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Effects of Engineered Carbon and Silver Nanoparticles on Gene Expression in Plutellaxylostella to Assess Toxicity

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ABSTRACT

The effect of pure single walled carbon nanotubes (SWCNTs) and silver nanoparticles (Ag NPs) on expression of CYP6BG1 was investigated in fourth in star of diamondback moth, Plutellaxylostella (DBM) larvae. To date there has been very little understanding of the effects of SWCNTs and Ag NPs on gene expression in insects; particularly on DBM.DBM is model organism for toxicity research because of its known resistance and sequenced genome. We investigated the effects of 138.24 µg/ml SWCNTs and 4.32 µg/ml and 8.64 µg/ml Ag NPs using artificial food. Control feed were prepared using distilled water. We measured the CYP6BG1 expression with Real-time PCR. CYP6BG1 was up regulated for 138.24 µg/ml SWCNTs and 4.32 µg/ml and 8.64 µg/ml Ag NPs compared to control. Our results suggest that DBM have very strong immunity and CYP P450 family potentially helps insects to metabolize toxic ENPs. De novo expression profile analysis due to ENPs exposure to DBM is now suggested to be required for ENP toxicity studies.

Keywords: *Plutellaxylostella, Engineered nanoparticles, Insects, Single-walled carbon nanotubes, Silver nanoparticles, Gene expression, Toxicity*

INTRODUCTION

Nanoparticles that result from human activities (e.g.,combustion)or are engineered for consumer products and new technologies are probably encountered by all organisms (Buzea *et al.*, 2007). According to the American Society for Testing Materials, and the Scientific Committee on Emerging and Newly-Identified Health Risks, engineered nanoparticles (ENPs) can be defined as manufactured materials having at least two dimensions between 1−100 nm. ENPs can be categorized into different classes; for example, metals, metal oxides, non-metals, polymer based, functionalized (Klaine *et al.*, 2008). ENPs can exhibit many novel properties and reactivity because they have high surface to volume ratio compared to other larger sized materials with similar chemical composition (Hochella *et al.*, 2008; Auffan *et al.*, 2009). ENPs have novel traits in terms of their form and function, unique physical and chemical properties, design, potentially complicated interactions with biological and environmental agents, potential bio-persistence in organisms and feed chains, quick dispensability, bioaccumulation, penetrability through tissue, and irreversible biochemical activities.

ENPs are increasingly used in a wide range of products and technologies; from electronic devicesto renewable energyto cosmetics and medicine (Fabrega*et al.*, 2011). According to consumer product inventories (CPI), there were 653 products in 2007 containing ENPs, and 1202 consumer products in 2014 containing NPs (Vance *et al.* 2015). This number is expected to increase significantly over time. However, there are no data that estimate ENP concentrations or distribution in the environment (Klaine *et al.*, 2008).

Because of their novel properties (size, shape, specific surface area, size distribution, chemical composition, and surface structure), some ENPs are thought to be potentially toxic (Sahu and Casciano, 2009; Sharifi *et al.*, 2012). Several researchers reported that nanoparticles are more toxic than their counterpart microparticles (Shi *et al.*, 2001; Yang and Watts, 2005; Borm*et al.*, 2006; Hund-Rinke and Simon, 2006; Powell and Kanarek, 2006). Since ENPs do not have any natural analog, it is difficult to forecast their fate, transport, reactivity, and toxicity in the environmental systems. Therefore, there are concerns about their potential negative effects

when released into the environment (Lowry *et al.*, 2012).

Carbon nanotubes (CNTs) (Iijima 1991) and single walled carbon nanotubes (SWCNTs) (Iijima and Ichihashi, 1993) were developed in 1991 and 1993 respectively. A CPI report (2013) showed that CNTs are the 3rd most common ENP after silver and titanium. CNTs are an allotrope of carbon, and there are three main types of CNTs: single-walled CNTs (SWCNTs), double-walled CNTs (DWCNTs) and multi-walled CNTs (MWCNTs). Raw SWCNTs are mainly hydrophobic, whereas purified SWCNTs are mainly hydrophilic because of the functional groups on their surfaces (Sun *et al*., 2002). There is controversy about whether purification of raw SWCNTs reduces (Sayes *et al*., 2006) or increases (Tian *et al*. 2006) their toxicity.

Another class of engineered nanoparticle that are becoming increasingly common are Ag nanoparticles (AgNPs). There is interest in their use for antimicrobial (Demir *et al*., 2011) and anti-inflammatory (Bar-Ilan *et al*., 2009) properties. In addition, AgNPs are effective against multidrug resistance strains of bacteria such as methicillin-resistant *Staphylococcus aureus*, *Pseudomonas aeruginosa* (Palanisamy*et al*., 2014), ampicillin-resistant *Escherichia coli* O157:H7, and erythromycin-resistant *staphylococcus pyogenes* (Shahverdi *et al*., 2007). Consequently, AgNPs have advantages over other antibiotics (Lara *et al*., 2011).Because of their properties, they are also increasingly incorporated into consumer products such as feed packaging (Edwards-Jones, 2009), deodorants, clothing materials, bandages (Chen and Schluesener, 2008), burn treatments (silver sulfadiazine), socks, soaps and detergents, water and air filters, washing machines, wet wipes, bedding, coating on surgical instruments and medical and industrial textiles (Buzea *et al*., 2007; Chen *et al*., 2007; Kumari *et al*., 2010; Liu *et al*., 2010).

With the use of high quantities of AgNPs, the accumulation in the environment and the exposure of living tissue of AgNPs are increasing (Chen and Schluesener, 2008). Some researcher believes that the lethal properties of AgNP to microbial cells are also responsible for their toxicity to eukaryotic cells (Buzea *et al*., 2007). There is some evidence that particle size or surface area is mainly responsible for AgNP toxicity, as Ag^{+} released from the NP surface after oxidation could enter into the body and interact with biological molecules (Moore, 2006; Lin *et al*., 2010; Park *et al*., 2010).

The cytochrome (CYP) P450 monooxygenases (p450s) are an abundant gene superfamily of heme-thiolate proteins, and this group of enzymes is found in almost all living organisms (Werck-Reichhart and Feyereisen, 2000). CYP genes can be categorized into 4 major clans; CYP2, CYP3, CYP4 and mitochondrial (Nelson, 1998). They are involved in the first step of drug metabolism, in detoxification of numerous xenobiotics and endogenous substances, and are essential for proceeding to second step of detoxification (Pelkonen*et al*. 1998; Martignoni*et al*. 2006; Fröhlich*et al*., 2010).

In an *in vitro* study Sereemaspun *et al*. (2008) reported inhibition of CYP1A2, CYP2C19, and CYP3A4 in heterologously expressed human p450s in insect cell membrane exposed to 15 nm sized AgNPs. In Rainbow trout, 10 nm sized AgNPs caused CYP1A2 induction in gill tissue (Scown *et al*., 2010). Kulthong *et al*. (2012) found no significant effect of orally administered AgNPs (~180 nm diameter; up to 1000 mg/kg) on CYP activities in vivo in Sprague-Dawley rat, but reported inhibition of CYP2C and CYP2D activities *in vitro*. SWCNTs inhibited CYP3A4BRactivity in a dose-dependent manner by choking the exit channel of substrate/products through a complex mechanism in Bactosomes (El-Sayed *et al*., 2016).

The CYP3 clan is the largest clan (Cui *et al*., 2017) incorporating with CYP6 and CYP9 gene families. They are found among insect p450 genes in large clusters (Feyereisen, 2006). Gene families from this clan play very important roles in insects via inactivation and metabolism of xenobiotic compounds such as insecticides and pesticides (Iga and Kataoka, 2012; lin *et al*., 2013). Genes from this clan are referred to as "environmental response genes" (Berenbaum, 2002).

Resistance to toxic chemicals in insects are often associated with one or more detoxifying genes; e.g., p450s, esterases and glutathione S-transferases (Niu *et al*. 2011; Martinez-Paz *et al*. 2012). CYP6B enzymes are believed to be mostly responsible for insecticide toxicity in caterpillars (Cohen *et al*., 1992; Berenbaum *et al*., 1996). In several insects, a large number of CYP6 genes have been identified, which are

associated with toxic chemical resistance. For example, CYP6A1 (Carino *et al*., 1994), CYP6D1 (Liu and Scott, 1998) and CYP6A12 (Guzov *et al*.,1998) in housefly; CYP6CM1 (Karunker, *et al*., 2008) in whitefly; CYP6CY3 (Puinean, *et al*., 2010) in peach aphid; CYP6B3, CYP6B4, CYP6B5 (Scott and Wen, 2001) in butterfly *Papiliopolyxenes*; CYP6BQ9 (Zhu *et al*., 2013) in red flour beetle; CYP6BQ23 (Zimmer *et al*., 2014) in pollen beetle; CYP6ER1 and CYP6AY1 (Bass *et al*., 2011; Ding *et al*., 2013; Bao *et al*., 2016) in brown planthopper; CYP6G1 (Hoi *et al*., 2014) and CYP6A2 (in *D. melanogaster*; CYP6AB11 (Niu*et al*., 2011) in *Amyeloistransitella*; CYP9G2 (Shen *et al*., 2004) and CYP6BF1 (Li *et al*., 2005) in diamondback moth (DBM). More than half of CYP P450 genes were upregulated in the resistance strain of Colorado potato beetle against imidacloprid pesticide (Zhu *et al*. 2016). Bautista *et al*. (2007) reported that, permethrin resistance at the fourth in star stage of DBM larvae was associated with CYP6BG1 over expression in resistant strains and inducible in their susceptible counterpart. Bautista *et al*. (2009) confirmed that CYP6BG1 over expression was due to increased metabolism for permethrin detoxification through RNA interference mediated gene silencing (RNAi).

Only a few studies have examined whether the CYP p450 gene is expressed differentially in animals exposed to, presumably, toxic concentrations of ENPs. El-Sayed *et al*. (2016) examined the effect of carboxylated SWCNT on animal CYP activity. Fröhlich *et al*. (2010), Lamb *et al*.(2010) and Warisnoicharoen *et al*.(2011) examined the effects of Ag NPs on CYP activity.

Our research examined if there was any evidence for detoxification of the ENPs? Specifically, were p450 genes up regulated or down regulated in DBM exposed to SWCNTs and AgNPs? We examined the effects of these ENPs on DBM because it is considered a model pest. It is a major pest on cruciferous crops (Talekar and Shelton, 1993) and can migrate and reproduce very quickly (Yu *et al*., 2015). It is remarkably resistant to insecticidal toxins (Sun *et al*., 1986; Sun, 1992; Talekar and Shelton, 1993; Scott and Wen, 2001; Furlong *et al*., 2013), and toxicity to ENPs would indicate that many insects might be negatively affected. For example, DBM was the first pest that became resistant to DDT (Ankersmit, 1935; Jhonson, 1953). In almost all countries they have evolved resistance against agricultural synthetic insecticides (Talekar*et al*., 1990), even the bio-insecticides based on *Bacillus thurengiensis spores* (Tabashnik*et al*., 1997).

Materials And Methods

SWCNTs (COOH-CNT) produced by the catalytic vapor deposition (CVD) process were purchased from NanoLab Inc. (Waltham, MA, USA) in powdered from. For this study, they were dispersed in 18MΩ deionized water to make the desired concentration (290 mg/L or 292 mg/L or 305 mg/L) with more than 95% purity and pH of 6.5 -7.5. Initially, 400 mg of SWCNT material were added to 1L of 18MΩ deionized water. A probe sonicator was used for dispersion (twice for 30 min with a 15 minutes' rest in between) followed by ultracentrifugation at 25,000g for 30 min. The supernatant collected from the centrifuge tubes and ultracentrifugation was repeated at 25,000g for 30 minutes. During this process, the concentration decreases due to the removal of amorphous carbon and unfunctionalized carbon material and finally yield an approximate 300 mg/L desired concentration. The SWCNTs have an approximate diameter of 1.5 nm, length of 1-5 µm and surface are of 1020.48 m^2/g.

Table1. *Primer design and properties used for qRT-PCR*

CYP6BG1		**Amplicon length**	**T_m**	**GC content**
Forward	ACCCTCGAGAAGGGTCTCCGA	111	61.7	61.9
Reverse	ATTCTCCGGCGAAAACCGATC		57.7	52.4
RPL32				
Forward	CAATTTACCGCCCTACCATC	91	53.4	50
Reverse	CGCCAGTTACGCTTTATTTTG		52.7	42.9

AgNPs (20 nm PELCO® citrate NanoXact™ AgNPs) were commercially obtained from Ted Pella Inc. (Redding, CA, USA). Their diameter was 18.5 ± 3.4 nm and their hydrodynamic diameter was 28 nm with 29.0 m^2/g surface area (TEM). The concentration of the Ag NP suspension was 0.021 mg/ml, with a particle concentration of 6.0E + 11 particle/ml. The AgNP has -43 mV zeta potential and 396 nm absorbance peak (λ_{max}) with 3.50 max Optical

Density/cm in a solution with 8.1 pH. The particle surface was sodium citrate and the aqueous carrier was 2 mM citrate. AgNPs were stored at 4ºC and away from light before use and they were used directly without any processing or change before use.

The lab benches were sterilized and cleaned with 75% ethanol before the preparation of the experimental feed to avoid any infection to DBM larvae through feed with any natural microorganism. The artificial feed was a dry mix purchased from Southland Products Inc. (Lake Village, AR, USA). The mix was specifically formulated for DBM (*P. xylostella*). The recipe for 250 ml feed was as follows: 40.5 g dry mix and 1.75 ml raw linseed oil and 232.5 ml deionized boiling water. For SWCNT feed preparation, 1.75 ml raw linseed oil was added to 40.5 g dry mix in an Erlenmeyer flask. Then SWCNT solution was added in 7.45 ml, 14.89 ml, 29.79 ml, 59.58 ml or 119.2 ml volume to generate 8.64 µg/ml, 17.28 µg/ml, 34.56 µg/ml, 69.12 µg/ml and 138.24 µg/ml SWCNT feed respectively. The mixture was then combined with deionized boiling water (225.05 ml, 217.61 ml, 202.71 ml, 172.92 ml and 113.3 ml respectively) on a magnetic stirrer hot plate. After mixing the final suspension for 2-3 minutes, the resulting semi-liquid feed was poured into labeled Petri dishes and left in room temperature about 15-20 minutes for solidification. The solidified feed mixture was then partitioned using a small corer (1.3 cm diameter) and the remaining feed was stored in the refrigerator at 4ºC.

To prepare 60 ml AgNP feed, 0.42 ml raw linseed oil was added to 9.72 g dry mix in a flask. Then AgNP solution was added in a volume of 12.34 ml and 24.69 ml to achieve a final concentration of 4.32 µg/ml and 8.64 µg/ml AgNP feed respectively. It was then combined with deionized boiling water (43.46 ml and 31.11 ml respectively). Other steps were the same as SWCNT feed preparation. Nanomaterial free feed were used as control feed.

Eggs of DBM were purchased commercially from Benzon Research Inc. (Carlisle, PA, USA). Eggs on aluminum foil were kept with artificial control feed discs in plastic feed boxes under diurnal cycle of 16 h light: 8 h dark to hatch and grow until they are 2ndinstar larvae. The lab temperature was 25 ± 3ºC with 65 ± 5 % relative humidity. The DBM laboratory is licensed by the United States Department of Agriculture to rear and distribute insects (USDA permit number: P26P-14-02726).

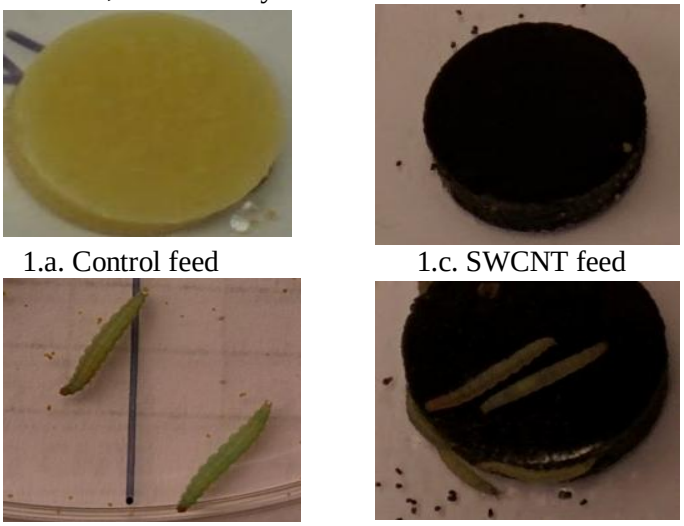
Fourth in star larvae (whole body) were collected and stored at -82ºC. Five larvae were collected from each of the five replications of control, and the 138.24 µg/ml SWCNT treatment and the 4.32 µg/ml and 8.64 µg/ml AgNP treatment arenas. For total RNA extraction, the larvae were placed in liquid nitrogen and crushed using a porcelain mortar and pestle. All the equipment's were cleaned with 70% ethanol and R Nase-Away (Molecular Bio Products Inc., San Diego, CA, USA) to eliminate contamination. The larvae were then homogenized in 750 µl Trizol Reagent (life technologies, Carlsbad, CA, USA). Total RNA was precipitated with 500 µl is opropanol and resuspended in 50 µl DEPC treated water.

Extracted RNA was purified with Qiagen RNeasy® Mini Kit (Thermo Fisher Scientific, Eugene, Oregon, USA) following manufacturer's guidelines. I used Qubit® 3.0 Fluoro meter and Qubit® RNA BR assay kits (Invitrogen, Eugene, Oregon, USA) to quantitate the RNA following the manufacturer's guidelines. The samples were then stored at -80ºC until next use.

Three samples containing RNA at high concentration were selected from each treatment and first strand cDNA was synthesized with Qiagen QuantiTect® reverse transcription kit (Chatsworth, CA, USA) following the manufacturer's guidelines. I also performed reverse transcription using both oligo dT and random primers, but the QuantiTect® kit provided the highest yield of cDNA. All RNA samples were reverse transcribed simultaneously to avoid variations in cDNA. The cDNA was then amplified by regular PCR and confirmed with agarose gel electrophoresis. cDNA was stored at -20ºC until next use.

Permethr in resistance gene CYP6BG,1 a member of the p450 gene family, was used as a target gene, and ribosomal protein L32 (RPL32) was used as a reference gene for qRT-PCR. The primer design for CYP6BG1 was reported previously by Bautista *et al*. (2007). Fu *et al*. (2013) and Gao *et al*. (2016) reported that RPL 32 is a reliable housekeeping gene. We used the Integrated DNA Technologies website to design the forward and reverse primer for RPL 32 (Table 1).

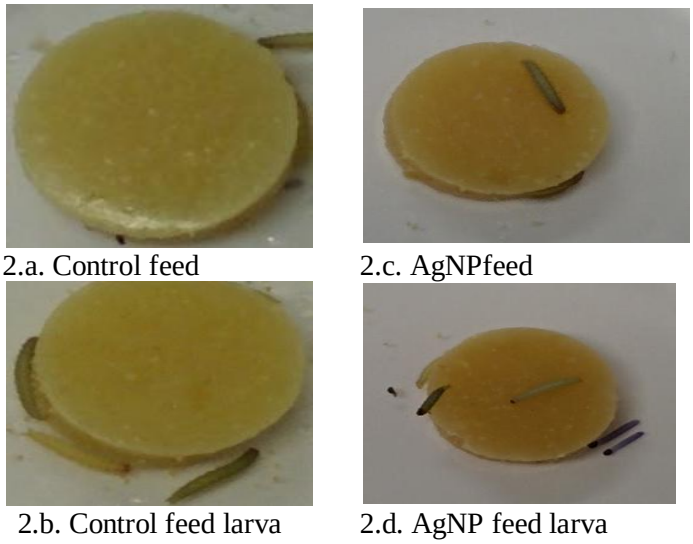
The qPCR was performed with GoTaq® qPCR master mix (Promega Corporation, Madison, WI, USA) in an Mx3000P thermal cycler (Agilent Technologies, Inc. Santa Clara, CA, USA) with the help of MxPro qPCR software. 96 well EU thin-walled PCR plates (BPCTiinc., Durham, NC, USA) were used for the reaction. To determine PCR amplification efficiency, standard curves were generated for both target and reference gene using 10-fold serial dilutions. Thermal cycling profiles used in this study were: 95ºC for 10 min, followed by 40 cycles of 95ºC for 30 sec, 55ºC for 60 sec, 72ºC for 60 sec. A dissociation step cycle at 95ºC for 60 sec, 50ºC for 30 sec and 95ºC for 30 sec was added as a final step to generated melting curves. The amplification reaction was done in three technical replicates for each biological replicate. No-template controls (NTC) were run for every sample to check for DNA contamination. The gene expression level was calculated based on cycle threshold (C_t) value by using Pfaffl method (Pfaffl *et al*., 2002).



1.a. Control feed 1.c. SWCNT feed

1.b. Control feed larva 1.d. SWCNT feed larva

Figure1. *DBM larvae exposed to artificial food.(1.a, b.) or SWCNT food The SWCNT fed are very much darker than control fed.(1.b.)Larvae feeding on control feed, clear gut (1.d.)Larvae fed on SWCNT food, accumulated SWCNT on their gut. Evidence that SWCNT were taken with food and accumulated in their body.*



2.a. Control feed 2.c. AgNPfeed

2.b. Control feed larva 2.d. AgNP feed larva

Figure2. *DBM larvae exposed to artificial food. (2.a,b.) or AgNP food. The AgNP feed are a little brownish compared control fed. (2.b.) Larvae feeding on control food, healthy and normal length. (2.d.) Larvae fed on AgNP food are dying and have shorter length.*

Results And Conclusions

Larva fed SWCNT clearly incorporated the ENP in their tissue (Figure 1), whereas it is not as clear, larva feed AgNP also have discoloration associated with the ENP (Figure 2). We performed qRT-PCR, and calculated relative gene expression levels using the Pfaffl method

to detect differences in the expression of the CYP6BG1 gene expression between treated and control DBM. The efficiency for reference and target gene was 97.5% and 83.8%, respectively. The expression level of CYP6BG1 gene was higher for all the ENP treatments (Figure 3), but they were not significantly different from control DBM (Table 1).We found that CYP6BG1 gene expression was higher in DBM feed ENPs than those feeding on feed with no ENPs. Even though our results were not statistically significant, further studies are warranted. Bautista *et al*. (2007, 2009) reported and confirmed up regulation of CYP6BG1 gene due to permethrin resistance in DBM. CYP P450 gene family have 36 to 180 genes in insect genome (Feyereisen, 2012; Zhou *et al*., 2014), which makes it very hard to target one or few genes for gene expression assessment due to ENPs exposure. Our study on CYP6BG1 gene expression emphasize that DBM have very strong immunity and CYP P450 family potentially helps insects to metabolize toxic ENPs. De novo expression profile analysis due to ENPs exposure to DBM is now required to advance understanding of toxicity of ENPs in insects due to changes in gene expression.

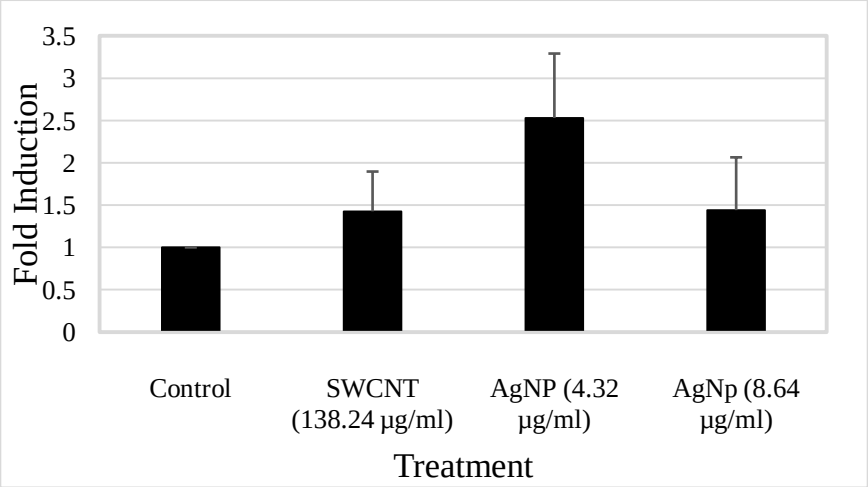


Figure3. *CYP6BG1 gene expression level in DBM feeding on different concentrations and types of ENPs.*

Table2. *Mean (±SE) gene expression of CYP6BG1 of DBMs feeding on artificial feed without or with SWCNT (138.24 µg/ml) and Ag NP (4.32 µg/ml and 8.64 µg/ml). Gene expression (df = 3; F = 1.43 and p = 0.252) wasn't significantly affected by the presence of SWCNT and AgNP in the feed.*

Variable	Treatment	Mean	SE Mean
Fold change of gene	0 µg/ml	1.0	0.0
	138.24 µg/ml SWCNT	1.43	0.47
	4.32 µg/ml AgNP	2.53	0.76
	8.64 µg/ml AgNP	1.44	0.62

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