Developmental effects of micro- and nanoscale tire particles and recycled rubber on zebrafish

By:
Taylor Mottern

A THESIS

Submitted to:
Oregon State University
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Honors Baccalaureate of Science in Bioengineering (Honors Associate)

Presented February 14, 2022
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Taylor Mottern for the degree of Honors Baccalaureate of Science in Bioengineering presented on February 14, 2022.
Title: Developmental effects of micro- and nanoscale tire particles and recycled rubber on zebrafish.

Abstract approved:____
Stacey Harper

Plastics have become an essential part of everyone lives, found in almost every product made today. Tires only make up a fraction of these products but as their use increasing so does the microplastics that flake off. Many studies have evaluated the toxic effects of tire particles on aquatic environments by testing the leachate they give off in water. However, no studies to date have investigated the specific effects of micro- and nanoscale tire particles themselves. With the increase of many toxic particulates being introduced into the ocean and other aquatic environments, there is serious concern as to how it could affect the life in these areas. This study looked at the effects of micro (1-20 μm) and nano-sized (<1 μm) tire and recycled rubber particles on developing zebrafish embryos. Zebrafish (*Danio rerio*) were selected for these studies because they are vertebrates that make for a efficient and cheap ecotoxicity indicator for potential impacts on fish in aquatic systems while providing insight into potential negative human health effects. Previous studies reveal that tire particles can cause mortality and sub-lethal effects in multiple fish species, while recycled rubber does not show any overt toxicity but can release polycyclic aromatic hydrocarbons (PAHs), some of which are known toxicants. In our studies, the highest mortality was caused by exposure to nanoscale tire particles (TPs). Micro-sized particles showed limited mortality and sub-lethal effects. Leachate exposures resulted in varying mortality occurrence but nothing that was significant, although, the leachate did show the most sub-lethal effects. These effects occurred at concentrations well above levels currently identified in environmental samples; however, the sampling techniques currently used in environmental sampling overlook any particles in the nanoscale range. Given that environmental concentrations are completely unknown, it is important to understand the levels at which micro- and nanoscale TPs and their leachates can elicit adverse outcomes for exposed organisms.

Key Words: Zebrafish, tire particles, nano, micro, fractionate

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I understand that my project will become part of the permanent collection of Oregon State University, Honors College. My signature below authorizes release of my project to any reader upon request.

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Developmental effects of micro- and nanoscale tire particles and recycled rubber on zebrafish

Abstract

Plastics have become an essential part of everyone lives, found in almost every product made today. Tires only make up a fraction of these products but as their use increasing so does the microplastics that flake off. Many studies have evaluated the toxic effects of tire particles on aquatic environments by testing the leachate they give off in water. However, no studies to date have investigated the specific effects of micron and nanoscale tire particles themselves. With the increase of many toxic particulates being introduced into the ocean and other aquatic environments, there is serious concern as to how it could affect the life in these areas. This study looked at the effects of micro (1-20 μm) and nano-sized (<1 μm) tire and recycled rubber particles on developing zebrafish embryos. Zebrafish (*Danio rerio*) were selected for these studies because they are vertebrates that make for an efficient and cheap ecotoxicity indicator for potential impacts on fish in aquatic systems while providing insight into potential negative human health effects. Previous studies reveal that tire particles can cause mortality and sub-lethal effects in multiple fish species, while recycled rubber does not show any overt toxicity but can release polycyclic aromatic hydrocarbons (PAHs), some of which are known toxicants. In our studies, the highest mortality was caused by exposure to nanoscale tire particles (TPs). Micron-sized particles showed limited mortality and sub-lethal effects. Leachate exposures resulted in varying mortality occurrence but nothing that was significant, although, the leachate did show the most sub-lethal effects. These effects occurred at concentrations well above levels currently identified in environmental samples; however, the sampling techniques currently used in environmental sampling overlook any particles in the nanoscale range. Given that environmental

concentrations are completely unknown, it is important to understand the levels at which micro- and nanoscale TPs and their leachates can elicit adverse outcomes for exposed organisms.

Introduction

In 2019, plastic production reached an estimated 368 million metric tons (Tiseo, 2021). Plastics that end up in the environment are slow to decompose, with time frames ranging from 58 years for plastic bottles to 1200 years for plastic pipes (Chamas et. al., 2020). Over time, plastics do break down into mesoplastics (5-40 mm), microplastics (1-5000 μm), and nanoplastics (<1 μm) (Peng et al., 2020). There are two main mechanisms that degrade plastics, physical and chemical. Physical changes can occur from cracking, embrittlement, and flaking while chemical changes occur by bond cleavage or oxidation (Gewert et al., 2015). Degradation can also be accelerated by microbial breakdown, heat, and/or light (Pathak, V.M., 2017). Plastic have continued to increase in versatility as more plastics and plastics combinations are created. The largest market of plastics are thermoplastic polymers which include polyethylene terephthalate (PET), high, low, and linear-low density polyethylene (HDPE, LDPE, and LLDPE), polyvinyl chloride (PVC), polypropylene (PP), polystyrene (PS), among others like polycarbonate (PC) (CUT, 2021).

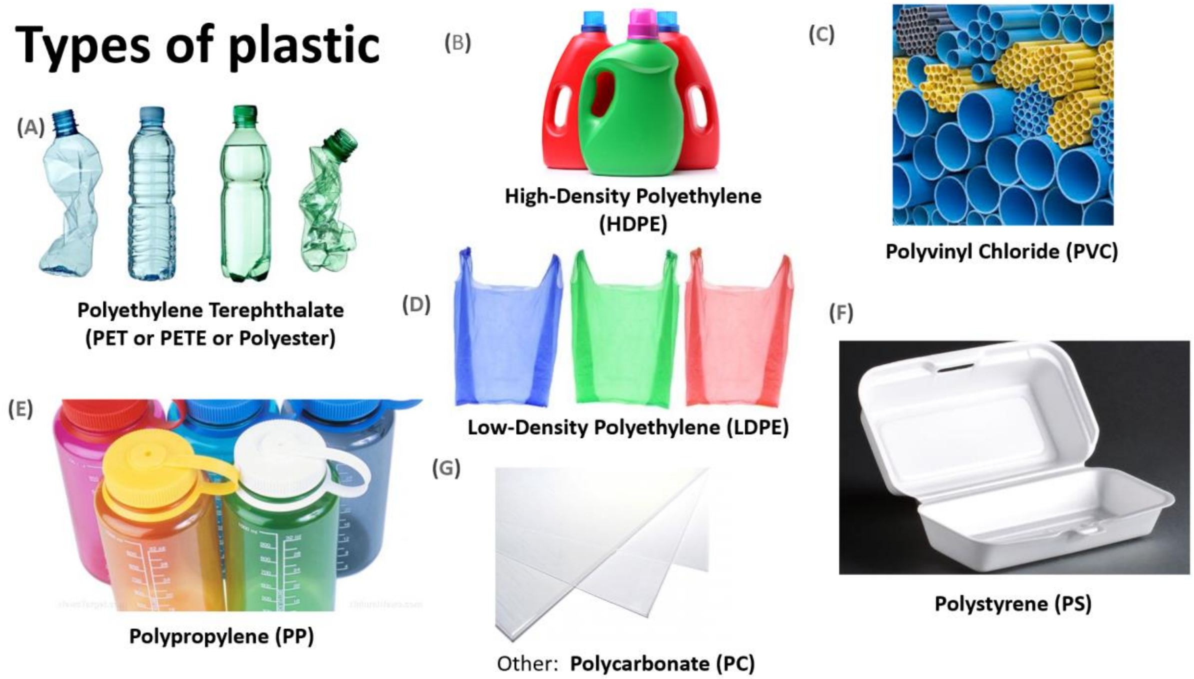

Figure [1]: Example products can be seen for each type of plastic. (A) PET used in water bottles, (B) HDPE used for laundry detergent bottles, (C) PVC used for underground pipes, (D) LDPE used for grocery bags, (E) PP used for reusable bottles, (F) PS used for single-use storage containers, and (G) PC used as a glass window replacement (CUT, 2021).

They have become a part of almost every aspect of life from construction to consumer goods. Some examples can be seen in Figure [1]. An estimated 58% of these plastics end up in landfills where rain can wash the micro- and nano-sized plastics, which are degraded from larger pieces, down into streams and rivers eventually making their way to the ocean where they mix with the plastics that have been directly thrown in (Geyer et al., 2017). Once plastic degrade into micro and nanoplastics, they can take different morphologies. They can form as fibers, fragments, films, spheres, foams, and pellets (Peng et al., 2020).

The effects of microplastics have become a rising concern as pollutants of the environment for numerous reasons. First, plastics in landfills and waterways can leach potentially harmful chemicals into the environment. These leachates are often additives that are known to be health hazards to organisms, including metals like cobalt, nickel, and zinc; as well as organics like BPA, phthalates, and brominated flame retardants (Gunaalan et al., 2020). Leachates are known to cause mortality in many different marine organism but toxicity (Capolupo et al., 2020)(Li et al, 2016)(Tian et al., 2021). Second, some of these plastic particles are porous and able to sorb environmental pollutants such as dichlorodiphenyltrichloroethane (DDT) and polychlorinated biphenyls (PCBs) to their surface or absorb the chemicals into their interior (Wright et al., 2013). Third, the smaller particles can enter all levels of the food web and transfer up the food chain as well. Plankton can ingest the lightweight particles that float at the surface while the particles that sink can be taken up by organisms living beneath the surface (Fossi et al., 2012). If these microparticles are small enough, 3.0 μm or smaller, they can even enter tissues other than the digestive system, like the cardiovascular system and liver (Baztan et al., 2016). Exposure to and uptake of micro and nanoplastics have various effects such as blockage and damage of digestive organs, inflammation, reduced reproduction, impacts on metabolism, among other negative health impacts (Peng et al., 2020).

Tires are one such item made of multiple different types of plastics, rubber, and metal. An all-season tire has about 30 types of synthetic plastics, 8 natural rubbers, and other components such as steel, carbon black, polyester, nylon, and on average 40 different chemicals (Table 1); However, the composition depends on the application of each tire (Wagner et al., 2018).

Table 1: General composition of tires (adapted from Wagner et al., 2018).

Category	Content [wt%]	Ingredients
Rubber/Elastomere	40-60	poly-butadine, neoprene, et al
Reinforcing agent (Filler)	20-35	carbon black, silica, silanes
Process Oils	12-15	mineral oils
Textile & metal net	5-10	ZnO, S, Se, Te
Vulcanization agent	1-2	Thiazoles, organic peroxides, et al.
Additives	5-10	Preservatives, anti-oxidants, et al.

Tire particles (TP) created from road abrasion have caused an estimated 5-10% of global plastics to end up in the ocean (Kole et al., 2017). They also contributed to 5-30%, depending on the type of tire, of non-exhaust emission caused by traffic (Wagner et al., 2018). The TP travels from the roads, urban and rural, where they go to wastewater treatment plants or collect in ditches and then flow into rivers that connect to the oceans. Once the TP is able to travel into aquatic environments it has the potential to affect the development of life in those areas. Much of the published research on the effects of TPs has focused on the effects elicited from tire leachate, the toxic components left after the tire particles are removed. Effects have been evaluated for plankton (*Daphnia*), decapods, fish and a few other aquatic species (Redondo-Hasserlerharm et al., 2018)(Khan et al., 2019)(Halle et al., 2020)(LaPlanca et al., 2020). In more recent studies, the effects of nano-TWPs have been investigated. One study looked at the benefit that zinc in these particles have on the growth of cucumbers (Moghaddasi et al., 2013). Only a few studies to date have looked into the effect nano-TWPs have on the aquatic environment(Triebskorn et al.,2019)(Shariati et al., 2020). The toxicity of the TWP varies between the procedure used to leach and the aquatic lifeform that is tested (Wik et al., 2009). Studies that used whole tire were less toxic compared to tests done with pulverized tire particles (tire crumb), which is due to the larger relative surface area caused by the degradation process (Wik et al., 2009). Some species, like *Daphnia,* were not affected by whole tire but were when exposed to tire crumb (Wik et al., 2009).

Recycled rubber (RR) is another component of tire that has been used in many applications to reduce and recycle tire waste. RR is made of natural and synthetic rubbers and used in the creation of asphalt, rubber mulch, and rubber pavers. RR pavers have been used for sidewalks, flooring, playgrounds, as well as sport fields (Llompart et al., 2013). Studies have been found that these products have toxicity risks caused by chemicals like polycyclic aromatic hydrocarbons (PAHs). (Llompart et al., 2013) A study on crumb rubber using two fish species mummichogs and fathead minnow, observed ingestion and breakdown of PAHs present in the rubber particles (LaPlaca et al., 2020). This is concerning as many children play in areas that use RR pavers and have the potential to enter waterways as the pavers degrade. Some PAHs, like benzo(a)-pyrene, are well known carcinogens, mutagens, and teratogens (Kim et al., 2013).

Zebrafish are a commonly used aquatic species for toxicity testing. This species is a vertebrate and is transparent to allow for easy assessment of development. The assessment of embryonic development when introduced to a contaminant can easily be evaluated non-invasively using a dissecting microscope. It is less expensive and more size efficient than other vertebrate models, such as mice(Zhang et al., 2014). Zebrafish also have similarities with humans at molecular and physiological levels (Krishnaraj et al., 2016). They have fast breeding cycles, high fecundity, diverse adaptability, and their genome has been thoroughly studied which make them suited for a broad range of research (Dia et al., 2014). The embryonic zebrafish model has been used in nanomaterial and chemical screening as well as drug discovery to evaluate the effects of a wide range of substances (Usenko et al., 2007). Moreover, zebrafish are used as indicators for cardiotoxicity, hepatotoxicity, and neurotoxicity (Zhang et al., 2014).

An embryonic zebrafish assay was used to analyze the effects of different sized tire and recycled rubber particles. Our objective was to gain a better understanding of the effects of tire particles (TP) and RR particles themselves in addition to the effects from leachate alone. Comparisons can be made to find out if these materials are toxic enough to warrant concern. The nanoscale particles are expected to be just as or more toxic than the leachate as they are able to penetrate the chorion surrounding the embryo in early stages of development which could lead to higher levels of particles and leachate inside of the protective chorionic barrier.

Material & Methods

Tire particles

TP was obtained from a new all-season tire that is designed for 40,000 miles of use. The particles were created using a cryomill (Retch Cryomill, Haan, Germany). This process used liquid nitrogen to go below the typical tire glass transition temperature of -65 to -50 ^{o}C, ceramic balls are then used to break the tire apart. To fit the tire into the chamber, tire from the inner portion of the tire tread was sliced off into 2-4 mm pieces with a clean stainless blade. The shaving were then cryomilled at 30 Hz for three 5 minute cycles separated by 5 minute pre-cooling cycles at 5 Hz.

Recycle rubber particles

RR was obtained as rubber mulch from Recycled rubber products LLC (Joliet, IL). It was milled through the same process as the TP material.

Particle suspension and fractionation

TP and RR were separated using a 20 μm sieve followed by a 1 μm mixed cellulose ester filter (Advantec, Chilliwack, BC Canada) to separate them into different sized fractions. A wet suspension with the addition of natural organic matter (NOM) (Suwanee River NOM, International Humic Substances Society, St. Paul, MN) was needed to keep the particles from agglomerating (clumping). Three grams of milled tire were combined with 300 ml of a solution containing 50 mg/L NOM and Milli-Q water in a flask and filtered through a 0.2 μm filter (Anotop, North Bend, OH). To further increase yield of smaller particles of RR, glass beads were added to the suspension, capped and autoclaved for 20 minutes, then placed on a shaker for 72 hours at 130 rpm to stabilize the suspension. Solution was then poured through a course strainer to remove the glass beads. The solution was then strained through a 20 μm standard mesh sieve to produce particles less than 20 μm in one dimension. A 47 mm syringe filter containing a 1 μm mixed cellulose ester filter (Advantec, Chilliwack, BC Canada) was used to filter the solution down to a suspension of less than 1 μm nanoparticles. The filter holder was then back flushed to collect a suspension of particles in a range of 1-20 μm. Mass filters of 3,000 KDa (VWR, Radnor, PA), 10,000 KDa (Corning, England), and 30,000 KDa (Amicon, Ireland) were put in a centrifuge (5430 Eppendorf, North America) to remove particles and separate the leachate into 3 different mass fractions.

Concentration characterization

The 1-20 μm particles were counted in triplicate sampling using a flow cytometry (Acurri C6 Flow Cytometer). The nanoscale particles were counted in triplicate using nanoparticle tracking Analysis (NTA) on a NanoSight instrument (NanoSight NS500, Malvern Instruments).

Scanning electron microscopy and particle characterization

A drop of each sample was placed on a 5 x 5 mm silicon wafer (Ted Pella Inc. Prod No. 16008) mounted to an aluminum specimen mount (Ted Pella Inc. Prod No. 16111). Samples were then placed on a hot plate set at 35°C for approximately 1 hour to dry. All samples were then coated with a layer of gold-palladium using a Cressington 108auto sputter coater (Cressington Scientific Instruments, Watford, UK) to prevent sample charging and improve signal-to-noise ratio during imaging. Random images of the polydisperse microplastic particle samples were taken with a FEI Quanta 600F environmental scanning electron microscope (FEI Company, Hillsboro, Oregon, USA) at 5 kV beam voltage. The FEI Quanta 600F is housed in the Oregon State University Electron Microscopy Facility (Corvallis, Oregon, USA).

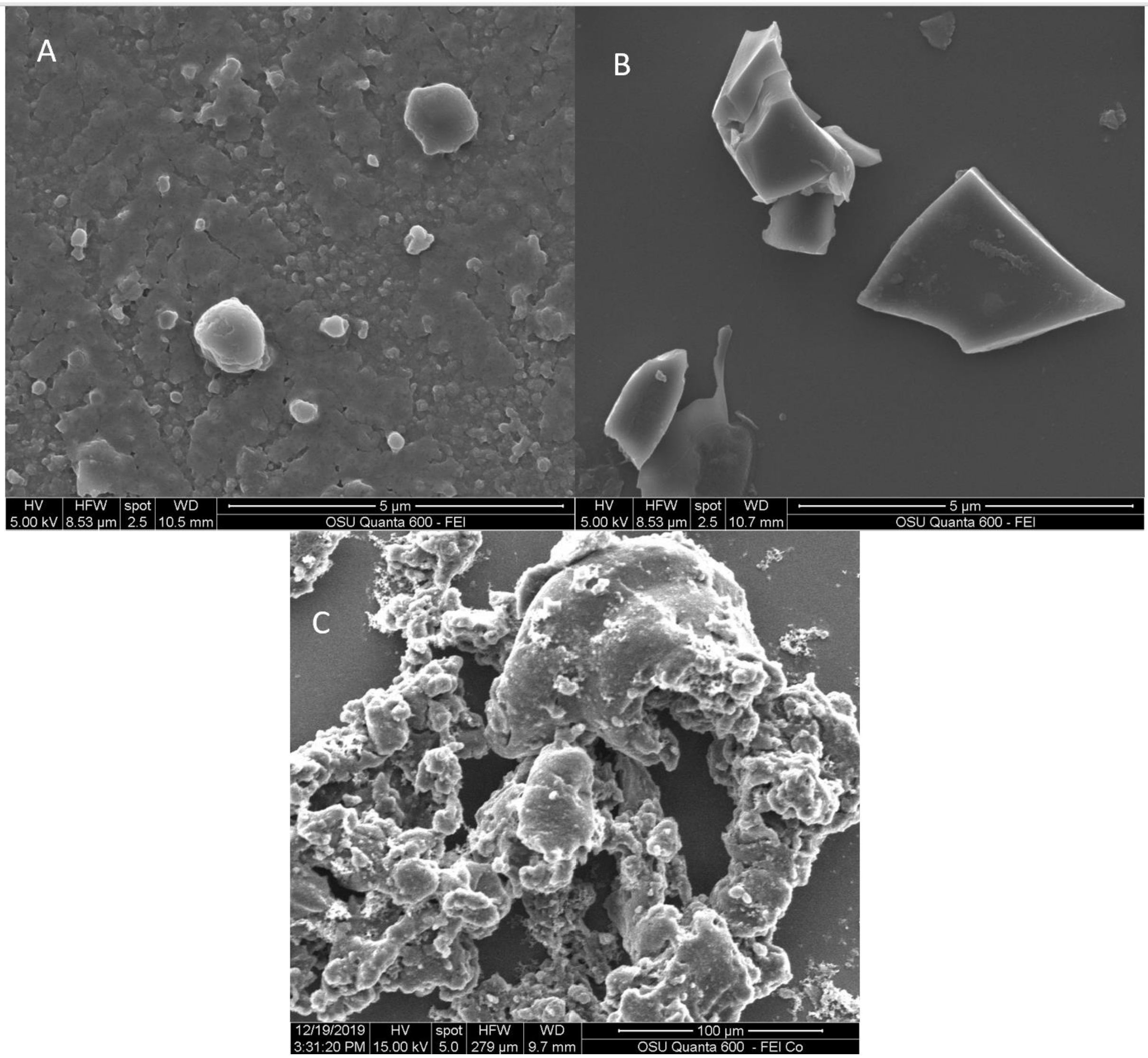

Figure [2]: SEM Images of (A) Nano tire sized less than 1 µm, (B) micro tire sized at 1-20 µm, (C) Recycle rubber that appears to have aggregated together. Nano tire particles are mostly spherical while the micro size tire particles are jagged chunks. The recycle rubber was sized at 1-20 µm but looks to have a rounder shape before aggregating.

Zebrafish exposures

The experiment was conducted in 96-well plates (falcon flat bottom polystyrene) with each row pertaining to a different concentration and each plate has a control row of fish water and 50 mg/L NOM only. Embryos were exposed from 8 hours post fertilization (hpf) until 120 hpf. Embryos were obtained from Sinnhuber Aquatic Research Laboratory at Oregon State University. At 24 hpf, each zebrafish was evaluated for spontaneous movement (a typical developmental behavior in zebrafish), normal growth (developmental progress), mortality, and hatching from their chorionic membrane. At 48, 72, and 96 hpf, mortality and hatching were evaluated. At 120 hpf, zebrafish were evaluated for morphological malformations of the body axis, brain, eyes, pectoral fin, pigment, circulation, jaw, snout, otic vesicle, trunk, and somites. These endpoints are commonly used in the study of developmental effects on zebrafish (Gonçalves et al., 2020). In addition, zebrafish were evaluated for pericardial or yolk sac edema, which is a swelling around the heart or yolk sac, respectively. All defects were recorded as present or absent observed through the use of a microscope (Olympus SZ61). Images were taken of significant effects using an imaging camera (SC100). The different tire samples that were tested are as follows: 1-20 micrometer particles, nanometer particles, leachate, leachate of 3 different molecular weights: 3 KDa, 10 KDa, and 30 KDa. Recycled rubber was looked at with samples as follows: 1-20 micrometer particles, nanometer particles, and leachate. The chorion was kept on to simulate their growth in the real environment and all samples were diluted with fish water and 50 ppm NOM. Exposures followed ACUP #5114 guidelines. Exposure concentrations for the nanometer and micrometer particles was done through a set of 10 dilutions but differed depending on the concentration characterization found and included a negative control of 50 mg/L NOM and Milli-Q water as well as an exposure using the stock concentration. Leachate fractions were done in a set of 11 from 0-100 % in exposures differing by 10%. Tricaine methanesuflonate (Syndel) was used to immobilize the zebrafish for observation of effects and were then euthanized according to our OSU-approved animal care and use protocol.

Statistical analysis

Statistical analyses were done using a Fisher's Exact Test to determine statistical differences ($p \leq 0.05$) between control, exposure, type, size, as well as the different concentrations when assaying mortality and sublethal effects (Sigma Plot, SPSS, Chicago, IL). The Fisher's Exact Test is used in this assay instead of Chi-squared test because of the small sample size, a frequency of at most 8 per group. The Chi-squared test is only an approximation of comparative distribution of categorical variables while the Fisher's Exact Test is an exact comparison between categorical variables (Bower, 2013).

Results

Exposure data was analyzed in sigma plot and graphed in excel to show if occurrence of development defects and mortality were significant for each concentration. In this case, the Fishers Exact test was used where any data found was significant at an $\alpha=0.05$ with a sample size of 8. Below, Figure [4] shows the mortality rates of the different size fractions. Data labeled Nano TP, 1-20 micro TP (standard), and tire leachate were obtained from Brittany Cunningham as standards to compare to this set of exposures. It can be seen that the nanometer fraction of TP showed greater mortality than the leachate with the micrometer particles showing no significant occurrence. The RR showed no significance in lethal effects on the zebrafish.

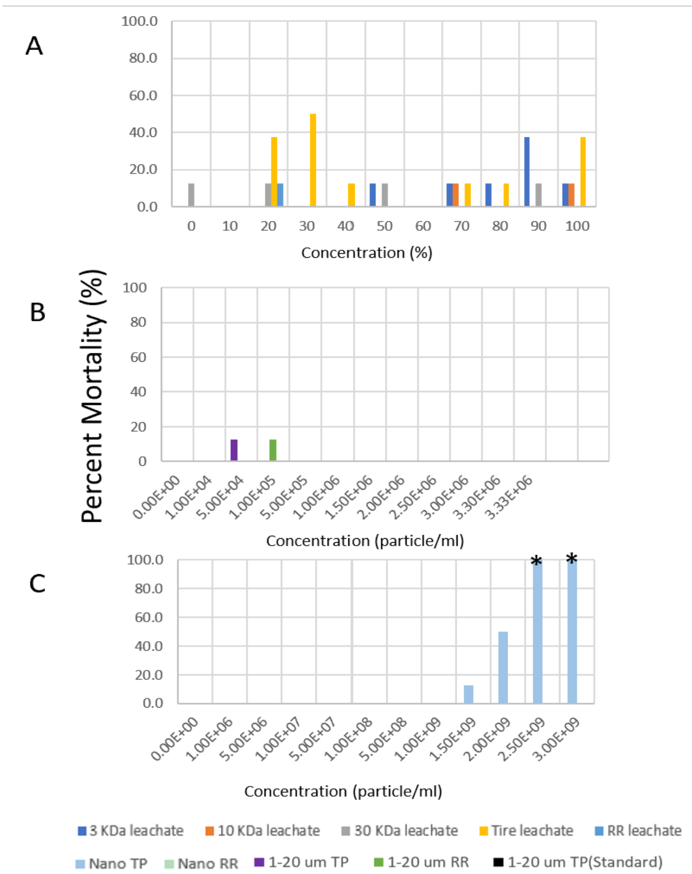

Figure [4]: Zebrafish mortality from 5-day exposure to (A) tire particle leachate, (B) 1-20 micrometer tire particles, and (C) nanoscale tire particle exposures.

When comparing the sub-lethal effects of the nano-scale particles, the only one that showed any significant occurrence for the RR was yolk sac edema (Figure [5]). The nano TP showed significant occurrence for multiple developmental areas at a concentration of $2*10^9$ particles/L, which are as follows: axis, trunk, touch response, caudal fin, pectoral fin, and pericardial edema. It should be noted that since there was 100% mortality for the TP at the two highest concentrations, no sub-lethal defects were observed. There were also impacts from TP on spontaneous movement at the 24 hpf, shown in Figure [5].

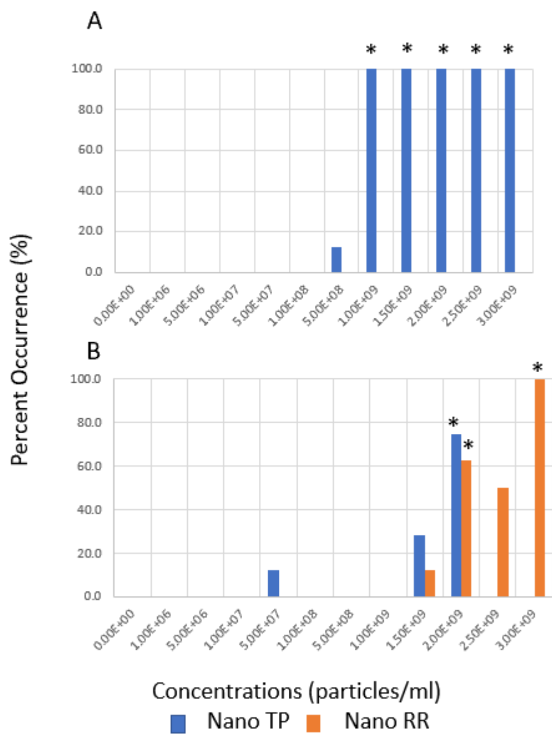

Figure [5]: (A) Nanoscale spontaneous movement particle exposure at 24 hpf for TP and RR, no effects were seen for the RR. (B) Nanoscale particle effects pertaining to yolk sac edema for different concentrations of exposures for TP and RR.

1-20 micrometer particles showed significant occurrence for only one developmental defect, yolk sac edema which can be seen in Figure [6]. The standard TP showed no significant occurrence for snout and jaw defects but the other micro-TP did show significance for the touch response. RR did show occurrence for touch response but only at the highest concentration. All datapoints where no adverse effects were observed are zero and cannot be seen on the graph.

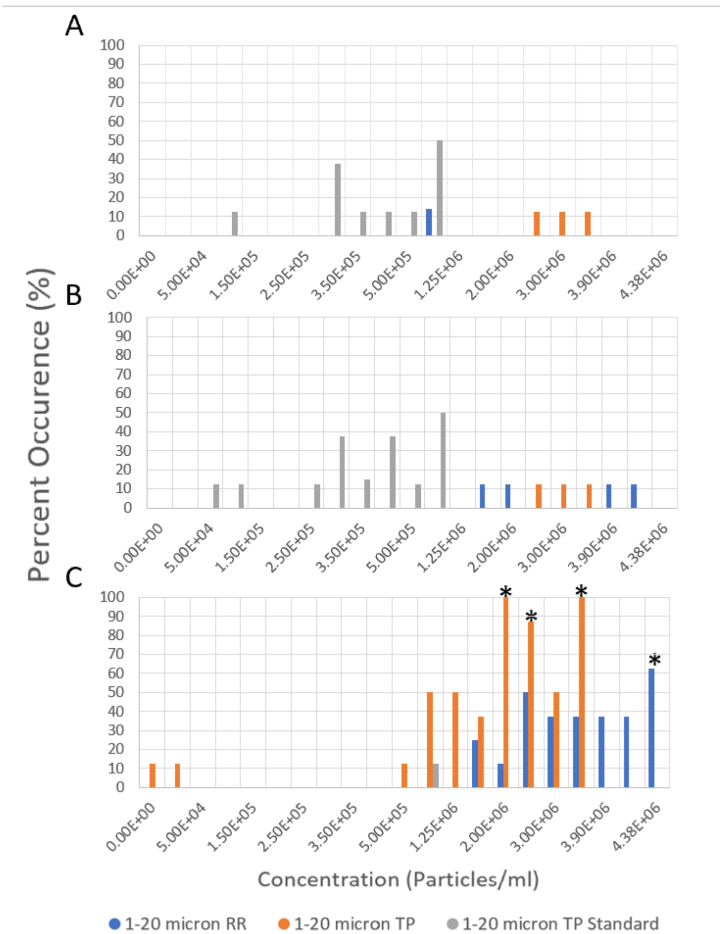

Figure [6]: Development defects of 1-20 micrometer exposures- (A) snout defects, (B) jaw defects, (C) touch response defects are shown above.

Leachate showed the most sub-lethal effects with brain, pigment, otic vesicle, caudal fin, and trunk not showing any significant occurrence. Figure [7] shows leachate effects of 4 of the development defects that showed the highest percent occurrence. The other graphs that did show effects from the leachate all occurred at 90% or higher. RR showed no significant occurrence and very little effects on the zebrafish. The other developmental defects not shown had significant occurrence at values 80% or above except for 24 hpf spontaneous movement had significant occurrence at 50% and above, shown in Figure [8].

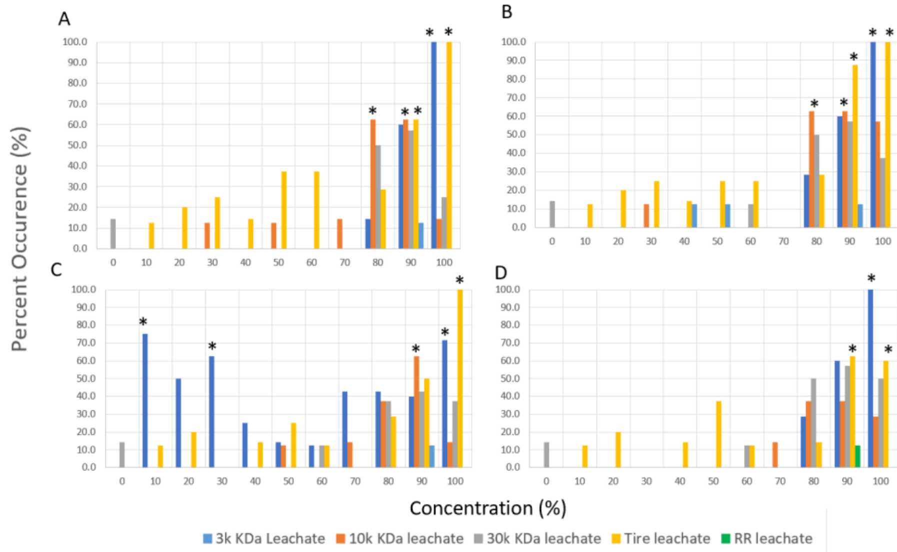

Figure [7]: Leachate exposures of mass fractionated leachate (3k, 10k, and 30k KDa), TP standard, and RR. (A) yolk sac edema, (B) pericardial edema, (C) touch response defects, (D) and jaw developmental defects are shown above.

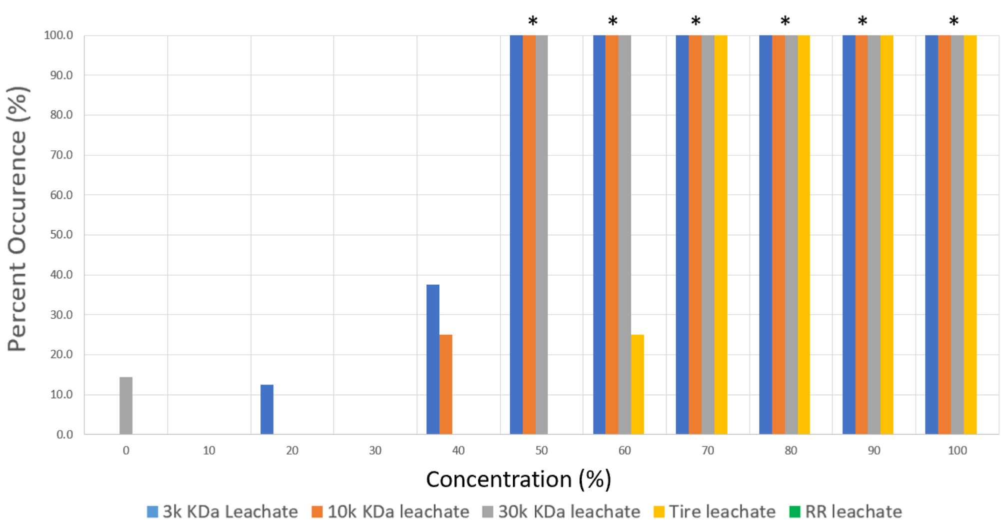

Figure [8]: Leachate exposure at 24 hpf for spontaneous movement. Exposures includes 3 mass fractions, leachate standard, and RR, which did not show any occurrence.

Discussion

Our analyses found that nanoparticles caused the most mortality while the leachate caused the most sub-lethal effects. The lethal effects that showed significance were limited to the highest concentration, which were well above environmentally relevant conditions. So, it can be inferred that the lethality of tire particles in zebrafish is low. Sub-lethal effects varied depending on increased concentration and type of particle. Recycled rubber showed almost no significance except for the nanometer particles that caused yolk sac edema at the highest concentration. Since RR is used in playgrounds and other areas where developing children spend time it is assuring that there is very little toxicity. All other potential developmental defects did not show any significant data for RR.

Leachate data found a general trend of 80% occurrence or more causing significance in the data for developmental defects. 10,000 kDa leachate showed the most significant effects for pericardial edema (PD) and yolk sack enema (YSE). 3,000 kDa fractionate had very little effect on the zebrafish so it is likely that these small molecules can pass through the zebrafish chorionic membrane without much harm to the fish. The 30,000 kDa fractionate also showed very little effect on the zebrafish. This indicates that the most harmful chemicals in leachate can be found in the 10k to 30k kDa range. Compositional analysis would be needed to find out if there are certain chemicals that are isolated in this size range or potentially a mix of leachate components in the tire particle that cause these effects. Additionally, other studies have found that TPs increase the toxicity when compared to leachate alone (Halle et al., 2020) (Chibwe et al., 2021). Nanoplastics have been found to cause absorption of PAHs into embryonic Zebrafish (Zhang et al., 2020). TP leachate is also known to contain PAHs which can cause yolk sac edema (Philibert et al., 2016), smaller jaws (Incardona et al., 2012), and eyes (Incardona et al., 2004) in zebrafish.

This could be a reason why the nanoparticles were the only ones to show any significant mortality. The micrometer particles showed the least amount of developmental defects to occur. Touch response was the only one to show a significant occurrence. The standard used to compare the results from this experiment did not show any significance occurrence. This is most likely because of the difference in concentration ranges but more research would be needed to increase the variability of the data. 96 well plates were not agitated once zebrafish were introduced so there is potential that particles settled out of solution while in the plates. Zebrafish that were near the top of each well could have been less effected by the particles. Each solution was shaken before being introduced to the wells to make sure each concentration was accurate. There was potential concern that polystyrene could have leach from the wells but since only a few particles could leach over five days the risk is low. One study that tested zebrafish with low concentrations of nanoscale polystyrene, less than 10 ppm, found that it had very little effect on their physiology except for a lower heart rate (Pitt et al., 2018). A comprehensive study that looked at microplastic accumulation in zebrafish found that larvae and adult fish had the same sites of accumulation so even once zebrafish start eating toxicity should not change increase (Bhagat et al., 2020). Another study that did toxicity test of tire particles on zebrafish found no consistency between tested concentrations and tire types (Wik et al., 2009). There is little comparison between their results and the results found in this paper. However, two other studies found that if leachate is extracted in different conditions, change in pH and temperature, the composition of leachate changes and therefore the toxicity (Gualtieri et al., 2005)(Marwood et al., 2011). Since our study used an incubator that was at 38^{o}C for the zebrafish, there is the potential that the leachate could have degraded causing more toxicity than normal environmental conditions. Another study found that a pristine tire particle suspension was more toxic than the worn tire suspension, except for the worn leachate (Halle et al., 2021). Since only a new all-season tire was used for this study, the toxicity for TP found was potentially higher than studies that used worn tires. A study done on other species, *Fundulus herteroclius* and *Pimephales promelas,* found zero and 40% mortality at the highest concentration used of micron tire crumb rubber, respectively (LaPlaca et al., 2020). Another study tested tire and road wear particles on algae, daphnia, and fish but found the particles to be low risk under acute exposure (Marwood et al., 2011). All this research shows that tire particles are of low risk for acute, severe toxicity and vary between species. Since these studies were done in lab there is still the question of how toxic these particles are in nature. However, it is difficult to obtain data on how tire particles in nature effect the development of aquatic species as water runoff that travels to aquatic environments contains many more toxins. One study that looks at storm water runoff and its effects on lateral line growth in zebrafish and salmon embryos found that its toxicity varied between years and seasons, summer being the most toxic (Young et al., 2018).

Conclusion

As tires and other products they are in continue to be used throughout the world, more of these particles are created and found in almost every part of the environment. This results in an increasing need to diminish the growth of these molecules or be faced with the irreversible effects they cause. In this study nanoscale tire particles showed the most mortality while the leachate elicited the most sublethal effects. Most of the effects were seen at higher concentrations so our data suggests that the acute lethality of tire particles are low, but the sub-lethal effects are of higher risk. Since this study was done up to 120 hours of development, these sub-lethal effects could lead to reduced fitness and early death. Recycled rubber was also tested but did not have any conclusive toxic effects. Environmental focus should be on improving treatment of contaminated water and soil to decrease theses contaminates. Further research should be to repeat the experiment to increase the plausibility of our hypothesis. Research also looking into the long-term effects that tire particles have on growth should be done, potentially looking at fertility of the zebrafish.

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