ABSTRACT

Polymer nanocomposites have diverse industrial and commercial uses. While many toxicity studies have assessed the individual materials (e.g., polymer, nanomaterial) comprising nanocomposites, few have examined the potential toxicity of the nanocomposite as a whole. Furthermore, products undergo machining during their manufacture and/or degradation as they age thereby resulting in potentially altered mixture exposures and differential effects compared to the parent nanocomposite. We assessed potential toxic effects of repeated oral exposure to silver nanoparticle (AgNP) embedded nanocomposites and compared them to individual component materials using a transparent strain of the fish model Japanese medaka (*Oryzias latipes*). This strain allowed for the comparison of morphologic alterations at three levels of biological organization: whole animal; organ/tissue by examination of histological sections; and, subcellular using transmission electron microscopy (TEM). Adult fish were exposed to AgNPs, silver nitrate, abraded PETG microplastics, or abraded nanocomposites via 7 oral gavages over the course of 2 weeks. *In vivo* observations showed alterations in the liver and gallbladder of fish exposed to pristine AgNPs and nanocomposites. When histologic sections of these same individuals were examined by light microscopy, hepatic and biliary alterations were observed. Similarly, alterations of head kidney were also observed in fish exposed to silver and its composites, with both tubules and glomeruli affected. In both of these organs, much of the changes occurred adjacent to large blood vessels, suggesting material translocated from the bloodstream to adjacent tissues. TEM of the liver supported histological findings, with increased glycogen as well as hepatocellular swelling and abundant lipid vesicles in exposure groups. Very few morphological changes at any level of biological organization were observed in fish exposed to the thermoplastic matrix alone. This transparent fish model proved advantageous in evaluating risks and potential human health concerns associated with the ingestion of silver nanocomposites.

Keywords: nanocomposites, AgNPs, ingestion, additive manufacturing, microplastics

1. INTRODUCTION

Advanced materials (ADMAs) are innovative materials with novel or enhanced properties that improve their performance compared to conventional products.[1, 2] The union of nanotechnology and polymer sciences has created a class unique materials with new properties dependent on the incorporated nanomaterials.[3] Nanomaterial incorporation leads to an improvement of the already useful properties of polymers including physical (*e.g.*, density, crystallinity), mechanical (*e.g.*, tensile strength), and biological (*e.g.*, anti-microbial, biodegradability) properties.[3, 4] As a result, polymer nanocomposites have been used in numerous products including automobile parts, packaging, and biomedical supplies.[3, 5]

The addition of nanomaterials to thermoplastics potentially changes the risks associated with it, leading to concerns about adverse health effects from particles released during fabrication and/or handling.[6, 7],[4] The discharge of ultrafine particles from manufacturing processes such as 3D printing and machining, or over the lifetime of a product, has the potential for occupational and residential exposure.[8, 9] Safety standards have been set by agencies such as NIOSH (*e.g.*, Pelley[10]) for using 3D printers including some guidelines that include metals.[11] However, the toxicities of pristine nanomaterials or microplastics must be used as points of reference[4, 12] as there are not many studies of the potential toxicities of specific nanocomposites.[4]

The enhanced antimicrobial properties of silver nanoparticles (AgNPs) make them attractive for use in a variety of medical and consumer product applications.[13] AgNPs also have physico-chemical properties such as high electrical and thermal conductivity, chemical stability, and catalytic activity that make them useful for various applications such as imaging and electronics.[14] Consequently, AgNPs are incorporated into a variety of polymers used in agriculture, medicine, textiles, and food packaging.[15, 16]

In humans, ingestion is the primary exposure route for silver and silver compounds,[17] and studies have estimated daily uptake of 20 µg/day to 80 µg/day.[18, 19] AgNPs leach from food and beverage packaging and from a variety of other consumer products (*e.g.*, plush toys, cleaning products,

humidifiers) made of AgNP-enabled polymer nanocomposites.[20-24] During the manufacture of silver nanocomposite materials, occupational oral exposure may occur via swallowing after inhalation and expectoration when the affected individual attempts to clear materials from oral and nasal passageways (*i.e.*, phlegm).[19, 25, 26] Due to the absorptive nature of the gut and its proximity to various organs and tissues, toxic effects may occur at very low concentrations after oral or dietary exposure.[27] AgNPs are known to cross biological membranes such as the gut wall,[28] mainly by endocytosis in epithelial cells.[29] Studies using fish models have shown bioaccumulation in tissues such as gills, brain, and liver.[30, 31] However, it is free silver ions (Ag+) that are generally reported as toxic.[28] A Trojan horse effect could occur following exposure to AgNPs where Ag+ is released inside tissues or cells.[32] Ag+ is also released upon association of the AgNPs with the surface of the cell's lipid plasma membrane as these ions are compatible for uptake by plasma membrane ion transporters.[29]

While there is an abundance of literature on the toxicity of AgNPs in multiple species, there are almost none on silver nanocomposites. This is concerning considering its increased use in a variety of fields (e.g., medical, technology) and consumer products.[33] Nanomaterials released over a product's lifetime comprise only part of the equation for potential exposure and toxicity of nanocomposites. Particles released during machining (*e.g.*, abrading, cutting), environmental degradation and/or aging of products are largely composed of the polymer matrix[4, 34-36] and, in the case of plastic nanocomposites, often in the form of microplastics (MPs).[37] Glycol-modified polyethylene terephthalate (PETG, $(C10H8O4)_n$) is one such polymer widely used for applications such as 3D printing due to its transparency, higher thermal resistance, durability, and high elastic modulus.[38, 39] Ingestion is also considered to be the major exposure route for MPs in humans.[40] Subsequently, particles can act locally and/or translocate to other tissues within the body.[40] Local effects have been shown in oral exposures using fish models, with various types of MPs altering gut morphology and negatively impacting intestinal health.[41-43] In turn, alterations in gut mucosa may increase permeability of epithelial barriers thereby facilitating translocation of particles, particularly to the liver and spleen.[40] In both instances, effects such as inflammation, cytotoxicity, and increased risk of neoplasia may result.[40] Because AgNPs are

78 incorporated into plastics largely for their antimicrobial properties, biological effects of AgNP
79 nanocomposites have predominately been studies of bacterial inhibition and cytotoxicity. There are very
80 few investigations on the potential toxicity of these materials on non-target organisms, particularly higher
81 taxa. Ours is one of the first studies that expand on the potential toxicity of nanocomposites, and
82 specifically 3D printed nanocomposites, in a whole organism.

83 Fish models, such as the Japanese medaka (*Oryzias latipes*) used in this study, have been a
84 valuable toxicological tool for the understanding of exposure in a whole organism.[44, 45] In particular, they
85 have been valuable predictive and experimental models in the study of disease formation and
86 pathogenesis over relatively short time spans.[46] As such, there is extensive descriptive morphology and
87 disease pathology available for multiple levels of biological organization.[47] Of interest for oral exposure
88 in humans, are the similarities between teleost and mammalian digestive tracts including general
89 architecture and processes of injury and inflammation.[48-50]

90 In this study, we used abraded PETG, pristine AgNPs, abraded AgNP nanocomposites (two
91 different nanoparticle loadings) that were created and fully characterized by Sipe et al.[51] The objective
92 was to describe and assess morphologic alterations at multiple levels of biological organization associated
93 with oral exposure in order to evaluate relative risks of released component materials compared to their
94 composites. For this, we used a transparent strain of medaka to directly image acute morphologic changes
95 to specific organs observed in the live animal and to compare them to tissue level alterations in histologic
96 sections and cellular ultrastructure on an individual basis. Importantly, the small size of this species
97 enabled whole body histologic sectioning so that multiple organs and tissues could be evaluated. Medaka
98 also have a single lobed liver that facilitated clean removal and processing of the entire organ for
99 transmission electron microscopy (TEM), avoiding issues of lobar distribution seen in some model
100 organisms.[52] This was of interest because studies have shown AgNPs and microplastics to impact the
101 liver.[53, 54] In addition, our recent work demonstrated the liver as the organ most impacted by oral exposure
102 to multi-walled carbon nanotube (MWCNT)-embedded nanocomposites.[55] Together, the investigation by

Sipe et al. [51] in combination with our study are the first to evaluate the mechanical breakdown of silver nanocomposites and their potential toxicity in a vertebrate.

2. MATERIALS AND METHODS

2.1 Materials

In depth descriptions and characterizations of the materials used in this study are detailed in Sipe *et al.*[51] Briefly, AgNPs were uncoated, spherical silver nanoparticles purchased from NanoDynamics, Inc. (ND30, Buffalo, NY) with a reported size of 30 nm. A Mastersizer 3000 laser diffractometer (Malvern, UK) in batch modes (1 g/L) was used to measure particle size distribution. AgNPs had a diameter ranging between 20 nm and 50 nm. , Zeta potential was measured using the Nanosizer NanoZS (Malvern Panalytical Ltd.). AgNPs were negatively charged, with a zeta potential of -19 mV ± 3 in distilled water, -20 mV ± 6 in freshwater (pH 7.4), and 4 mV ± 10 in artificial saltwater (pH 8.4). A typical TEM image of these pristine AgNPs is shown in Figure S1.

Neat PETG filament and AgNP-polymer nanocomposite filaments were prepared from virgin filament grade PETG polymer pellets (ChasePlastics, Clarkston, MI) and AgNPs. Mass fractions of AgNPs in nanocomposite filaments were either 2 % or 0.5 %. First, filaments were made by mixing PETG matrix pellets with the powdered AgNPs. Then these mixtures were extruded (Eurolab XL 16 twin-screw extruder) three times to ensure even mixture of the nanoparticles with the polymer matrix. Neat and nanocomposite PETG filaments were 3D printed into cylinder pucks (diameter of 50.8 mm and thickness of 5 mm; printer settings in Supplementary Materials). PETG matrix and nanocomposite materials were abraded as described below to simulate wear and degradation during of a product during its lifecycle. Hereafter, the abraded neat PETG matrix will be referred to as PETG, pristine AgNPs as AgNPs, and abraded nanocomposites as either 2 % composite or 0.5 % composite.

An abrasion machine designed and custom built at Duke University as described by Bossa *et al.,*[37] Sipe *et al,.*[56] and Scott *et al.* [57] was used to create abraded polymer and nanocomposite materials for this study (Figure 1). The machine included a spinning abrasion platform with P100 sandpaper that was able

to maintain a constant abrasion with the same power and abrasion rate. An airtight chamber enclosed the sample holder and allowed for collection of particles generated during the abrasion process. The full characterization of these particles is also detailed in Sipe *et al*. [51].The presence of AgNPs within a composite did not affect the abrasion process, but although each puck underwent the same abrasion process, the presence and concentration of AgNPs affected the resulting wear particle size (Mastersizer 3000). The D50 particle size (*i.e.*, mean diameter of 50 % of particles) by volume was 695.5 µm ± 230.8, 39.8 µm ± 0.2, and 143.8 µm ± 4.4 for PETG, 0.5 % composite, and 2 % composite, respectively. SEM with EDS imaging confirmed the presence of AgNPs on and in abraded nanocomposite particles. Zeta potentials of PETG, 0.5 % composite, and 2 % composite were -33 mV, -21 mV, and -17 mV, respectively, in distilled water. Zeta potentials were within a standard deviation of each other, signifying that the incorporated AgNPs (free or at the surface) did not affect its behavior in water. It should be noted that at neutral pH, elemental silver has very little solubility and at pH values greater than of 8, approximately 80 % of any dissolved silver precipitates as silver oxide or silver sulfide.[58] The exact pH of the gut of agastric fishes is difficult to determine and data are often highly variable.[59] There is some recent work in zebrafish, a very similar fish model to medaka, that indicates that gut pH is likely above 7.5.[60]

Dissolution of silver for each of the materials was determined at two time points, 1 h and 24 h. These time points were selected to represent the times after preparation for which the fish was dosed (1 h) and the amount of time that the material likely stayed within the gut before egestion (24 h). All solutions were prepared in MilliQ water (ddH_2O; EMD Millipore, Burlington, MA) in triplicate using the same methods and concentrations that would be used for dosing fish (section 2.3). Next, each solution was allowed to sit at room temperature in the dark for either 1 h or 24 h. After the allotted time, all samples including procedural controls were filtered using a 0.2 µm cellulose acetate membrane syringe filter (VWR International, Radnor, PA). Then 0.5% trace metal grade nitric acid (HNO_3; Millipore Sigma) and 0.5% trace metal grade hydrochloric acid (HCl; Millipore Sigma) were added to each. Samples were than quantified for silver concentration (µg/L) by inductively coupled plasma-mass spectrometry (ICP-MS;

Agilent 7700X ICP-MS equipped with an Octopole Reaction System) in the Pratt School of Engineering at Duke University (Durham, NC) using using hydrogen reaction gas at a H2(g) flow rate of 6 mL/min with a silver NIST standard. Results of this test can be found in Table S1.

2.2 Medaka culture

Transparent, Quintet mutant strain medaka (Strain ID: MT829; Sasado et al. [61]) were maintained at Duke University in a recirculating system (Pentair Aquatic Eco-Systems, Apopka, FL) at 24 °C, with a pH 7.4 and a 14:10 light:dark cycle. Colony fish were fed three times per day with Otohime β1 commercial dry diet (200 µm to 360 µm, Pentair Aquatic Eco-Systems). The first two feedings were supplemented with *Artemia* nauplii (90 % Great Lakes Strain, Pentair Aquatic Eco-Systems). All procedures were approved by Duke University Institutional Animal Care and Use Committee (A062-15-02 and A031-15-01).

2.3 Exposure regime

AgNP solutions were made fresh just prior to each dosing. For this, a stock solution was prepared by placing 1 mg AgNP powder into 100 mL autoclaved ddH_2O. Next, this stock solution was sonicated (80 % pulse; BioLogics Inc. 3000 ultrasonic homogenizer, Manassas, MA) for 30 minutes on ice to disperse particles. Immediately following sonication, the solution was diluted with autoclaved ddH_2O to the test concentration and immediately administered to fish as described below. All abraded materials were suspended in autoclaved ddH_2O and vortexed to distribute particles just prior to administration to each fish. A preliminary range-finding assay using 3 doses of AgNPs was completed. From these data, 1000 µg/L AgNPs was selected as the test concentration. This concentration is consistent with consumer product leaching data, specifically the high AgNP release in media similar to that which we used (*i.e.*, water and saliva).[23] We also took into consideration concentrations used in three studies that tested chronic or repeated dietary AgNP exposure in adult fish. Lacave *et al.*[62] exposed zebrafish (*Danio rerio*) to 100 µg/L of 5 nm polyvinyl pyrrolidone/polyethylenimine (PVP/PEI)-coated AgNPs via *Artemia* spp. nauplii for 21 days. Merrifield *et al.*[63] fed zebrafish diet spiked with 500 mg/kg of uncoated 59 nm AgNPs

for 14 days. Wang and Wang [64] exposed marine medaka (*Oryzias melastigma*) to 200 µg/L or 1000 µg/L of 20 nm AgNPs stabilized with Tween20 (0.025 g/mol or 20 µM) via *Artemia* spp. nauplii for 28 days.

For comparison, doses of abraded materials (PETG, 2 % composite, 0.5 % composite) were calculated to achieve the same AgNP mass dose of 1000 µg/L. $AgNO_3$ was tested at 65 % of the concentration of AgNPs to account for silver ion (Ag+).[65-67] Concentrations (µg/L) of all materials were: 1000 AgNPs; 650 $AgNO_3$; 49,000 PETG; 50,000 2 % composite; or 200,000 0.5 % composite.

Adult fish (8 mo. old) were selected from the colony and briefly examined to ensure there were no gross internal abnormalities. Five fish (3 females, 2 males) per group were randomly divided into 7.5 L glass aquaria in a dedicated room at 24 °C and a 14:10 light:dark cycle. Aquaria contained batch water consisting of 1 g/L of aquarium salt (Instant Ocean, Blacksburg, VA) in reverse osmosis (RO) treated water that was mixed and oxygenated for at least 16 hrs before use. Each aquarium was equipped with independent heaters for temperature control, pumps for mechanical filtration, and ceramic beads taken from the colony system for biological filtration (Figure S2). Aquaria were siphoned twice daily to remove fish waste, eggs, and uneaten food. Filters were rinsed with DI water once per day and changed for new every 3 days. Water quality (e.g., temperature, pH, ammonia, nitrite, nitrate) was tested daily with an API Freshwater Master Test Kit and 30 % water changes conducted once per week or as needed to maintain stable water quality. Each experimental group had dedicated instruments (nets, siphons, etc.) to prevent cross-contamination. Fish remained on the breeding colony feeding regime (section 2.2) during their 3-day acclimation period.

Experimental fish were administered material every other day for 14 days, resulting in a total of 7 doses. Procedural controls received the suspension media (*i.e.*, autoclaved ddH_2O) only. Fish were fasted for 24 hrs prior to each dose to clear the gut of digestate, and then doses were administered using oral gavage as a method to deliver precise quantities of material to the digestive tract of each individual. Methods for oral gavage followed exactly those detailed by Chernick *et al.*[55] In short, individual fish were anesthetized by submersion in 0.024 % MS-222 (Pentair Aquatic Eco-systems), pH 7.4, and then carefully placed into a trough cut into a soft sponge (L800-D, Jaece Industries, North Tonawanda, NY)

moistened with batch water such that the head faced up and opercular movement was not obstructed. Next, a 13 mm length of micro-renathane catheter tubing (MRE-025, Braintree Scientific, Inc., Braintree, MA) was placed over a 31G, 8 mm needle of a sterile syringe (BD Biosciences, San Jose, CA) such that approximately 5 mm extended past the end of the needle and 10 µL of dosing solution was drawn up into the tubing. Tubing was gently inserted into the oropharynx, just caudal to the branchial chamber. Material was injected slowly and then the tubing removed. Fish were constantly monitored for normal opercular movements and gill color, heart rate, and responsiveness. Following oral gavage, each fish was rapidly examined for obvious internal alterations (e.g., hemorrhaging, gallbladder color change, enlargement/darkening of liver) and then placed into a large bowl containing batch water where it was monitored for recovery and possible regurgitation. Following recovery from anesthesia, the fish was returned to its original tank and the procedure repeated with the next individual.

Twenty-four hours after the last dose, fish were euthanized with an overdose of MS-222 (300 mg/L; Pentair Aquatic Ecosystems) and then photographed (Olympus Corp., Center Valley, PA) in both lateral and dorsal recumbency. The latter was facilitated by placing the fish in the same type of sponge used for gavage. During this time, *in vivo* alterations were recorded as both descriptions and presence/absence data. These observations were later verified in images. Fish were then either fixed for histology (section 2.4) or livers were dissected for cellular ultrastructure (section 2.5). Specimens, liver and whole animals, were stored in individually labeled containers so that results from each could be compared to *in vivo* images on an individual basis.

2.4 Histology

Four fish (2 females, 2 males) in each group were fixed for histology immediately after imaging and for *in vivo* evaluation according to Chernick *et al.*[55] In brief, to facilitate fixation of deep tissues, a ventral incision was made from the anus to near the pectoral girdle. Then 10 % neutral buffered formalin (10 % NBF; VWR International) was very gently flushed into the incision, making sure not to displace internal organs. Next, 10 % NBF was flushed into the buccal cavity such that it could enter the branchial cavity, pharynx and foregut. Finally, each fish was immersed in 10 times their volume of 10 % NBF,

placed on an orbital shaker (≈5.2 rad/s or 50 rpm) overnight, and then stored at 4 °C until processing. Prior to processing, specimens were brought to room temperature, the tail removed just caudal to the abdomen using a single edged razor blade, and the specimen submerged in fresh 10 % NBF.

These fish were processed, embedded, sectioned, and stained by the Histology Laboratory, Department of Population, Health and Pathobiology, College of Veterinary Medicine, North Carolina State University, Raleigh, NC. First, fish were decalcified by placement in 10 % formic acid for 48 hrs. Next, they were placed in an automated tissue processor (Thermo Shandon Path Centre, Grand Island, NY) and dehydrated by passage through a graded ethanol (EtOH) series and then cleared with Clear-Rite 3 (Richard Allen Scientific, Kalamazoo, MI). Processed fish, in right lateral recumbency, were individually embedded in paraffin. Parasagittal sections were cut at 5 µm thickness using a Leica 2135 rotary microtome (Leica Biosystems Inc., Buffalo Grove, IL) and then mounted on glass histology slides. Slides were routinely stained with hematoxylin and eosin (H&E) and coverslipped.

All labeling of slides were covered to ensure sections were examined blindly. Examination took place using a Nikon Alphaphot II compound light microscope. The presence or absence of various alterations (Table 1) was recorded for each individual and calculated as proportion of individuals within a group showing a change. Definitions for each of the categories of change followed those published in supplementary materials in Chernick *et al.*[55] In this way, relative occurrences of each alteration were established in each treatment group. Representative images were taken using a Nikon E600 compound light microscope fitted with a Nikon DXM1200 digital camera and Nikon NIS-Elements 3.10 software (Nikon Instruments Inc., Melville, NY).

2.5 Transmission electron microscopy

Reagents and consumables were purchased from Electron Microscopy Sciences (Hatfield, PA). All stains were filtered (syringe filter, nylon, 0.2 µm, VWR International) prior to use so that stain particulates would not be confused with AgNPs. Specimen preparation and processing followed Chernick *et al.*[55] For each group, a single female fish was imaged as described above and observations under *in vivo* evaluation recorded. Dissected livers were then immediately placed into 2.5 % glutaraldehyde buffered

with a cacodylate-sucrose solution (0.1 mol/L sodium cacodylate and 0.1 mol/L sucrose, pH 7.6) and fixed at 4 °C. Just prior to processing, each liver was cut into 4 or 5 relatively equal sized pieces using a single-edged razor blade and each piece was placed in a labeled 20 mL borosilicate glass vial (Wheaton, VWR International). After washing twice in 1X PBS (10 min/wash), specimens were stained with 1 % osmium tetroxide (OsO_4) in the dark for 1 hour. Then samples were again washed with 1x PBS and rinsed with acetate buffer (10.17 g/mol (124.43 mM) sodium acetate, 1.5 g/mol (25 mM) acetic acid) before staining with 0.5 % uranyl acetate for 1 hour. Next, specimens were rinsed with acetate buffer and dehydrated by passage through a graded EtOH series. Samples were then placed in propylene oxide (PO) three times for 10 min each. Spurr's resin was prepared (16.4 g cycloaliphatic epoxide resin (ERL-4221), 23.6 g nonenyl succinic anhydride (NSA), 5.72 g Dow epoxy resin (DER 736), 0.4 g 2-Dimethylaminoethanol (DMAE)), and samples were immersed in a Spurr's:PO (1:2) mixture in a chemical fume hood for 4 hours with the vial caps off and then overnight with the caps on. The next day, the mixture was replaced with a Spurr's:PO (1:1) mixture and cured for 8 hours in a chemical fume hood with vial caps off. This mixture was then replaced twice with new, pure resin and incubated in a 60 °C oven for at least 10 minutes each time. For embedding, fresh resin was placed in a flat multi-well embedding mold with liver portions positioned near the tips and were allowed to cure in a 60 °C oven for 24 hours. These blocks were thin sectioned at 70 nm to 90 nm thickness, placed on 150 square mesh copper gilder grids (#G150-CU, EMS), and then stained with lead citrate. Thin sections were examined and imaged at 80 kV using a FEI Tecnai G^2 Twin transmission electron microscope with a single tilt stage, and a spot size of 2.

2.6 Data Analysis

Morphologic alterations were recorded as presence/absence data for *in vivo* (*i.e.*, whole animal) and in histology (Table 1; n=4). For these data, Pearson Chi-squared pairwise tests were conducted using the categorical response analysis tool in JMP Pro 14 (SAS Institute, Cary, NC) comparing incidence of individual changes between experimental groups. For clarity, statistical differences and associated p-values described within the text of section 3 (Results) are comparisons to control fish. Percentages

reported herein represent incidences of fish within a group with the alteration. A full comparison, including proportions of affected fish and any statistical differences between other treatment groups, for histological alteration can be found in Table 1. P-values of ≤ 0.05 were considered to be statistically significant.

3. RESULTS

3.1 In vivo *observations*

An incidental single mortality of a male fish occurred in the 1000 µg/L AgNP group on day 4 (after 2 doses). No other mortalities or behavior that would suggest stress (*e.g.*, cowering, gasping) were observed in other fish during the experiment including prior to mortality in the single male fish. Fish were not observed to regurgitate gavaged material upon recovery from anesthesia.

In vivo assessment showed that control fish had no alterations and exposed fish had a few types of alterations (Figures 2-4). Discoloration, specifically lightened patches, of the head kidney was observed following exposure to AgNPs but not in a significant number of individuals (Figure $2C_1$). Enlargement and/or discoloration (*i.e.*, darkened or mottled appearance) of the liver was observed in 75 % of fish exposed to AgNPs (Figure $3C_1$) and 60 % of fish exposed to 2 % composite (Figure $3D_1$) ($p=0.0177$ and 0.0384, respectively). It should be noted that had the other male survived in the AgNP group and had a normal liver, this group would still have had a statistically significant difference. Gallbladder alterations (*i.e.*, darkened or altered color and/or enlargement) were not observed until after the sixth dose in any group and persisted until after the final dose (Figure 3). The 2 % composite group had significantly more fish with this change than the control group ($p=0.0098$, Figure $3D_1$) and the 0.5 % composite group ($p=0.0384$). Half of the fish in the AgNP group had this change (Figure $3C_1$), which was not statistically significant. Changes in the midgut (*i.e.*, large clear areas around a bolus, indicating distention or discoloration of walls), or fluid accumulation in the peritoneal cavity, were not observed in any fish.

307 *3.2 Histologic evaluation*

308 Proportions of individuals affected with various types of change are listed in Table 1. Alterations
309 within the branchial chamber were observed within all groups including control, which may have been the
310 result of anesthesia or the gavage procedure. While the exposure route was dietary, epithelial lifting on
311 primary and secondary lamellae in some groups suggested a small amount of silver may have interacted
312 with gill tissue. $AgNO_3$ and AgNPs caused damage to apical cells of the mucosal gut folds ($p=0.0285$ and
313 0.0082, respectively). The oval body of the swim bladder was significantly affected in fish exposed to
314 $AgNO_3$ ($p=0.0285$), with vacuolated, swollen, and injured cells observed. While not statistically
315 significant, it is worth noting that the only other treatment group that had any incidence of this change
316 was AgNPs, with injured cells in the swim bladder.

317 The head kidneys showed a variety of changes in exposed fish of each treatment group (Figure 2,
318 Table 1). Tubules were altered in all groups except control ($p<0.05$; Figure 2). Tubule alterations included
319 altered tubular epithelial cells, dilated lamina of tubules, and increased tubulogenesis. Alterations in
320 tubular epithelial cells were characterized by increased intercellular spaces, intermittent degeneration or
321 damage, and loss of contact with the basement membrane (Figure 2). The proportion of individuals with
322 this change was higher after exposure to $AgNO_3$ ($p=0.0285$), AgNPs ($p=0.0082$), 2 % composite
323 ($p=0.0285$), and 0.5 % composite ($p=0.0047$). It is noteworthy that the proportion of fish with altered
324 tubules was the only statistically significant change for fish exposed to PETG. Dilated tubules were
325 typically adjacent to large veins within the head kidney and occurred in all groups except control and
326 $AgNO_3$ but incidence was not statistically significant ($p>0.05$). Calcifications in the tubule lumens were
327 observed in fish exposed to PETG ($p=0.0136$) but were small and infrequent. When encountered,
328 alterations in the trunk kidney were infrequent and minor. However, these alterations occurred in more
329 fish exposed to $AgNO_3$ ($p=0.0285$).

330 Altered glomeruli were observed in fish exposed to $AgNO_3$ ($p=0.0285$), AgNPs ($p=0.0082$), 2 %
331 composite ($p=0.0047$), and 0.5 % composite ($p=0.0047$). Alterations within the glomerular tuft included
332 congestion of capillaries, calcifications, an abundance of eosinophilic material, altered Bowman's

capsule, and/or necrosis of glomerular cells (Figure $2C_2$, Table 1). Except for congestion, there were no statistical differences between treatment groups when these individual types of changes were analyzed independently. All fish exposed to 0.5 % composite had congestion in the capillaries of the glomeruli ($p=0.0047$). The only other group to have this change was AgNPs (33.3 %) although it was not statistically significant. Alterations in Bowman's capsule were characterized by basophilic epithelium within the visceral layer (*i.e.*, podocytes) and/or changes in Bowman's space that included expansion or reduction and instances of fibrous material that extended through the space (Figure $2C_2$, $2D_2$). The latter change is an unusual finding that only occurred in fish in composite groups (1 fish in each). The number of fish that had alterations in Bowman's capsule was significantly higher in the 2 % composite and 0.5 % composite groups ($p=0.0285$ in both groups).

When grouping all types of liver alterations together, both hepatocellular and biliary, fish exposed to silver and composites were most affected. Fish in the PETG group were more similar to controls. While a couple of the PETG exposed fish had either perivascular or peribiliary fluid accumulation, it was minor, and they exhibited very few other liver changes. 75 % of fish exposed to $AgNO_3$ had liver alterations ($p=0.0285$) while all fish exposed to AgNPs and composites had liver alterations ($p=0.0047$). Hepatocellular alterations included swelling and shrinking, vacuolated hepatocytes, pyknotic nuclei, necrosis, spongiosis hepatis, and/or perivascular fluid accumulation (Figure 3, Table 1). Several of these changes often occurred within the same individual. Hepatocellular swelling was, by far, the most extensive change both in terms of extent/severity within individuals and proportion of fish affected within treatment groups. Swollen hepatocytes had rounded or irregular margins, and a majority of the cytoplasm showed little staining or staining was restricted to a perinuclear region (Figure $3B_2$, $3C_2$). 75 % of fish in the $AgNO_3$ group had this change ($p=0.0285$) as well as all fish exposed to AgNPs, 2 % composite, or 0.5 % composite ($p=0.0082$, 0.0047, and 0.0047, respectively). Areas of altered livers exhibited shrunken hepatocytes, often with pyknotic nuclei, typically in the region closest to the foregut and porta hepatis (Figure $3B_2$). Interestingly, shrunken hepatocytes only occurred in fish exposed to composites, and to all fish of these treatment groups ($p=0.0047$). For half of the individuals in the 2 % composite group, the

overall size of the liver appeared slightly to moderately larger, likely a result of these changes in cell volume (Figure S3B). Scattered necrotic hepatocytes were observed in all treatment groups except for control and PETG; all fish exposed to AgNPs had necrotic hepatocytes. Spongiosis hepatis was found in fish exposed to 0.5 % composite (75 %, p=0.0285). This alteration was characterized by hepatocyte swelling such that cells became very large with nuclei displaced to the periphery by fluid and/or protein-like material, forming lesions with sharp margins. This alteration may have given the liver a mottled or lighter appearance in these individuals *in vivo*.

Biliary alterations included reactions around biliary structures, alterations of biliary epithelial cells, and/or peribiliary fluid accumulation (Figure $3D_2$, Table 1). Fish in the $AgNO_3$ and 0.5 % composite groups had more instances of these alterations (p=0.0285 and 0.0047, respectively; Figure 3). However, individual types of biliary responses did not show significant differences (Table 1). That said, it is notable that only fish in the 0.5 % composite had alterations of biliary epithelial cells. All groups containing silver had at least one individual that showed reactions adjacent to or within biliary structures. While no statistical differences were found in incidence of peribiliary fluid accumulation, it is notable that the AgNP group was the only one apart from the control that did not have this change.

Inflammation was another major finding of this experiment and appeared to primarily be the result of AgNP exposure, both in its pristine form as well as that associated with composites. For the purposes of our analysis, we defined "inflammation" as a cellular response (*i.e.*, white blood cells) because humoral components of the immune system are not visible in histologic sections. An inflammatory response was observed in fish exposed to PETG or $AgNO_3$, but it was minor, and the number of individuals with it was not significantly different from control. Inflammation was found in more fish exposed to AgNPs (p=0.0082, Figure $4B_2$), 2 % composite (p=0.0047, Figure $4C_2$), and 0.5 % composite (p=0.0285). When looking at specific locales of inflammation, some patterns emerged. More fish in the 2 % composite (p=0.0047, Figure $4C_2$) and 0.5 % composite (p=0.0285) groups had inflammation within the liver, either perivascularly or peribiliary (p=0.0047 and 0.0285 for 2 % composite and 0.5 % composite, respectively). Macrophage aggregates were present in a small number of

385 fish exposed to AgNPs and were generally small in size (≤200 μm) and extent. Inflammation also
386 occurred within the mesentery, specifically on the serosal surface of the gut and margins of the liver
387 (Figure $4A_2$, $4B_2$). This only occurred in groups exposed to silver, with all fish in the AgNP group
388 ($p=0.0082$) and 75 % of fish in the 2 % composite group ($p=0.0285$) having this response.
389 *3.3 Liver ultrastructure*
390 TEM of liver from the control fish (Figure 5A) had normal structure, with uniform hepatocytes,
391 each with a single nucleus containing a defined nucleolus; rough endoplasmic reticulum (RER) with
392 straight parallel cisternae with narrow cisternal spaces; and, hepatocytes arranged around bile canaliculi
393 with small, normal biliary epithelial cells. In addition, the space of Disse was narrow and sinusoids
394 contained abundant red blood cells; mitochondria were numerous, oval shaped with normal, irregularly
395 oriented cristae and narrow lumens; secondary lysosomes were rare and small, and lipid vacuoles were
396 moderately sized and few. Glycogen was present in small deposits toward the periphery of hepatocytes.
397 The liver from the PETG group was very similar to control. The only difference was an increase
398 in glycogen (Figure 5B). The $AgNO_3$ exposure group also had increased amounts of glycogen in their
399 livers and portions of RER had transformed into myelin-like bodies (Figure 5C). We did not observe
400 nanoparticles within the liver from fish exposed to AgNPs. However, when not in large aggregates, their
401 size, shape, and electron density can be similar to granules within glycogen deposits. It is possible that
402 some of these granules were actually AgNPs. Livers from the AgNP treatment group were the most
403 altered. While hepatocytes had increased amounts of glycogen similar to that observed in fish exposed to
404 PETG or $AgNO_3$, the deposits had wide, clear spaces between glycogen granules (Figures 5D). In
405 addition, plasma membranes and intercellular spaces were indistinct (Figures 5D), indicating
406 hepatocellular swelling. Lipid vesicles were also abundant and large throughout these cells (Figure 5D). 2
407 % composite exposure did not result in hepatocellular or biliary change apart from additional glycogen
408 (Figure 5E). However, macrophages were observed adjacent to biliary and sinusoidal spaces (Figure 5E).
409 Liver from the 0.5 % composite treatment (Figure 5F) was most similar to that of the liver from the PETG
410 treatment, showing only increased glycogen (Figures 5F).

4. DISCUSSION

Our study is the first to evaluate toxicity resulting from thermoplastics embedded with AgNPs in a vertebrate. The benefits and risks associated with the use of AgNPs in various fields have been studied for decades.[68] More recently, the number of studies on the impacts of MPs has surged due to increased interest from regulatory agencies, non-profit organizations, and the public among others. As such, the potential adverse impacts of MPs on humans and organisms the environment is becoming better characterized.[69, 70] These effects may occur through the plastics, chemicals internally incorporated in them, and/or materials sorbed to their surface during their lifecycle.[71-73] However, exposure risks associated with the generation of silver-containing nanocomposites has not been well studied despite the incorporation of AgNPs into many industrial and consumer products, including plastics. In addition, the oral exposure route is poorly understood.[74] The transparent medaka strain proved a useful tool for the comparison of responses in individuals at multiple levels of biological organization and provided insights into potential risks of oral exposure to AgNP nanocomposites in humans.[45, 75]

Tissues composing the gut are both the first line of defense and potentially the most directly impacted by oral exposure. Histology showed $AgNO_3$ and pristine AgNPs to cause changes in these tissues. AgNPs are known to cross biological membranes such as the gut wall and distribute to multiple organs.[28, 76, 77] Macromolecules and nanoparticles are taken up by the gut epithelium via endocytosis and transported to macrophages in the lamina propria and into circulation[78] For example, Scown *et al.*[79] demonstrated that increased concentrations of silver in the liver were likely the result of uptake by gut epithelium during exposure to 10 µg/L to 100 µg/L AgNPs (49 and 65 nm, unspecified coating). Likewise, high concentrations of silver in the intestine as well as liver and bile of common carp (*Cyprinus carpio*) were found following aqueous exposure to 0.01 µg/L and 0.01 µg/L AgNPs (35 nm, unspecified coating).[80] As fish gut anatomy and function are comparable to that of humans,[81] this suggests similar uptake may occur as a result of analogous exposures to silver in humans. A study of observed an increase in goblet cell density in adult zebrafish exposed to chitosan AgNP nanocomposites via diet for 30 and 60

436 days.[82] This is the only other study of gut morphologic changes in fish following AgNP nanocomposite
437 exposure.

438 Studies suggest that the kidney is one of the major sites for nanoparticle accumulation and
439 adverse effects following oral exposure.[77, 83, 84] We observed a variety of changes in head kidneys, mostly
440 adjacent to large blood vessels that suggested injurious components systemically carried by blood.
441 Tubular epithelial cells were affected in all test groups except controls and were notably one of the few
442 changes observed in fish exposed to PETG. Cell damage and tubular necrosis is a common type of
443 xenobiotic-induced kidney damage in teleosts as there is a high capacity for membrane transport in these
444 cells as well as concentration of compounds in the tubule lumen.[85] In a dietary AgNP exposure in adult
445 zebrafish, Yazdanparast *et al.*[86] reported similar findings including damage to kidney cells and increased
446 Bowman's space. Flattening or fusion of the pedicels within Bowman's space was an unusual finding
447 unique to fish exposed to nanocomposites in our study. Podocytes with swollen and elongated pedicels
448 have been reported in male rats following intraperitoneal injection of AgNPs (2000 mg/kg; 20 nm to 65
449 nm, unspecified coating).[87] This change could have reduced filtration area and hydraulic permeability[85]
450 and may have been linked to the congestion of glomerular capillaries observed in fish in our study. Such
451 reductions in glomerular filtration rate (GFR) and plasma flow are also signs of malnutrition. These renal
452 changes can also result from perturbations in transmembrane signaling transduction,[88] which may have
453 occurred from silver interacting with cell membranes.[89] Boudreau *et al.*[77] reported significant
454 concentrations of silver in kidneys of rats exposed via oral gavage to AgNPs, higher even than the Ag+
455 control (as AgOAc). Within the kidney, they found AgNPs to remain intact and localize to renal tubular
456 epithelium while control AgOAc silver localized to basement membranes of glomeruli. This is in line
457 with the results of our study. Nanocomposites have been used as therapies to address kidney injury and
458 dysfunction. However, to date, there are no reports for kidney injury resulting from AgNP nanocomposite
459 exposure.

460 We observed substantial changes to the liver, consistent with reports in both mammals and fish
461 for AgNP accumulation and resultant damage. The liver has been shown to be a site of bioconcentration

462 of AgNPs in both adult medaka[90] and zebrafish,[91] and it is the most likely route of nanomaterial
463 excretion.[92] When adult zebrafish were exposed to aqueous AgNPs (25 nm, unspecified coating, 23.7
464 µg/L to 331.8 µg/L) for 14 days, hepatic parenchyma was less homogenous, with cells in loose contact or
465 irregular in shape.[93] Livers of Siberian sturgeon (*Acipenser baerii*) exposed to aqueous AgNPs (8 nm,
466 unspecified coating, 100 µg/L to 1500 µg/L) for 21 days developed dilations and blood congestion in
467 vessels as well as hepatocyte shrinkage and enlargement.[94] Vacuolated hepatocytes and necrotic foci were
468 also reported following dietary exposure of AgNPs (5 nm, PVP/PEI coating, 100 ng/L and 100 µg/L) via
469 *Artemia* in zebrafish.[62] When Wu and Zhou[95] exposed adult medaka to aqueous AgNPs (30 nm, PVP
470 coated, 0.05 mg/L to 0.5 mg/L) for 14 days, they found congested blood vessels, enlarged hepatocytes,
471 dilation of sinusoidal space, focal necrosis, focal inflammation, and areas of vacuolation in the liver.
472 These findings correspond with what we observed in fish exposed to silver, either pristine or composite.
473 However, it is worthy of mention that some of these studies lack a Ag+ treatment group, so it is unclear if
474 their findings were the result of the nanoparticle or Ag+. There are also no AgNP nanocomposite studies
475 that focus on liver morphology that we can compare to our findings.
476 We observed hepatocellular alterations in most or all fish in treatment groups containing silver. It
477 was interesting that hepatocellular shrinkage was only observed in fish exposed to nanocomposite
478 materials, specifically near the porta hepatis. It is also noteworthy that we also observed shrunken
479 hepatocytes in our recent study of oral exposure to MWCNT-embedded nanocomposites.[55] This
480 morphology can be indicative of nutrient deprivation,[96] and it can be a symptom of cytoplasmic
481 alterations and aggregation of organelles and is characteristic sign of apoptotic cell death.[97] Importantly,
482 the fish in the control and PETG groups in our study were fasted for the same amounts of time as those in
483 other groups, and this change was not observed suggesting that the composites were the agent of this
484 change. Conversely, necrotic hepatocytes occurred with exposure to AgNPs but not nanocomposites,
485 suggesting there may be a threshold concentration of AgNPs released from composites to cause this type
486 of significant change. This is a possibility as AgNPs can have strong interactions with their polymer
487 matrices. [14] There is evidence *in vitro* from rainbow trout (*Oncorhynchus mykiss*) hepatocytes that AgNPs

488 inhibit cellular metabolic activity, reduce membrane integrity, and increase reactive oxygen species
489 (ROS) in a dose-dependent manner.[98] Swollen hepatocytes with clear or lightly staining cytoplasm in
490 histologic sections is indicative of glycogen accumulation but cannot be confirmed by H&E staining
491 alone.[88] This is where analyses of multiple levels of biological organization proved valuable. TEM
492 provided confirmation of glycogen accumulation, showing large areas of glycogen in hepatocytes of
493 exposed fish. In teleosts, hepatic intracellular glucose is channeled into glycogen and becomes a major
494 store of carbohydrates.[99] Increased glycogen such as that in fish in our study is a metabolic situation
495 typically associated with food deprivation[99], and supported our findings in kidney and liver tissues.
496 Also prevalent in fish of our study was widespread immunological responses. There are a number
497 of studies of toxicant-induced immunotoxicity, but the mechanisms underlying changes are not well
498 understood.[100] In our study, inflammation appeared to be the result of AgNP exposure, both in its pristine
499 form as well as that released from nanocomposites. Many metals are known to be immunotoxic, typically
500 in the disruption of ion influx or reactive oxygen formation.[101] AgNPs can penetrate the serosal layer of
501 the gut and activate serosal macrophages located in the peritoneal cavity[101] as suggested by the
502 macrophages we observed in these areas in histologic sections. Once inside macrophages, lysosomal fluid
503 can increase the amount of silver content on the nanoparticle surface and increase the rate of Ag+
504 production from AgNPs, a contributor to metallic cytotoxicity.[76] Additionally, lysosomal membrane
505 potential (LMP) is accompanied by the release of cathespin B, initiating NLRP3 inflammasome assembly
506 and caspase-1 activation.[102] This process leads to the maturation of IL-1β and IL-18 and a subsequent
507 inflammatory response.[102] The ability of AgNPs to initiate this inflammatory pathway via LMP disruption
508 has been demonstrated in human liver[103] and lung cells[104] as well as oyster (*Crassostrea virginica*)
509 hepatopancreas tissues.[105]
510 Accumulation of white blood cells are indicative of sites of inflammation, reactive oxygen
511 formation, and lipid oxidation.[101] The adaptive immune system in fish shows a slower response than that
512 of mammals, sometimes taking weeks instead of days. *In vitro* studies have shown AgNPs to induce an
513 inflammatory response of which an early stage is the cellular secretion of pro-inflammatory cytokines.[106]

514 It is possible that the inflammation that we observed was in the early stages and a more chronic exposure
515 may be necessary to determine full effects of AgNPs on the immune system. Future investigations should
516 include testing of humoral elements, as well as gene alterations for cell regulation, classes of
517 inflammatory and stress responses.[106]

518 Considering the commonalities in toxicity of AgNPs across diverse biological systems, cross-
519 species extrapolations may be possible.[80] AgNPs have been shown to distribute, accumulate, and affect
520 more organs compared to other types of nanoparticles, such as gold nanoparticles (*e.g.*, Yang *et al.*[107]).
521 AgNPs are added to various matrices (*e.g.*, polymers) so that their release provides bactericidal and
522 bacteriostatic effects.[108] This incorporation has been viewed as a way to reduce silver toxicity to humans
523 while still imparting antimicrobial benefits.[109] But to date, abrasion studies such as we have conducted
524 have not been done to support this statement. The observations made in our study showed that exposure
525 to pristine AgNPs resulted in the most morphological change as did $AgNO_3$, although to a lesser extent.
526 This suggests that silver is more toxic as a nanoparticle rather than AgNPs being a delivery mechanism
527 for Ag^+. This was supported by our dissolution data, which showed that while dissolution increased
528 slightly over the course of 24 hours for AgNPs, the concentration of dissolved silver did not reach that of
529 equivalent $AgNO_3$. Additionally, the material matrix of the nanocomposites protected from total AgNP or
530 Ag^+ release. Interestingly, the different responses we observed in nanocomposite exposure groups
531 indicate that these advanced materials should be viewed as unique mixtures that may alter the toxic
532 effects of the silvers particles incorporated on the material matrix.

533 **5. CONCLUSION**

534 Our study is the first to evaluate morphologic changes in a vertebrate resulting from oral exposure
535 to abraded AgNP nanocomposites, a unique mixture of nanoparticles and a plastic polymer. AgNPs and
536 AgNP composites affected a wide range of organs and prompted an immune response. These systemic
537 responses indicate that ingested silver was able to penetrate the gut wall and distribute throughout the
538 body. Effects in both the kidney and liver suggested much of this distribution occurred through the

bloodstream. While very few effects were observed with the PETG matrix alone, this component of nanocomposites should not be disregarded as it may have impacted other mechanisms (*e.g.,* microbiome[110], reproduction[111]). While effects of nanocomposites containing higher loads of AgNPs were most similar to exposures with pristine AgNPs, we also observed nanocomposites caused different morphological changes within some tissues suggesting unique mixture effects.

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Conflicts of Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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