NANOSILVER AND CNT-NANOCOMPOSITE TOXICOLOGY IN AN IN VIVO MODEL, D. MELANOGASTER

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NANOSILVER AND CNT-NANOCOMPOSITE TOXICOLOGY
IN AN IN VIVO MODEL, D. MELANOGASTER

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ABSTRACT

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The use of nanomaterials has increased exponentially over the past decade. There has long been a need to quickly, cost-effectively, and efficiently test their toxicity. Development of an in vivo fly model enables rapid toxicity assessment of life history parameters unavailable in in vitro models capable of finer discrimination of nanoparticle toxicity (Posgai et al. 2011).

Carbon nanotubes have been used in generating nanocomposites with incredible structural strength, but under industrial applications may leach, creating the potential for human exposure. Silver nanoparticles have been used as surface antimicrobial agents in a number of applications, including water purification and undergarments. Their ingestion raises the concern of that they could alter the composition and/or function of
gut microbiota as well as engender side-effects on eukaryotic tissues. Here these aspects of nanoparticle toxicity are assessed in a *Drosophila* nanotoxicology model.
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CHAPTER 1

LITERATURE REVIEW

1.1 Statement of Interest

Nanomaterial based technologies are used in most every known industrial sector worldwide, and continue to grow year to year. Their current use includes textiles, healthcare, food/agriculture, electronics, and environmental applications [1]. Thus far scientific toxicity testing (and legislative regulation) has not been able to keep pace with industrial output. This has created a potentially toxicological hazard in many of the products we use and foods we eat every day. This thesis focuses on four separate projects with the underlining theme of toxicity testing of nanomaterials whose use entails human exposures.

A nanomaterial is simply a physical object with at least one dimension in the nano scale (1 meter = 1,000,000,000 nanometers). Nanoparticles (NPs) are singular small object that behaves as a whole unit with respect to its transport and properties. Nanocomposites are made from two or more materials, at least one of which is a nanoparticle.
The interest in nanomaterials stems from their nanoscale properties that differ from their properties in bulk. For example, bulk gold has an unmistakable yellow hue. However, gold nanoparticles change color based on size; small NPs are red, medium sized ones are purple, and large ones are blue. The differences are not purely visual. In stark contrast to their bulk counterparts, gold nanoparticles are good catalysts, have a decreased melting temperature, are reactive, magnetic, and change from a metal to a semiconductor [2]. Toxicity is also impacted by size. A harmless material in bulk can become toxic at the nanoscale [3]. Another aspect of nanomaterial toxicity is their sensitivity to synthesis method, seemingly minor changes, batch-to-batch, can have drastic impacts of the resulting nanoparticle. These changes include size and shape changes, coatings, and charges [4, 5].

1.2 Carbon Nanotubes

Carbon nanotubes are cylinders, with one or more layers of graphene (SWCNT or MWCNT)\(^1\), that are can have open or closed ends [6]. SWCNTs typically have a diameter of 0.8-2 nm compared to 5-20 nm for MWCNTs. Both can be as long as several centimeters, however, the short diameter qualifies as being a nanomaterial [7].

Documentation of ‘hollow carbon nanofibers’ was first made in the

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\(^1\) SWCNT = Single-wall Carbon Nanotube; MWCNT = Multi-wall Carbon Nanotube
1950s, but real interest came with the introduction of MWCNTs in the 1980s and widespread research in the 1990s. Since 2006 CNT production has increased 10-fold along with CNT related publications and patents (Fig 1) [8, 9]. In 2010 the global CNT industry turned over $669.3 million; $631.5 million of that was from SWCNT and only $36.8 million from MWCNT. The combined global financial contribution of CNTs is expected to hit $1 billion in 2015 [10].

1.2.1 Uses and Properties

The industrial uses of CNTs typically take advantage of their mechanical properties, conductivity, and/or absorbent and capillary capability [9].

*Strength and hardness:* CNTs are the strongest and hardest material ever discovered with regards to tensile strength\(^2\) and elastic modus\(^3\). Typical CNTs have been tested to have a tensile strength of approximately 100 GPa or 15,000,000 psi. Taking into account density, their specific strength is 46,000+ kN*m/kg making it 180x stronger than stainless steel, titanium, and aluminum or magnesium alloy, and 18x stronger than Kevlar. [11, 12] A new special SWCNT synthesized under extreme pressure (SP-SWCNT) was found to have a bulk modulus\(^4\) of around 500 GPa which is higher than the 420 GPa for diamonds [13]. This makes CNTs a suitable replacement for plastics, fiberglass, and steel for things like bikes, boats, airplanes, rockets, and spaceships.

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\(^2\) Tensile Strength: maximum stress that a material can withstand while being stretched or pulled before failing or breaking

\(^3\) Elastic modus: resistance to being deformed elastically (i.e., non-permanently) when a force is applied to it.

\(^4\) Bulk modulus: resistance to uniform compression.
**Conductivity:** The geometry of CNTs play a major role in their electrical conductivity\(^5\). Small variations can shift conductivity behaviors from metallic to semi-conductive or essentially non-conductive [14]. Conductivity and ampacity\(^6\) are two measures looked at for electrical potential. Graphene has been shown to be a better electrical conductor than silicon, and will likely replace silicon in computer applications in the next few years [15]. SWCNTs and MWCNTs are not great conductors on their own, however, attaching other metals such as copper can greatly increase their ampacity [16]. Single-walled CNTs and graphene also have extremely high thermal conductivity\(^7\) making them useful for thermal management applications [17, 18, 19]. This is not the case with bulk CNTs.

**Absorbent and capillary capability:** CNTs have a unique ability to interact with the environment without any intervention. MWCNTs are able to absorb microwaves, and they neither reflect nor scatter visible light, which could make CNT-composite aircrafts nearly invisible to radar and to the naked eye at night [20]. The addition of sulfur\(^8\) to the CNT synthesis process produces a three-dimensional, ultra-low density (6 mg/ml) amorphous form of CNT with highly contorted, unusually long CNTs that are extremely selective in absorbing toxic organic solvents [21]. CNTs as biosensors hold potential given the dimensional and chemical compatibility of CNTs with biomolecules such as DNA [22] and proteins, capable of low detection limits based on changes in their

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\(^5\) Electrical conductivity: measure of a material’s ability to conduct an electric current. Inverse of resistance
\(^6\) Ampacity: maximum amount of electrical current a conductor or device can carry before sustaining immediate or progressive deterioration
\(^7\) Thermal conductivity: property of a material to conduct heat. (materials of low thermal conductivity are used as thermal insulation)
\(^8\) A common practice known as defect-engineered CNTs
electrical impedance [22] and optical properties [23] upon binding. CNTs can also be internalized by the cell [24, 25] for in vivo imaging [26, 27, 28] and drug delivery [29, 27].

1.3 Nanosilver: Uses and Exposure

Silver has long been used for its antibacterial properties. The famous historian Herodotus first wrote about the use of silver to keep water fresh in the 5th century BC and Hippocrates, the father of medicine, was using silver to treat wounds and ulcers around the same time [30]. The prevalence of nanosilver has increased significantly, but it is still the antibacterial properties that make it attractive. Nanosilver has been added to paints, clothing, children’s toys, cosmetics, food storage and handling containers, and much more in order to prevent bacterial contamination [31, 32].

The use of silver for wound care has been approved since the early 1950s and nanosilver has been on the USEPA priority pollutant list since 1977 [33]. The recent escalation of nanosilver has raised questions about bioaccumulation, chronic exposure toxicity, and cumulative effects that were previously low priority, and many public advocates seek government intervention to regulate to use of nanosilver [33]. A study in 2007 estimated that 128 tons of nanosilver (457T before treatment), are released into the waterways of the USA each year [34], which like comes back to use through drinking water. The other common NP exposure route is through diet [35]. Silver is considered a supplement by the FDA and thus is not under stringent regulation in comparison to food additives. Current efforts like the NanoRelease Food Additive (NRFA) project is working to determine how much nanosilver is actually being consumed [36].
1.4 Toxicity

There is evidence that the toxicity attributed to nanosilver should actually be attributed to silver ions (Ag⁺). One study found that in AgNP suspension with silver ion fraction greater than 5.5%, the AgNPs did not contribute to toxicity compared to the supernatant (Ag⁺) alone [37]. In a rat ingestion model, the amount of silver in organs correlated very highly with Ag⁺ and not AgNP [38]. There remain contradictory reports as to the role of the ion vs. the nanoparticle in toxicity; the entire AgNP vs Ag⁺ argument may be a moot point because the source of the silver ions are AgNPs [39]. The release of Ag⁺ has been shown to be dependent on the size, shape, and coating of the AgNP [40].

There are several inter-related mechanisms for the bactericidal properties of nanosilver. AgNPs have been shown to damage the cell wall and membrane [41, 42, 43]. From there AgNPs concentrate in the center of cell and physically interact with the DNA and prevent inhibit replication [43]. They can also inhibit the 30s ribosomal subunit protein S2 which can then affect all TCA proteins leading to death by lack of ATP production [44, 45]. Interactions with respiratory enzymes can lead to accelerated generation of reactive oxygen species [46].

The mechanisms of toxicity for eukaryotes is very similar to those outlined for prokaryotes. AgNP alone (no Ag⁺) was shown to actually accelerate cell proliferation and induce

![Mechanisms of AgNP toxicity](image)
abnormal cellular morphology in a human model [47] and decrease the mitotic index in a plant model [48]. Other models have shown oxidative stress to be the driving force in toxicity [4, 49]. Figure 2 shows the various proposed mechanism for AgNp toxicity [50].

The mechanisms of CNT toxicity has not been thoroughly investigated and is not well understood; in vitro studies have been done often disagree with each other. For example, as stated previously, CNTs are able to cross the cell membrane [24, 25]; some in vitro studies report no toxicity [51, 52], others report toxicity only when modified [53], and many others report toxicity to lung related tissues [54, 55].

There have been very few in vivo studies and many of those use artificial exposure methods such as injection. Current knowledge of in vivo toxicity find that longer CNTs are more toxic than shorter ones [56]. The needle-like structure of CNTs resemble asbestos and inhalation studies in mice induced inflammation, nodular lesions (granulomas) and scarring in the mesothelial lining, symptoms consistent with asbestos
inhalation phenotypes [57, 58]. Oxidative stress may also result from CNT exposure, Figure 3 [59].

The lack of agreement with respect to the mechanism and impact of physical nanomaterial variables (size, shape, coating, etc.) calls for studies such as presented here.

1.5 Model Organism

*D. melanogaster* provides a rapid, inexpensive *in vivo* model to research nanoparticle toxicity. *D. melanogaster* contains 700 counterpart genes known to cause disease in humans, including, but not limited to, genes for neurological, immunological, cardiovascular, auditory, visual, developmental and metabolic disorders [60]. Many of the genetic and biochemical pathways that underlie disease when compromised are shared by both humans and fruit fly. For example, immune pathways such as Imd and STAT, the production of antimicrobial peptides (AMPs) or reactive oxygen species (ROS) upon infection are basic immune defense mechanisms used by both humans and *D. melanogaster*. Thus, *D. melanogaster* offers relevant model to triage nanoparticle toxicity. Its short generation time permits more sensitive toxicity assays of exposure effects on the whole organism, including developmental rate, survivorship, and reproductive effort that are unavailable *in vitro* or impractical in mammalian models [4, 49].
CHAPTER 2

DEVELOPMENT OF A CONCEPTUAL FRAMEWORK FOR EVALUATION OF NANOMATERIALS RELEASE FROM NANOCOMPOSITES: ENVIRONMENTAL AND TOXICOLOGICAL IMPLICATIONS

2.1 Introduction

The following chapter was part of a larger collaborative effort with Dr. Alex Orlov at SUNY Stony Brook. The purpose of this project was two-fold: to look at environmental factors contributing to the degradation and subsequent release of CNTs and to establish a framework to effectively test the toxicity of the released CNTs \textit{in vivo}. \textit{In vitro} studies cannot provide a complete understanding of toxicity given that \textit{in vitro} toxicity does not necessarily predict \textit{in vivo} toxicity [4]. \textit{In vivo} toxicity studies have not yet been performed on nanocomposites or nanocomposite byproducts during manufacturing or environmental weathering.

The work presented here is the preparation and \textit{in vivo} toxicity testing of CNT-epoxy nanocomposites, a portion of this larger study [61].
2.1.1 CNT Exposure

Exposure to CNTs can occur during three stages of the CNT life: manufacturing, use, and/or disposal stage. Contact during the manufacturing stage comes from handling raw CNTs, the manufacturing process itself, and exposure through operating and maintaining equipment. The ‘use’ stage presents the most risk for the average consumer. Nanocomposites can breakdown over time due to physical abrasions, UV exposure, and several other factors depending on the specifics of the nanocomposite. The ‘disposal’ stage is difficult to account for, given the multitude of elements at work. The popular disposal methods of landfill disposal, water disposal, and incineration, open the door to pollution to the land, air, and water and toxicity to all organisms who could come in contact with these disposal products [61]. Most literature has focused on the manufacturing stage.

Among one of the promising nanocomposites involving CNTs are those containing epoxy. Epoxy polymers are commonly used for aerospace, infrastructure, and consumer products because of their mechanical properties and chemical and hydrolytic resistance [62]. When CNTs are added to the epoxy base those properties are enhanced [63]. The added strength and resistance the epoxy provides could reduce degradation during the ‘use’ stage where it provides the greatest risk of human exposure.

Epoxy-CNT composites have been shown to be less prone to UV compared to similar SiO₂ nanocomposites [64]. Additionally, chemical, physical, and structural properties of the polymer/nanofiller interface⁹ play a crucial role in the degradation of the

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⁹ In this case where the epoxy and CNTs come together.
composite. The key for reducing risk during the manufacturing stage could lie in several modifications\textsuperscript{10} that could minimizing degradation and thus toxicity \cite{65}.

**2.2 Materials and Methods**

**2.2.1 Nanomaterials, Preparation and Visualization**

“Free” carbon nanotubes (MWCNTs, with and without amine group amendments) and “embedded” CNT-epoxy nanocomposite were supplied by Dr. Alex Orlov (SUNY Stony Brook) and tested for toxicity in a fly ingestion test. Free MWCNTs were grinded with mortar and pestle without any aid. Epoxy embedded CNTs were frozen with liquid nitrogen and ground into micron sized particles with diameters between 5 and 50 μm with mortar and pestle. Ground nanocomposite particles were suspended in ethanol to facilitate uniform dispersion.

Images of free and embedded MWCNTs were taken with the Hitachi H-7600 Transmission Electron Microscope (TEM) and Hitachi S-4800 High Resolution Scanning Electron Microscope (HRSEM).

**2.2.2 Ingestion Exposure**

OreRS flies obtained from Bloomington Stock Center, Bloomington, IN were used in toxicity tests. Toxicity tests were performed by incorporating nanomaterials into standard Cornmeal-malt-yeast medium using 60x15 mm petri plates\textsuperscript{11}. Nanocomposites

\textsuperscript{10} Chemical modification of the CNTs (\textit{i.e.}, functionalization or defect-engineered), or by using surfactants to moderate the physical interactions between the carbon nanofillers and matrix

\textsuperscript{11} Recipe taken from Bloomington Stock Center website
were added to the media while cooling and decanted into petri plates. Control plates contained the same media with an equivalent volume of ethanol.

Fifty *D. melanogaster* embryos were laid on each plate and allowed to feed *ad libitum* through pupation. Percent survivorship was calculated as the number of embryos that pupated divided by the total number of embryos. Developmental rate was assessed as the time from first larval instar hatch to pupation [4]. The experiment was carried out at 25°C under 12 hour day-night cycles. The mean and standard deviation of percent survivorship and time to pupation were calculated and statistically analyzed using a two-tailed t-test with a cutoff for statistical significance of p < 0.05.

### 2.3 Results

#### 2.3.1 Microscope Images

Figure 4 A, B, and C shows epoxy embedded CNTs after freezing and grinding. Image A shows surface CNT sizes from 1-5 μm, they are heterogeneous in both size and shape. Image B shows free CNT ends sticking out from the epoxy matrix. In contrast, image C shows most CNTs remain embedded in the epoxy mesh after grinding. Figure 2D shows free MWCNTs after grinding.
2.3.2 Toxicity

Figures 5 and 6 shows survivorship and developmental rate in *D. melanogaster* following CNT ingestion. A t-test was used to compare treatment to control for each concentration. Figures 5 B and 6 B show that the amine-CNTs affected developmental rate and survivorship at all concentrations measured, with embedded amine-CNTs showing less toxicity (Figures 5D and 6D). CNTs without the attached amine group were not toxic, whether embedded or not (Figures 5 A,C and 6 A,C).
Figure 5. Proportion of larvae surviving to pupation (mean ± SdV) is presented for control and treatment lines as described in experimental section in detail. A) Plain CNTs, B) Amine- CNTs, C) plain CNT-epoxy composite, and D) Amine-CNT epoxy composite. *Significant difference under 2-tailed t-test (p < 0.05).

Figure 6. Mean and standard deviation for time until pupation for surviving larvae presented for control and treatment lines as described in experimental section in detail. A) Plain CNTs, B) Amine- CNTs, C) plain CNT-epoxy composite, and D) Amine-CNT epoxy composite. *Significant difference under 2-tailed t-test (p < 0.05).
2.4 Discussion

The TEM and SEM images show structural differences in CNTs resulting from encapsulation. Figure 4 shows that the epoxy limits the number of free CNT ends, the basis of CNT toxicity (Fig. 4 A,B,C vs D). The epoxy is considerably stronger and more durable, evidenced by the fact that even after rigorous freezing and grinding cycles, the nanocomposite remains in relatively large agglomerates with few free ends (Fig. 4 A) and the CNTs remain anchored (Fig. 4 B,C) compared to the free CNTs which broke apart very easily (Fig. 4D).

Free CNTs with an amine group attached (Figs 5 B and 6 B) showed toxicity at all concentrations measured by both survivorship and developmental rate. Encapsulation with epoxy reversed much of the toxicity (Figs 5D and 6D). This is likely due to the fact that embedding nullifies the high aspect ratio of the CNTs which has been shown to be heavily associated with toxicity [66].

The statistically significant increase in survivorship seen in Fig. 5D at 10 μg/ml is likely an aberration, but it is worth noting that in the plant *Cicer arietinum*, treatment with 6.0 μg/ml CNT increased plant growth due to increased water retention [67]. Next to determine is if the CNTs cross the gut and accumulate in fly tissues. This would determine if the encapsulation reduced toxicity through a size effect that limited their ability to cross the gut wall. It would also determine if the greater toxicity of the amine-CNTs compared to non-amended CNTs (Figs 5A,B and 6A,B) can be attributed to the increase in cellular uptake due to the amine group [68].

The impact of solvent in CNT preparation is also in need of further study. Ethanol was chosen over other common solvents such as acetone, ethanol, toluene, or N, N-
dimethylformamide because it is less toxic and more readily available. Solvent selection typically does not change the shape or size of the CNT but it can influence the hardness and the flexural strength [69]. Ethanol is completely soluble in water while being non-polar enough to keep all CNTs well dispersed in solution, and so is ideal for dispersing CNTs in fly media. However, the differences in ethanol concentration across the different CNT concentrations means that developmental rate and survivorship cannot be compared across treatments. That said, the highest ethanol concentration for any plate is <10.8%, and is likely much lower given that the ethanol-CNT mix (or just ethanol for controls) was added when the agar containing media was liquid, approximately 85°C. Given ethanol’s boiling point of 78°C, it is likely that some, if not all, the ethanol evaporated off prior to any fly embryo being laid. Previous literature has looked at the role of ethanol on *D. melanogaster* and found that they actually develop faster and have higher survivorship in media containing 0.5-9% ethanol [70].

As stated in the introduction, the purpose of this project was two-fold: to look at environmental factors contributing to the degradation and subsequent release of CNTs and to establish a framework to quickly, cost-effectively, and effectively test the toxicity of the released CNTs *in vivo*. Epoxy-embedded CNTs are non-toxic, removing a barrier to their use in industrial applications.
CHAPTER 3

DIFFERENTIAL TOXICITY OF EPICATECHIN VS BOROHYDRIDE REDUCED SILVER NANOPARTICLES

3.1 Introduction:

Nanosilver is primarily used for their bactericidal properties [32, 71] which often entail eukaryotic exposures. Previous studies have shown silver nanoparticles to be toxic to eukaryotes both in vivo and in vitro [72, 73, 74, 75]. This is unfortunate given the nano-industrial boom and inevitability that humans and other eukaryotes will be exposed to them.

One proposed strategy to curb the toxicity of nanoparticles without losing their industrial usefulness is to alter the way in which they are synthesized. Residues from reactants can bind NPs and affect toxicity [76]. Traditional Ag NPs are synthesized through chemical processes that use strong reducing agents. Recently, there has been interest in the synthesis of nanoparticles using plant extracts as reducing agents that do not involve harmful chemicals and are environmentally friendly. Several studies have examined the synthesis of Ag NPs using various plant extracts including alfalfa (Medicago sativa) and lemongrass (Cymbopogon flexuosus). Green tea (Camellia sinensis)
extracts have specifically been used in Ag NPs synthesis as reducing and stabilizing agents [77, 78].

The goal of this work was to compare traditional borohydride synthesized AgNPs and ‘green’ epicatechin synthesized AgNPs to determine the effect of synthesis method on their toxicity to prokaryotes and eukaryotes in vivo and in vitro. It was found in prokaryotic tests that epicatechin synthesized AgNPs were as effective as borohydride synthesized AgNPs as an antimicrobial agent, but were slower in exhibiting their toxicity [79]. It was also found that the epicatechin AgNPs were non-toxic to human keratinocytes whereas borohydride synthesized AgNPs were toxic in in vitro cell culture tests [79, 80]. The fly provided the in vivo eukaryotic model to determine if synthesis method affected in vivo eukaryotic toxicity.

3.2 Materials and Methods

3.2.1 Epicatechin-reduced Silver Nanoparticle Synthesis

1.3502g of Epicatechin (SID 24894431) was dissolved in 20 ml ethanol. 0.6262g silver nitrat (SID 24852543) was dissolved in 80 ml MQ-H₂O. The ethanol-epicatechin solution was added to the silver nitrate solution drop wise by burette at a rate of approximately 1 drop per sec. The newly precipitated nanoparticles were left to stir at a moderate speed at room temperature for 90 minutes. After 90 minutes the reaction was terminated by cooling to 4°C. The nanoparticles were washed several times by aliquoting 1.5 ml of nanoparticles into micrcentrifuge tubes and centrifuging at 16,000g for 60 min. Once washed, the nanoparticles were re-pooled and probe sonicated (Branson Sonifier 150) at 10W for 15 minutes. Nanosilver concentration was obtained using inductively
coupled plasma mass spectrometry (ICP-MS); size and shape characteristics were obtained using transmission electron microscopy (Fig 1).

Nanoparticles were stored at 4°C until needed, and were briefly sonicated at 5 W to disassociate clumped particles for 1 min before use.

### 3.2.2 Traditionally Synthesized Silver Nanoparticles

To generate traditionally synthesized nanoparticles a chemical reducing agent sodium borohydride is used. Sodium borohydride reduced nanoparticles were obtained from Dr. Dan Goia (Clarkson University Center for Advanced Materials Processing, Potdam, NY - UN Ag (Goia)), and Nanostructured & Amorphous Materials Inc. (stock# 0478YD -UN Ag (NAM)).

### 3.2.3. Toxicity Testing

Toxicity tests were carried out using 60x15 mm petri plates, standard Cornmeal-malt-yeast medium\(^\text{12}\), and OreRS flies obtained from Bloomington Stock Center, Bloomington, IN. Nanoparticles were added to the media while cooling and decanted into petri plates. Fifty *D. melanogaster* embryos were laid on each plate, three plates (150 embryos total) were used for each treatment. Percent survivorship was calculated as the number of embryos that pupated divided by the total number of embryos. Epi Ag and UN Ag (Goia) were evaluated at 10, 20, 30, 40, 50, 100, 100 μg/ml. UN Ag (NAM) were evaluated at 20, 50, 100, and 200 μg/ml.

\(^{12}\) Recipe taken from Bloomington Stock Center website
Developmental rate was assessed as the time from first larval instar hatch to pupation [4]. Experiments were performed at $25^\circ$C with 12 hour day-night cycles. The mean and standard deviation of percent survivorship and time to pupation were calculated and statistically analyzed using a one-tailed t-test with a cutoff for statistical significance of $p < 0.05$.

### 3.3 Results

All the tested nanoparticles showed uniform shape and size distribution with an average diameter of approximately 15 nm. Notice the uni-modal size distribution and lack of agglomeration (Fig 7 + inset)

Figure 7. TEM of UN Ag (NAM) (A) and Epi Ag (B). Insets show size distribution

Figure 8 shows the survivorship of *D. melanogaster* with respect to the controls. The UN Ag (Goia) survivorship was significantly lower than Epi Ag and UN (NAM) in
20-50 μg/ml concentrations. There was no significant difference between the Epi Ag and UN (NAM) at any concentration.

Figure 9 shows the percent change in developmental rate with respect to controls. The UN Ag (Goia) developmental rate was significantly higher than Epi Ag and UN (NAM) at all concentrations. There was no difference between the Epi Ag and UN (NAM) at any concentration.

Pigmentation changes in the fly cuticle following nanosilver ingestion has been shown to indicate toxicity and oxidative stress [4, 81]. The UN Ag (Goia) treatment
showed changes in pigmentation at all concentrations while Epi Ag did not show any changes at any concentration (Fig 10).

Figure 10. Developmental rates of D. melanogaster when reared on plates containing different NPs with respect to control. Significance is indicated by star (n>= 48)

Figure 9. Pigmentation of control OreRS fly (A), OreRS fly reared on 30 μg/ml UN Ag (Goia) (B), and OreRS fly reared on 200 μg/ml Epi Ag
### 3.4 Discussion

The fact that epicatechin nanoparticles are not toxic is encouraging. Epicatechin is also a natural antioxidant, which may be why AgNPs synthesized with epicatechin as the reducing where not toxic to eukaryotes [79, 80]. The differences in toxicity between UN Ag (Goia) and UN (NAM) needs explanation, but highlights the batch-specific effects we and others find in nanoparticle syntheses (Table 1). There are several possible causes for this:

1. **Effect of Ag**: As mentioned in Chapter 1, many studies have found that silver ion alone causes toxicity to eukaryotes. Though this is unlikely to be the cause of the difference between Goia and NAM particles given there was ample opportunity for both nanoparticle sources to reach AgNP-Ag equilibrium (48hr in oxidation conditions like in open air exposed food [82] and 40 days in storage conditions [83]) and, in solution, the Goia AgNPs had an ion concentration of 2 ppm and the NAM nanoparticles were at 2.5 ppm; not a large enough discrepancy to make a biological difference.

2. **Agglomeration could bind 10nm AgNPs into micron sized agglomerates**, making them less toxic. Each source was sonicated to break up any

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**Table 1. Variability of toxic AgNP concentration**

<table>
<thead>
<tr>
<th>Toxic Concentration</th>
<th>AgNP Characteristics</th>
<th>Model System</th>
<th>Citation</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.4 ng/ml (powder)</td>
<td>60-100 nm</td>
<td><em>D. magna</em></td>
<td>[82]</td>
</tr>
<tr>
<td>10 ng/ml (solution)</td>
<td>13 nm w/ citrate coat</td>
<td><em>D. magna</em></td>
<td>[82]</td>
</tr>
<tr>
<td>1.5 μg/ml</td>
<td>30-50 nm w/ 0.2% PVP coat</td>
<td><em>A549 lung cells</em></td>
<td>[37]</td>
</tr>
<tr>
<td>8.75 μg/ml</td>
<td>15 nm</td>
<td><em>C 18-4 Stem Cells</em></td>
<td>[137]</td>
</tr>
<tr>
<td>2.7 μg/ml</td>
<td>20 nm</td>
<td><em>L929 fibroblasts</em></td>
<td>[138]</td>
</tr>
<tr>
<td>5-50 μg/ml</td>
<td>10 nm w/ citrate coating</td>
<td><em>C. elegans</em></td>
<td>[139]</td>
</tr>
<tr>
<td>25 ng/ml</td>
<td>30-40nm w/ 0.2% PVP</td>
<td><em>D. rerio</em></td>
<td>[140]</td>
</tr>
<tr>
<td>0.34 μg/mL</td>
<td>20 nm</td>
<td><em>HEK</em></td>
<td>[141]</td>
</tr>
<tr>
<td>30-40 μg/mL</td>
<td>10 nm and 60 nm polysaccharide and plain</td>
<td><em>D. melanogaster</em></td>
<td>[4, 49]</td>
</tr>
</tbody>
</table>
agglomerations, as revealed by TEM, before being added to the fly food. In future studies, nanoparticles should be examined in the media by TEM to see if they significantly agglomerated in the short period time after they were added and before the agar solidified.

The goal of this project was to demonstrate the effectiveness of epicatechin as a reducing agent and compare the toxicity of these ‘green’ synthesized NPs to traditionally synthesized NPs. The fact that Epi AgNPs were non-toxic to human keratinocytes (data not shown) and in vivo to D. melanogaster while the UN Ag (Goia) were, indicates that we successfully showed ‘green’ synthesized NPs hold potential for keeping bactericidal properties without harming humans or other eukaryotes.
CHAPTER 4

USING SENSITIZED FLY LINES TO DETERMINE THE ROLE OF OXIDATIVE STRESS IN NANOSILVER EXPOSURE

4.1 Introduction

One documented mechanism of nanosilver toxicity is oxidative stress [4, 49, 46]. Oxidative stress is known to play a role in neurodegeneration [84], diabetes [85], and aging [86]. Oxidative stress is caused by the formation of reactive oxygen species (ROS). ROS are chemically reactive molecules that contain oxygen. In Figure 4.1 this is represented by an oxygen radical; other examples of ROS are hydroxide radical and peroxide, among others. ROSs can have an exogenous origin, for example from radiation, drugs, or smoke, or an endogenous origin from natural cellular processes like oxidative phosphorylation [87]. In this model, AgNP acts as an exogenous source by shedding electrons, creating

![Figure 11. Mechanisms of Oxidative Stress. Boxed are the proteins of interest SOD and Catalase [87]](image-url)
oxygen free radicals as well as Ag$^+$ [88].

This research focuses on the anti-oxidant proteins superoxide dismutase (SOD) and catalase. SOD is the first step in combating ROSs by changing the oxidative state of the free radical to form O$_2$ or H$_2$O$_2$. In the case of hydrogen peroxide formation, catalase or glutathione peroxidase will change the oxidative state to give water or water and oxygen (Fig 11) [87]. In cases when radicals are not immediately removed, they can cause DNA damage, protein misfolding, and mitochondrial damage, resulting in apoptosis or the development of malignancies [89].

The rational for this project stems from prior work in our lab by Posgai et al (2011) and Ahamed et al. (2010). Flies exposed to toxic levels of UN Ag (Goia) showed up-regulation of p53 and p38, two DNA repair genes, and caspase-3 and caspase-9, two apoptotic genes [49]. Biochemical tests also showed that the UN Ag (Goia) exposure increased levels of SOD and catalase compared to controls while glutathione peroxidase levels were lower$^{13}$. All of which is very indicative of oxidative stress. Additionally, when ascorbic acid (Vitamin C) was added to food with toxic levels of Un Ag NPs, there was complete rescue of the survivorship, developmental rate, and phenotype [4]. Vitamin C is a natural antioxidant that specializes in containing reactive oxygen species.

The goal here is to test nanosilver toxicity in fly mutants in which SOD or Catalase levels are up or downregulated, to determine if there is an interaction between this oxidative stress genes and nanosilver toxicity.

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$^{13}$ SOD and catalase are proteins so increased levels indicate a response to ROSs. Glutathione peroxidase is not a protein and is used up in response to ROSs.
4.2 Materials and Methods

4.2.1 Model

A GAL-4/UAS system was used to express SOD or catalase in a gain of function study, or SOD or catalase RNAi in a loss of function study, as illustrated in Figure 4.2. The GAL4 line was graciously provided by Dr. Amit Singh. The catalase and SOD lines were obtained from the Bloomington Stock Center, Bloomington, IN (catalase stock#24621; SOD stock # 33605).

Briefly, fly larvae are kept at the restricted temperature 18°C until ready for testing. Upon testing flies are moved to the permissive temperature 36°C for 1 hour to activate the heatshock response 2) Transcription factors bind to HSP promotor and activate flippase 3) flippase flips out the stop cassette 4) expressing GAL4 via the actin promotor 5) GAL4 binds to the upstream activation sequence (UAS) and activates either SOD or catalase gene or RNAi expression 6) GFP is also produced for validation purposes. This sequence is shown in Fig 12.

Figure 12. Schematic of genetic manipulation for activation of SOD and catalase. Modified from [142].
4.2.2 Experimental Design

Virgin GAL4 female flies and UAS males of either SOD+ (33605) or Cat+ (24621) (or RNAi lines respectively) were mated in conical fly tubes. Embryos were collected on embryo collection media\(^{14}\) each morning and afternoon over a span of no more than 4 hours for developmental staging. Embryos were transferred to petri plates containing either standard cornmeal-malt-yeast fly food\(^{15}\) (control) or standard food laced with 100 μg/ml, 15nm silver nanoparticles, from Nanostructured & Amorphous Materials Inc (stock# 0478YD).

For each gene, there were four treatment groups: 0hr Control, 0hr AgNP, no heatshock-control, no heat shock-AgNP. The ‘0hr’ and ‘never’ refer to stage in life when heatshock is activated, 0 hours would be immediately after embryo collection and never would be not ever activated.

Developmental rate and survivorship was recorded as before and 1-tailed students t-test was used for statistical analysis. Time permitted only gain of function SOD and catalase experiments were performed, tests of RNAi lines remain.

4.3 Results

The developmental rate and survivorship graph between gain of function catalase (Fig 13) and SOD (Fig 14) lines are nearly identical. Developmental rate under silver treatment was higher that controls, regardless of SOD or catalase activation.

Surprisingly, SOD activation reduced survivorship under silver treatment.

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\(^{14}\) Recipe on Bloomington Stock Center Website
4.4 Discussion

If oxidative stress was a component of nanosilver toxicity, increased levels of catalase and SOD should alleviate silver-induced oxidative stress. Yet we find the opposite result, SOD sensitized flies to silver ingestion. Confirmation of this result is important given its implications for our understanding of nanosilver toxicity. Biochemical assays to verify up-regulation of SOD, and additional tests at different time points using RNAi lines as gain of function lines would determine if this result is due to nanosilver, or reflects a more general effect of chronic SOD expression on development. SOD plays an important role in cuticle differentiation, for example, and its up-regulation in a nanosilver background may impact this or other aspects of normal development and physiology.
CHAPTER 5

DESTABILIZATION OF THE GUT MICROBIOTA DUE TO NANOSILVER INGESTION

5.1 Introduction

Prokaryotes and eukaryotes have co-existed for nearly 1.2 billion years, and with the advances in sequencing technology and software, we are just now starting to unearth many subtleties for this long standing, complex symbiotic relationship. The majority of the human microbiome\textsuperscript{15} is harmless or beneficial to the host. Collectively, it protects against pathogens [90, 91], provides us with nutrients and energy [92] [93], and fosters development [94]. However, disruptions to this delicate symbiosis lead to less biodiversity which has been associated with decreased neutrophil and lymphocyte activity [95], obesity [96], certain cancers [97], autoimmune diseases [98, 99, 100], and many behavioral and psychiatric issues [101, 102, 103].

The mechanism by which the gut community (gut microbiota) induces its affect is not through translocation, but rather dysbiosis\textsuperscript{16}. This process occurs naturally as a result of aging. Aerobic bacteria dominate the gut at birth and is altered in the first weeks to

\textsuperscript{15} Microbiome: the totality of microorganisms and their collective genetic material present in or on the human body or in another environment

\textsuperscript{16} Dysbiosis: microbial imbalance on or inside the body
form an anaerobic dominated environment [104]. By adolescences the gut has the highest proportion of *Bifidobacteria* and *Clostridia* that it ever will and will then begin to stabilize throughout adulthood [105]. In old-age the gut community shows a decrease in *Bifidobacteria* (genus) and *Bacteroidetes* (phylum), an increase in *Firmicutes* (phylum), and overall shows an increase in the number of facultative anaerobes [106]. When the gut composition changes, so does the metabolites released systemically. Many of these metabolites (Ex: SCFAs\(^\text{17}\) and GCPRs\(^\text{18}\)) interact directly with organ systems and specialized cell types which allows a seemingly minor change in the gut to have major phenotypic changes in the liver or brain [107].

The changes that occur over a lifetime can be uncontrollable, like the infant gut decreasing oxygen content over time, or from outside controllable forces like environment, pollution, and antibiotics. Diet is the most important of controllable outside influences to the gut microbiota. Our daily diet has the ability to artificially select microbes by providing substrates that favor one microbe over another [96]. In mice, switching from a low-fat, plant polysaccharide–rich diet to a high-fat, high-sugar “Western” diet shifted the structure of the microbiota, changed the representation of metabolic pathways in the microbiome, and altered microbiome gene expression in a single day [108]; and in one week changed the mouse’s enterotype\(^\text{19}\) classification [109]. In humans, the microbiota can change slightly from day-to-day, but it takes longer than 10 days to completely change enterotypes [110].

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\(^{17}\) SCFA: Short Chain Fatty Acids

\(^{18}\) GCPRs: G-Coupled Protein receptors

\(^{19}\) Enterotype: classification of living organisms based on its bacteriological ecosystem in the gut microbiome
The gut is an ecosystem that has stable states, not unlike a coral reef or rain forest. It will remain stable and healthy until disturbances knock it out of that state. For a coral reef these disturbances would be increased temperature, ocean acidification, or habitat destruction; for the gut these influences are diet, antibiotics, and pollution. These disturbances have a snowballing effect that eventually leads to a new unhealthy stable state, and positive and negative feedbacks that make it difficult to correct. Fig 15 shows the transition from a stable healthy state to an unhealthy stable state.

Silver nanoparticles are widely used in the food industry for their anti-bacterial properties so intestinal exposure is almost inevitable [32]. It stands to reason that the ingested nanoparticles can act as the ‘acute disturbance’ or ‘persistent stressor’ (Fig 15) to drive the gut to the degraded stable state. Similarly, it may also hold potential as a
method to reverse the gut back to a healthy stable state. To date, there have been very few studies looking at the effect nanosilver ingestion has the gut microbiota.

5.2 Materials and Methods

5.2.1 Model Organism

Two populations of flies were used: 1) OreRS flies supplied by Bloomington Stock Center, Bloomington, IN and 2) Wild-caught flies. OreRS flies were reared on cornmeal-malt-yeast medium\(^\text{20}\) containing tegosept and propionic acid as preservatives and were kept at 25°C on a 12 hour day-night cycle. The wild-caught fruit flies were caught at the Oakwood, OH home of Dr. Jayne Robinson and were reared on Dole brand organic bananas. They were kept at room temperature with natural and artificial light as they would receive in their natural environment.

The *Drosophila* gut shares many similarities with mammalian guts including: morphology (Fig. 16), development, disease pathways, and molecular, cellular, and tissue structures [111]. The *Drosophila* gut has approximately \(10^5\) organisms comprised of 20-60 different species compared to \(10^{14}\) and 500-1500 species for humans [112, 113, 114]. The functional metagenomics (gene expression of the host gut bacteria) between humans and drosophila are very similar and

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\(^{20}\) Recipe taken from Bloomington Stock Center website.
the major constituents are also analogous; *Firmicutes, Proteobacteria*, and *Bacteroidetes* are the most abundant phyla of both [111, 114, 115, 116, 117].

### 5.2.2 Organic vs. Non-organic

All wild-caught flies were pooled, and 10 were removed for dissection and DNA extraction (discussed in more detail below). The remaining flies were separated into two fresh fly jars containing either organic or non-organic Dole bananas. This group is the “parental” group. After the first pupae of $G_1$ appeared (approximately 10 days later) all parent flies were discarded. All $G_1$ flies were removed, and 10 from each treatment (organic or non-organic) were removed for dissection and DNA extraction and the rest were transferred to fresh fly jars with fresh bananas. This process was repeated through 5 generations.

### 5.2.3 Epicatechin vs. Un Ag NPs on OreRS Lab Flies and Wild-Caught Flies

Epicatechin reduced silver nanoparticles were the same batch used in chapter 3. The traditionally synthesized uncoated nanoparticles were obtained from Nanostructured & Amorphous Materials Inc (stock# 0478YD). Media was prepared by hand pureeing a Dole organic banana and adding either epicatechin or uncoated silver nanoparticles to give a final concentration of 100 μg/ml. Wild-caught flies (approximately 50) were added. All flies were removed before the first sign of eclosion from the progeny (15-20 days) and once enough of the progeny hatched the flies were dissected. The organic banana flies from the generational study above acted as control.
Meanwhile, the two silver nanoparticles were added to two different aliquots of standard cornmeal-malt-yeast media to give a final concentration of 100 μg/ml. The media was poured into 60x15 mm petri plates and allowed to cool. OreRS fly embryos were added directly onto the media and once enough of the progeny hatched the flies were dissected. OreRS embryos on standard cornmeal-malt-yeast media without any nanoparticles acted as the control.

5.2.4 DNA Extraction

Five *D. melanogaster* males and five females were dipped in 2.5% bleach and rinsed with sterile dH2O to reduce contamination from external bacteria on the exoskeleton. Each fly gut was dissected out in TBE buffer and pooled together in phosphate-buffered saline (PBS pH 7.4).

The DNA extraction method was modified from Tang et al. [118]. Fly guts were homogenized then centrifuged at 15,000g for 10 minutes and the supernatant was removed. The pellet was then washed twice with acetone. 1 ml of acetone was added, the sample was vigorously vortexed, and centrifuged at 15,000g for 10 minutes. After the final wash, the supernatant was discarded and a 200ul aliquot of 5% (w/v) of Chelex-100 was added along with 20ul of 20% (w/v) of proteinase K. The sample was vortexed again and incubated at 56°C for 30min in a water bath. After the incubation, the sample was vortexed again and centrifuged for 10,000g for 5 min. The supernatant was transferred to a sterile tube and stored at -20°C until needed.

The concentration and purity of nucleic acids was determined spectrophotometricly using Thermo NanoDrop 2000. A 1ul aliquot was analyzed at 260
nm for nucleic acid concentration and at 280 nm for ratio of DNA to protein impurities [119]. The sample was further tested by gel electrophoresis to ensure nucleic acids were in fact bacterial DNA. Each sample was amplified by Polymerase Chain Reaction using 16s rRNA primers [120]. 5 ul of amplified DNA was loaded, into a 1% agarose gel and electrophoresed at 150V for approximately 45 or until the dye front had traveled at least ¾ of the gel. The gel was stained with ethidium bromide [121]. Images were obtained using UVP Imaging System.

5.2.5 Next Generation Sequencing and Analysis

DNA was sequenced by Michigan State University Research Support Technology Support Facility – Genetics Core. The V4 region (515/806R) of the 16s was amplified using the method described by Caporaso & Knight [122]. After PCR the reaction products were normalized using Invitrogen SequalPrep DNA Normalization plates; products recovered from the normalization plate were pooled. After Ampure XP cleanup the pooled sample was QC’d and quantified. The pool was loaded on an Illumina MiSeq v2 flow cell and sequenced in a 2x250bp format using a standard v2 reagent cartridge (500 cycles). Base calling was performed by Illumina Real Time Analysis (RTA) v1.18.54 and output of RTA was demultiplexed and output to FastQ files by Illumina Bcl2fastq v1.8.4.

Library preparation was done by Dr. Scott Dowd at Mr. DNA Lab. The Q25 sequence data derived from the sequencing process was processed using a proprietary analysis pipeline (www.mrdnalab.com, MR DNA, Shallowater, TX). Sequences are depleted of barcodes and primers then short sequences < 200bp are removed, sequences
with ambiguous base calls removed, and sequences with homopolymer runs exceeding 6bp removed. Sequences are then de-noised and Operational Taxonomic Units (OTUs) were defined clustering at 3% divergence (97% similarity) followed by removal of singleton sequences and chimeras [123]. Final OTUs were taxonomically classified using BLASTn against a curated database derived from GreenGenes, RDPII and NCBI (www.ncbi.nlm.nih.gov, http://rdp.cme.msu.edu) and compiled into each taxonomic level [124].

For the generational data, a two-tailed Pearson’s Correlation test was used to see if any of the classes changed with respect to generation (α=.05). A one-way ANOVA test was used to see if there was a difference between the treatments on wild-caught flies on banana and Ore flies on standard lab food (α=.05). A Students T-Test was then used to pinpoint which classes changed (α=.05). Species richness\(^{21}\), number of reads\(^{22}\), and effective number of species\(^{23}\) was calculated for each treatment and an ANOVA was run to find differences and any significant result was investigated using the Students T-Test (α=.05). Species richness is simply the number of species regardless of abundance. (20 \textit{E. coli} and 10,000 \textit{S. aureus} both count as 1). The number of reads is the number of sequences observed taking into account coverage, so one sequence isn’t read twice. It can be thought of as the number of organisms (20 \textit{E. coli} counts as 20 and 10,000 \textit{S. aureus} count as 10,000). The effective number of species is a measure of diversity. It takes into

\(^{21}\) Species Richness = number of different species \\
\(^{22}\) Number of reads = Total number of different organisms present \\
\(^{23}\) Effective Number of Species = Measurement of diversity
account evenness\textsuperscript{24} and richness. An effective number of species of 3.5 is interpreted as this sample is as diverse as one with 3.5 equally distributed species [125].

5.3 Results

5.3.1 Combined Diversities

Species richness and number of reads, as well the diversity measure of effective number of species for all samples is shown in Figure 17. The species richness and number of reads is statistically the same for all samples (ANOVA: $\alpha=.05$). The only statistical significance regarding the effective number of species is that Ore control is significantly higher than all other treatments other than Ore + 100 µg/ml epicatechin nanosilver. (p<05).

\textsuperscript{24} Evenness = How even distributed the species are. A sample with 50% \textit{e. coli} and 50% \textit{s. aureus} is more even than one with 10% \textit{e. coli} and 90% \textit{s. aureus}. 
5.3.2 Organic vs Non-organic

Figure 18 shows the change in class proportions from parents to G5 for the non-organic data set. A Pearson’s Correlation statistical test was run and found statistically significant correlations between gut class changes and generation for the classes *Bacilli, Alphaproteobacteria, Clostridia, Gloeobacteria*, and ‘other’. The test determined no difference between the organic data set with regard to generation for the organic data set (Fig 19).

![Pie charts showing gut class proportions from generation to generation for non-organic treatment](image)

*Figure 16. Change in class proportions from generation to generation for non-organic treatment. n=1 for each pie chart.*
Table 2 shows that even though several classes changed with respect to generation, the species richness, number of reads, and effective number of species did not (Pearson’s Correlation Test).

![Figure 17. Change in class proportions from generation to generation for organic treatment. n=1 for each pie](image)

<table>
<thead>
<tr>
<th></th>
<th>Non-organic</th>
<th></th>
<th>Organic</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Generation</strong></td>
<td>P</td>
<td>G1</td>
<td>G2</td>
</tr>
<tr>
<td>Species Richness</td>
<td>230</td>
<td>208</td>
<td>249</td>
</tr>
<tr>
<td>Number of reads</td>
<td>27281</td>
<td>28209</td>
<td>38438</td>
</tr>
<tr>
<td>ENS</td>
<td>4.13</td>
<td>2.93</td>
<td>3.51</td>
</tr>
</tbody>
</table>

Table 2. Change in species richness, number of reads, and effective number of species (ENS) separated by generation. P-value is the return on Pearson’s test for Correlation.
5.3.3 Epicatechin vs Un Ag NPs on OreRS Lab Flies and Wild-Caught Flies

P to G₅ for the non-organic data and P to G₄ for the organic data above were combined and the class averages for organic + 100 μg/ml Epicatechin nanosilver and organic + 100 μg/ml uncoated traditionally synthesized nanosilver are presented in Fig. 20. The combined non-organic and two nanosilver treatments are very similar to each other while the combined organic data looks visually different, however, no class in any set was statistically different from the rest (ANOVA α=.05).

Figure 18. Average class values for all wild-caught D. melanogaster on banana treatments. N=5 for Non-organic and organic and n=2 for both nanosilver treatments
Figure 21 shows the class averages OreRS flies raised on standard lab food with no nanoparticles added (control), on lab food with 100 μg/ml Epicatechin nanosilver, or 100 μg/ml uncoated traditionally synthesized nanosilver. The ANOVA returned only two classes in which there was a difference between treatments: Betaproteobacteria and Gammaproteobacteria. Further T-tests revealed that the Gammaproteobacteria Ore control was significantly higher than Ore + 100 μg/ml uncoated traditionally synthesized nanosilver.
5.4 Discussion

The overall goal for this project was to see how the gut microbiota would change as a result of NP ingestion and to see how that compared to a diet switch from organic to non-organic bananas. The species richness and number of reads did not change for any sample meaning that any changes we saw were a result of changes to the distribution or evenness.

There were 5 classes of bacteria that changed in correlated with generations for the non-organic data set: Bacilli, Alphaproteobacteria, Clostridia, Gloeobacteria, and ‘other’, while there was no correlation between generation and class for the organic data set. Bacilli had a decreasing trend while the rest of the classes increased. It has been shown that earthworms living in herbicide rich soil have a higher proportion of alphaproteobacteria because they breakdown 2-methyl-4-chlorophenoxyacetic acid, a common herbicide [126]. We see a similar trend indicating a potential response to the herbicides in the non-organic banana. An increase in Bacilli and decrease in Clostridia is consistent with a gut population associated with type 2 diabetes [127]. The phylum firmicute decreased over the 5 generations of non-organic banana diet (data not shown). Increased firmicute population has been associated with obesity and several other problems in mice [108].

While trends can have predictive powers, there was no difference between classes when we compared each generation of non-organic to the cumulative organic or the cumulative non-organic to cumulative organic (Fig. 5.5). This could mean that we need more generations to compare or we need more trials at each data point to tighten the data up.
It is unclear what differences exist between organic and non-organic bananas. In the future it will be important to see if there is a nutritional difference between the two and to see whether pesticides are able to permeate the thick banana rind. A comprehensive study by Barański et al. showed that there is no nutritional benefit to organic over non-organic foods, but organic foods have higher concentrations of antioxidants and a lower concentration of pesticide residues [128]. It was not stated which type of foods this test was done on.

Given silver nanoaparticle’s bactericidal properties it was postulated that their ingestion would be deleterious, yet there was no effect for the wild-caught flies on banana. On the other hand there was an effect for lab flies on lab food (Betaproteobacteria and Gammaproteobacteria decreased), indicating a nanosilver – food interaction. Increased levels Gammaproteobacteria is associated with bowel inflammation and irritable bowel syndrome in humans [129]. Adding nanosilver to the fly diet decreased the level of Gammaproteobacteria, and uncoated silver was much more effective than epicatechin silver.

In a similar study ingestion of Ag-NPs had no effect on the microbiota of zebra fish, but it was hypothesized that this was due was due to the immediate precipitation of Ag⁺ ions due to chloride in the fish intestinal fluid [130]. In mice, the microbiota and host cell gene expression changed with AgNp and silver nitrate ingestion. Notably, there was a decrease in Firmicutes (phyla) and Lactobacillus (genus) [131]. A AgNP ingestion study using the Japanese quail found that there were no discernable differences between control and treatments except at high doses, where there was an increase in lactic acid bacteria (Lactobacillus) [132]. To further add to the Lactobacillus conundrum, our Ore +
Epi AgNP flies showed an increase of *Lactobacillus* and the Ore + Epi AgNP flies, predictably, showed a decrease.

The fact that we only saw changes in lab flies fed lab food could be telling. The lab food had added preservatives to it to get rid of outside microbes and agar to stabilize the media. The banana treatments had neither of those and thus the media changed quite drastically from day 1 to day 30. A change in viscosity could cause the distribution of the NPs to change or the nanoparticles could have changed in some way related to changes in the banana (enzymatic degradation, changes due to pH changes, direct interaction with microbes, etc). It is also possible that the chemical make-up of the banana renders the nanoparticles non-toxic, for example, -SH residues in decomposing organic food actively seek out the Ag+ ions which decreases toxicity to bacteria [133].

The fact that lab flies on lab food was much more diverse than wild caught flies on banana could indicate that the lab food promotes a more diverse gut or it could say something about the initial gut composition of the respective populations. In the future, wild caught flies should be reared on lab food and lab flies should be reared on banana. If the lab fly’s microbiota is simply more robust than the wild-caught’s, then we would see a similar highly diverse community in that population and a similar low diversity microbiota in the wild-caught population. If the food acts a disturbance leading to a degraded stable state (as seen in Fig 15) then there would be a sharp decline in diversity for the lab fly when switched to banana.

There are major concerns with regards to this type of testing in general. For one, nanoparticles are naturally occurring in many food products so determining the relevance of particles vs. food is very difficult [134]. It is also unknown what a relevant
concentration would be and there is no FDA obligation to disclose the presence of nanomaterials in any product. The NanoRelease Food Additive project attempts to identify these engineered nanomaterials but there are major hurdles like differences in the definition of ‘nanomaterial’ and inaccurate self-reporting [135]. Additionally, there are many several secondary sources for ingesting nanoparticles like frying pans and silverware. (Side note: In the above zebra fish experiment, the dose used was 500 μg/g)

Determining what happens after ingestion just raises more questions. A Sooresh et al. has developed a 7 step procedure for artificially replicating the human GI tract to accurately replicate post-ingestion (Fig. 22) [136]. Even this model is probability over simplistic when we consider how nearly impossible it is to represent the entire gut community in vitro and how many seemingly innocuous variables are left out of the model.

Figure 20. Post-indigestion modification of nanoparticles [35]
There is considerable room for growth in this field of research and at the very least we provide a reasonable protocol for testing this complex question quickly and cost effectively. The lack of/unreliability of discernable patterns is likely due to small treatment groups, but the agreeability of the samples overall is a positive and should give credence to similar studies going forward.
CHAPTER 6

GENERAL CLOSING DISCUSSION

The use of nanomaterials has increased exponentially over the past decade. There has long been a need to quickly, cost-effectively, and efficiently test their toxicity. My research shows that chemical amendments, synthesis method, genetic background, and vehicle of exposure all impact toxicity. The number of variables contributing to toxicity indicate generalizations based on single students lack explanatory power and testing of the specific particle in its intended use is needed to determine safety.
BIBLIOGRAPHY


[38] M van der Zande et al., "Distribution, elimination, and toxicity of silver nanoparticles and silver ions in rats after 28-day oral exposure.," *ACS Nano*, vol. 6, no. 8, pp. 7427-7442, 2012.


[52] K Pulskamp, S Diabaté, and HF Krug, "Carbon nanotubes show no sign of acute toxicity but induce intracellular reactive oxygen species in dependence on contaminants,"


[73] R Foldbjerg et al., "PVP-coated silver nanoparticles and silver ions induce reactive oxygen species, apoptosis and necrosis in THP-1 monocytes.," *Toxicology Letters*, vol. 190, no. 2, pp. 156-162, 2009.


[90] M Candela, et al, "Interaction of probiotic Lactobacillus and Bifidobacterium strains with human intestinal epithelial cells: adhesion properties, competition against


[127] N Larsen et al., "Gut Microbiota in Human Adults with Type 2 Diabetes Differs from Non-Diabetic Adults," PLOS One, 2010.


[136] A Sooresh et al., "A physiologically relevant approach to characterize the microbial response to colloidal particles in food matrices within a simulated gastrointestinal tract," *Food and Chemical Toxicology*, vol. 50, no. 9, p. 2971–2977, 2012.


[140] K Bilberg, MB Hovgaard, F Besenbacher,and E Bastrup, "In Vivo Toxicity of Silver Nanoparticles and Silver Ions in Zebrafish (Danio rerio)," *Journal of Toxicology*, 2012.
