^{-1} for both PC and polystyrene nanoparticles. MPO = degranulation of neutrophil primary granules measured as myeloperoxidase exocytosis compared to control (HBSS+, $n = 18$; Oxidative burst = cumulative production of oxygen radicals compared to control (HBSS+, $n = 17$); NET = neutrophil extracellular trap release from fathead minnow neutrophils compared to the negative control (HBSS+, $n = 23$). Data are shown as mean ± standard error of the mean, and the level of statistical significance is indicated: $^{*}p < 0.05$, $^{\#}p < 0.01$, $^{\S}p < 0.001$, $^{\$}p < 0.0001$.

organisms that incorporate them. Because nanotechnology is developing quickly and the nanoparticle concentration in the aquatic environment is expected to increase, more research is needed to provide information about the safety and sustainability of nanotechnology to identify possible toxic effects for the environment [28]. The nanoplastic concentration has not yet been determined in aquatic ecosystems for technical reasons [15], and estimates of nanoplastic contaminant concentrations are currently based on microplastic concentrations. Because there is a strong increasing trend in plastic production [2], we selected a wide range of concentrations to be used during in vitro exposure to establish a reference point for future studies that would be relevant in case of possible increases of nanoplastic in the environment. Furthermore, the concentrations used in the present study are similar to those previously reported in fish either directly or through bioaccumulation [9].

The fact that the small plastic particles interact with phagocytic cells is not without precedent as, after all, fluorescently labeled polystyrene microspheres are used for calibration in flow cytometry. Previously, it was shown that the rate of phagocytosis of polystyrene particles is highest for particles possessing diameters between 1.7 μm and 3 μm [29,30]. However, the phagocytosis rate was influenced not by the physical size of polystyrene particles but by the characteristic features of membrane ruffles of phagocytic cells as well as the attachment process [29]. This means that the microsized aggregates created by small nano-sized polystyrene particles through rapid aggregation will be favored for phagocytosis by phagocytic cells. Because the interaction of nanoparticles with the compounds in the circulatory system plays an important role in identifying possible toxicological effects, it is important to study the nanoparticle behavior not only in laboratory media but also in biological fluids such as plasma. In extracellular fluids the surface properties and size of nanoparticles play an important role in the binding process of proteins to nanoparticles, leading to the formation of protein coronas [31]. Positively charged PSNPs with a protein corona showed increased attachment and cellular uptake in endothelial cells compared to negatively charged protein-coated PSNPs [31]. The obtained size for PSNPs in fish plasma, in the present study, may enhance corona formation and stimulate the complement system, leading to the priming of nanoparticles for phagocytosis [32]. The present results demonstrated PSNP and PCNP behavior suspended in fish plasma (Figure 2). The present study is, to our knowledge, the first time that the behavior of nanoplastic particles in plasma of a nonmammalian organism (fish) has been studied, and these data may facilitate future research regarding the effects of nanoplastics in aquatic organisms.

The absorption of plastic particles is also highly influenced by their surface properties. Positively charged particles seem to react more readily with living cells because of electrostatic interaction, hydrogen bonding, and hydrophobic interaction [19]. It has been hypothesized that positively charged nanoparticles preferentially interact with the negatively charged cell membrane to result in facilitated cellular uptake. The particles utilized in the present study had a positive surface charge, which might have enhanced the phagocytic activity of the neutrophils leading to the significant responses to both plastic types in the assays (Table 1). The size distribution of PSNPs and PCNPs did not limit neutrophil phagocytosis (Figure 2). The average size of aggregates that were formed in the HBSS+ solution had diameters of 1710 nm (polycarbonate) and 1007 nm (polystyrene), whereas neutrophils can phagocytize aggregates up to 3 μm [33]. A study investigating the effects of nanoplastic on the function of human neutrophils, macrophages, and monocytes found similar results compared with the present study with fish cells [34]. Carboxyl polystyrene particles of various sizes (20–1000 nm) have induced oxidative burst and degranulation of primary granules in immune cells. However, with an increase in size the effect on granulocytes (neutrophils) was more pronounced [34].

The neutrophil extracellular trap release and the degranulation of primary granules suggest an ability of nanoparticles to induce a stress response of the immune system of fish. This is in accordance with recent findings of severe behavioral and metabolic effects in fish fed with PSNPs, showing reduced feeding activity, decreased overall fitness, and impacts on aquatic food webs [9].

The polycarbonate particles did not trigger significantly higher immune responses than polystyrene, even though a different toxicity could have been expected because of the different material characteristics. Previous studies with nanoparticles did not show acute toxic effects [35], yet sublethal effects of metallic nanoparticles on the innate immune response had serious consequences during a disease challenge [36]. Because plastic is not a naturally occurring material in the aquatic environment, it can act as an additional stressor to biota. Besseling and colleagues [20] showed that exposure of *Daphnia magna* to PSNPs had severe impacts on reproduction. The number and body size of neonates were

lower and the neonate malformation rate rose to 68% of the individuals in the next generation after exposure [20]. The impaired reproduction could also be associated with a depressed immune function [37], and life history traits of aquatic organisms can serve to identify the chemical toxicity or ecological stress potential of substances [38].

Our in vitro study on immunological responses reveals a potential target and suggests further implications of possible nanoparticle effects on aquatic organisms. The results clearly demonstrate that neutrophil function assays can serve as a valuable tool to assess the impact of nanoparticles on the innate immune response and could help to further investigate the effect of different plastic compounds. In that sense in vivo feeding experiments would be the next step to study the role of nanoparticles and to quantify their potential to cause changes in host–pathogen relationships [36].

The effects of nanoplastic can be related to particle toxicity, but plastic-associated chemicals and absorbed environmental chemicals can also have toxic effects. Plastic fragments found in marine habitats have been shown to absorb persistent organic pollutants (POPs). Polystyrene absorbs polycyclic aromatic hydrocarbons to a greater extent than other common plastic types such as polypropylene, polyethylene terephthalate, and polyvinylchloride [39]. Data for POP absorption to polycarbonate are not available. In the present study the absorption of POPs to polystyrene and polycarbonate particles was not used to be able to establish a baseline for the particle toxicity itself rather than the toxicity of the adsorbed chemicals or a combination of the 2. With regard to the high probability of POP absorption to plastic particles, further research should include PSNPs and PCNPs exposed to aquatic environments as well as virgin plastics functionalized with additives.

CONCLUSION

In conclusion, PSNPs and PCNPs can act as stressors to the innate immune response of fish. Both plastic types have activated neutrophil function as evidenced by phagocytosis, degranulation, neutrophil extracellular trap release, and, to a small extent, increase in oxidative burst. Therefore, nanoplastics could potentially interfere with innate immune responses in fish populations by altering organismal defense mechanisms.

Acknowledgment—The authors thank J. Miljatović for technical assistance with neutrophil function assays and microscopy and T. Schmidt for technical assistance with fish care and maintenance. The present study was partially funded by the CIG project of D. Palić (TITOXPATH, no. PCIG13-GA-2013-618152) and the LMU startup funding provided to Chair of Fish Diseases and Fisheries Biology.

Data availability—Data are available on request from the corresponding author (d.palic@fisch.vetmed.uni-muenchen.de).

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