ELUCIDATING THE MECHANISMS OF NICKEL AS AN ANIMAL MUTAGEN

by

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ABSTRACT

Nickel is an essential metal for many organisms. Nickel occurs naturally from volcanism and weathering of stone. Recently production of nickel from anthropogenic sources has overtaken natural production. Combustion of fossil fuels, metal alloy use and production, welding, and electroplating are all human causes of nickel in the environment. Nickel is a confirmed carcinogen and exposure to nickel causes an increased risk cancers. Nickel affects DNA but it is not clear if nickel affects DNA, either directly as a genotoxic agent, indirectly as an enabler of mutagens, or both. This experiment will demonstrate whether nickel affects DNA directly or indirectly.

C. elegans are developmental genetic model nematodes, with a rapid four-day life cycle. This experiment uses chemical mutagenesis of *C. elegans* to show the effects of nickel on DNA. We will subject *C. elegans* to exposure to four treatments: a control group not subjected to mutagens, ethyl methanesulfonate (EMS), $NiCl_2$, and EMS and $NiCl_2$ together. The F_2 generation or grandkids of exposed animals will be examined for easy to identify morphological mutations. The mutations resulting from each treatment will be counted and categorized. If nickel acts as a mutagen, $NiCl_2$ treated animals will accrue mutations and maybe compared to the known mutagen EMS to determine its relative mutagenicity. This will confirm nickel as an animal mutagen. If the number of mutations from the joint EMS and $NiCl_2$ treatment is significantly greater than the sum of the mutations from EMS and $NiCl_2$ alone, then nickel is acting synergistically with EMS. Thus, nickel is indirectly affecting the DNA, perhaps as an enabler of the known mutagen. If the number of mutations from the EMS and $NiCl_2$ treatment is equal to or less than the sum of EMS and $NiCl_2$ alone, then nickel is directly affecting the DNA independent

of other mutagenic agents. Our preliminary results suggest nickel is acting as a mutagen at least in part indirectly as an enabler of other mutagens.

This experiment will shed light on many important aspects of nickel toxicity. First, while nickel has been shown as a mutagen and genotoxic agent in prokaryotes, conclusive evidence of this in animals has been difficult. Based upon our results, nickel does appear to be an animal mutagen. Second, nickels mode of action by acting either directly on DNA, as an enabler of other mutagenic agents, or both remains unresolved. Based upon our results to date, nickel may act at least in part as an enabler of other environmental mutagens. If it is discovered how nickel affects DNA, directly or indirectly, then further study may help reveal solutions concerning how to prevent nickel's heritable toxic effects.

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INTRODUCTION

Nickel is an important metal for organisms and occurs in nature in the atmosphere, soil and water. Nickel is the 24th element in order of natural abundance in the earth's crust. It is an abundant metal and appears in significant quantities in nature. (Kasprzak, Sunderman Jr., & Salnikow, 2003). Historically, volcanic emissions and erosion of rocks and leaching from soils were the source of environmental nickel. Aqueous nickel comes from nickel compounds in airborne dust and from soluble nickel containing compounds leaching from sediments dissolving in the water. In recent years, human industrialization has dramatically increased the amount of nickel in the world's atmosphere and water. Almost 150,000 metric tons of nickel per year is naturally released from volcano emissions and erosion; in contrast, anthropogenic sources introduce 180,000 metric tons per year (Lu, Xianglin, Costa, & Huang, 2005). Combustion of fossil fuels, welding, alloy production, and electroplating are all human causes of nickel in the environment (Lu, Xianglin, Costa, & Huang, 2005). As a consequence of these sources there is an unprecedented increase human exposure and studies have shown that nickel is both a major allergen and carcinogen.

Nickel affects human health. In 1990, The International Agency for Research on Cancer classified nickel as a group 1 confirmed carcinogen (Lu, Xianglin, Costa, & Huang, 2005). Inhalation and consumption exposure to nickel compounds increases risk of nasal and lung cancer (Huffnagle, et al., 2013). Additionally, many people are allergic to nickel and can have skin irritation or rashes from exposure.

Nickel can cause DNA damage in many ways and induce mutations. It may promote damage indirectly to act as a carcinogen by enabling other mutagens (Lu, Xianglin, Costa, &

Huang, 2005). It may cause problems in chromatin remodeling, DNA methylation, and histone acetylation (Ke, 2006). Nickel may cause DNA to unwind and enable other mutagens to attack to the DNA (Dubins, 1986). It may also affect the signaling pathways that cause transcription factors to alter gene expression or other signal pathways (Gartner, 2008). Alternatively, nickel may result in the formation of reactive oxygen species which damage DNA. Nickel may possibly cause issues with intercellular communications (Miki, 1987). Lastly, nickel may damage DNA directly because nickel is positively charged while DNA is negatively charged.

In this work, nematodes will be used for investigating nickel toxicity. Two nematode species used in the Rudel laboratory, *Caenorhabditis elegans* and *Pristionchus pacifics*. This study uses *C. elegans.* Nematodes are a superb animal model system for toxicology studies. Nematodes are incredible environmental indicators and have been used to show the effects of different metals (Korthals, 1996). Nematodes are the most abundant multicellular animals and they populate almost every environment (Huffnagle, et al., 2013). Nickel elicits a toxicological response in animals, including nematodes. In most animals, cells that are damaged by a toxic agent undergo apoptosis or programed cell death, a form of cell suicide that insures that the damaged cell is removed and does not harm its' surrounding undamaged colleagues (Huffnagle, et al., 2013). The nematode is an excellent organism for these sorts of toxicology studies because a number of tools have been fabricated to analyze apoptotic cells and the complete cell lineages is known. In nematodes, if a cell undergoes apoptosis, it can be easily found and observed.

Studies have already shown that nickel can cause severe problems for nematodes. Sediment that contained nickel caused the nematodes to have decreased survival from larvae to adults and adults had a shorter lifespan (Rudel, Douglas, Huffnagle, Besser, & Ingersoll, 2013).

Nickel exposure comes in different forms and they can have different effects. Nickel also decreases fecundity in adult animals (Rudel, Douglas, Huffnagle, Besser, & Ingersoll, 2013).

A further study showed that *C. elegans* that are exposed to $NiCl_2$ lead to high programmed cell death (Huffnagle, et al., 2013). This study also showed a linear increase in damaged DNA with increased $NiCl_2$ exposure (Huffnagle, et al., 2013). Thus nickel elicits a complex physiological response and as a result nickel may cause cell deaths that interfere with assaying heritable genetic damage.

This work uses chemical mutagenesis of nematodes through exposure to $NiCl_2$, ethyl methanesulfonate, and $NiCl_2$ and ethyl methanesulfonate to better explain and describe how nickel acts as a mutagen/carcinogen.

Nickel is a carcinogen in solid and aqueous forms that affects DNA. This experiment will help determine if the carcinogen is directly affecting the DNA of organisms. Another possibility is that nickel is an enabler and allows other molecules to go in and damage the DNA directly. This means it could be disrupting the packaging of DNA, and the repair of DNA thus making the DNA more available to exposure to other environmental genotoxic agents. If the mechanisms by which nickel is affecting DNA are better understood, then putative solutions to the problem of nickel-induced DNA damage could be found and treatments to stop nickel from causing cancer developed.

This experiment addresses the mechanisms by which nickel is affecting DNA. Is it directly damaging DNA or is it an enabler? And if these questions are answered the next question is how can nickel's affects be prevented so that the organism will not be affected, e.g. induce cell death or get cancer?

METHODS AND MATERIALS

C. elegans are the model organisms used in this experiment. Two types of strains are used N2, the wild type laboratory strain and KX84, a homozygous *ced-3* loss of function mutant strain that inhibits apoptosis. Both strains were subjected to the same treatments and procedures. The worms were grown in a 20ºC fridge on NGM agar plates that were seeded with OP50 bacteria for food (Brenner, 1974). *C. elegans* is a self-fertilizing hermaphrodite with the potential for out-crossing with males and has a three and a half day life cycle at 20ºC. *C. elegans* progresses through four larval stages L1-L4 before reaching adulthood, each larval stage is characterized by distinct morphological features. In our screens, L4-larvae are used as they have begun to produce gametes (sperm and eggs) but have not produced self-progeny and are also not yet competent to be mated.

General method

The four treatments were conducted with the same general method. The P0 generation worms were subjected to one of the four treatments, either PBS control, EMS, $NiCl_2$, or EMS and $NiCl_2$ together. Figure 1 shows a diagram of the general procedure. Following treatment either 10 or 20 L4 larvae from the P0 generation were picked and moved to an individual agar and allowed to grow for three to four days plate to give birth to the F1 generation. Then after 10 L4 larvae from the F1 generation plates were moved to separate agar plates and allowed to grow for three to four days to give birth to the F2 generation. F1 animals are potential heterozygous for mutatinons, and F2 animals are potentially homozygous for the mutation allowing viewing of recessive phenotypes. The F2 generation worms were screened for morphological mutants, and the mutant animals were isolated to start new mutant cultures and categorized.

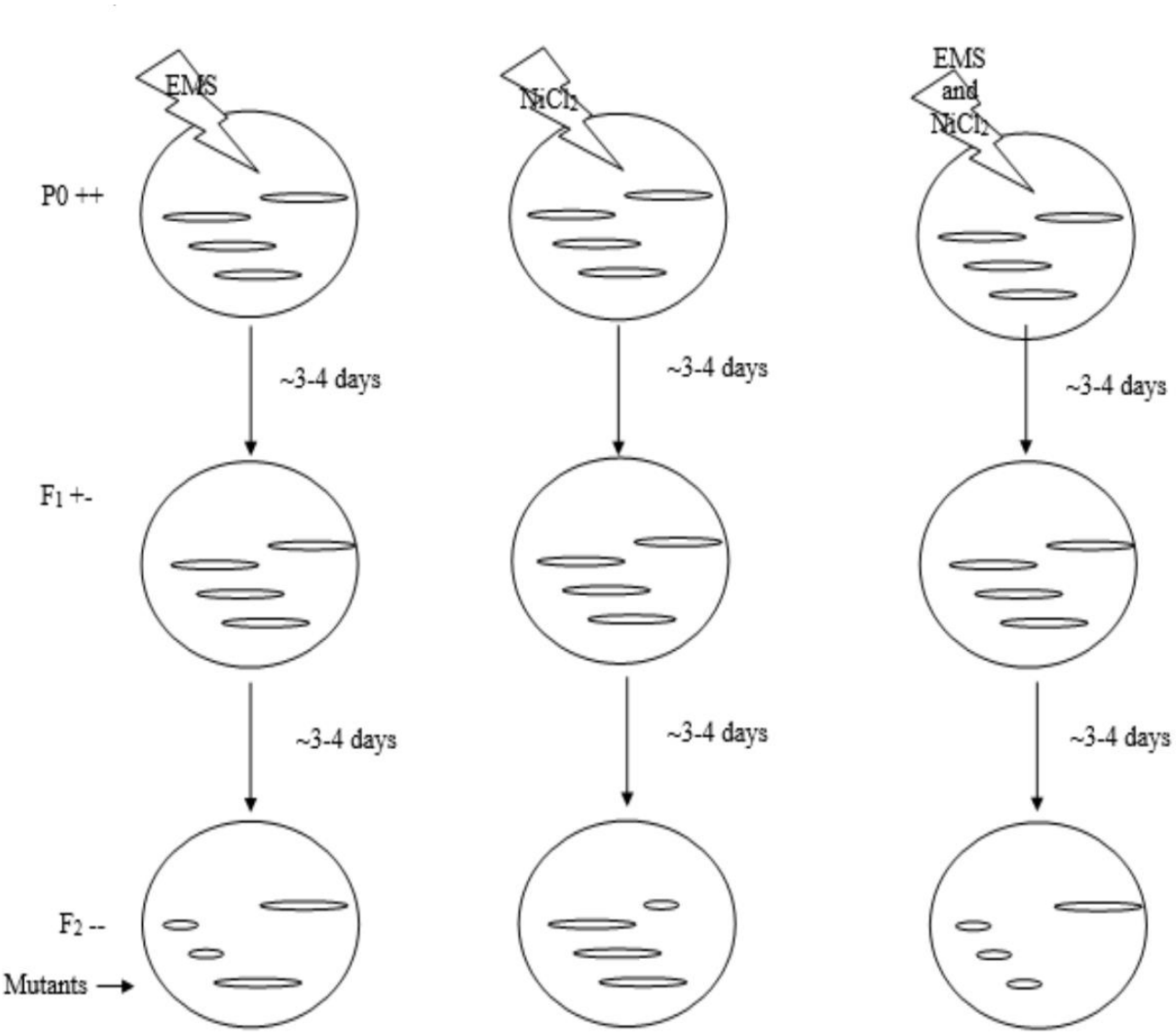

Figure 1. Diagram of the general procedure. (Atkinson, 2016)

Control

For the control, the P0 worms were washed off the plate with PBS into a conical tube, which was centrifuged at 6ºC at 1200 rpm for five minutes. The nematodes were subsequently washed 3 times with PBS and centrifuged at 1200 rpm in the same conical tube. The worms were then rocked for 4 mL of PBS for 4 hours, washed 4 times in PBS at same temperature, time, and rpm as before and given an hour to recover on an agar plate. Then the general screening method above was followed.

EMS

The P0 worms were washed off the plate with PBS into a conical tube, which was centrifuged at 6ºC at 1200 rpm for five minutes. The nematodes were subsequently washed 3 times with PBS and centrifuged at 1200 rpm in the same conical tube. Then the worms rocked in 4 mL of 0.2 M EMS in PBS for 4 hours, washed 4 times in PBS at same temperature, time, and rpm as before and given an hour to recover on an agar plate. Then the general screening method above was followed.

$NiCl_2$

The P0 worms were washed off the plate with 25 µg/L $NiCl_2$ PBS into a conical tube, which was centrifuged at 6ºC at 1200 rpm for five minutes. The nematodes were subsequently washed 3 times with 25 µg/L $NiCl_2$ PBS and centrifuged at 1200 rpm in the same conical tube. These worms were placed onto a seeded 25 µg/L $NiCl_2$ NGM plate and placed in the 20ºC fridge for 48 hours. The P0 worms were washed off the plate with 25 µg/L $NiCl_2$ PBS into a conical tube, which was centrifuged at 6ºC at 1200 rpm for five minutes. The nematodes were subsequently washed 3 times with 25 µg/L $NiCl_2$ PBS and centrifuged at 1200 rpm in the same

conical tube. Then the worms rocked in 4 mL of 25 µg/L $NiCl_2$ PBS for 4 hours. Afterwards, the worms were washed 4 times in regular PBS at same temperature, time, and rpm as before and given an hour to recover on an agar plate. Then the general screening method above was followed.

EMS and $NiCl_2$

The P0 worms were washed off the plate with 25 µg/L $NiCl_2$ PBS into a conical tube, which was centrifuged at 6ºC at 1200 rpm for five minutes. The nematodes were subsequently washed 3 times with 25 µg/L $NiCl_2$ PBS and centrifuged at 1200 rpm in the same conical tube. These worms were placed onto a seeded 25 µg/L $NiCl_2$ NGM plate and placed in the 20ºC fridge for 48 hours. The P0 worms were washed off the plate with 25 µg/L $NiCl_2$ PBS into a conical tube, which was centrifuged at 6ºC at 1200 rpm for five minutes. The nematodes were subsequently washed 3 times with 25 µg/L $NiCl_2$ PBS and centrifuged at 1200 rpm in the same conical tube. Then the worms rocked in 4 mL of 0.2 M EMS in 25 µg/L $NiCl_2$ PBS for 4 hours. Afterwards, the worms were washed 4 times in regular PBS at same temperature, time, and rpm as before and given an hour to recover on an agar plate. Then the general screening method above was followed.

RESULTS AND DISCUSSION

Two strains of *C. elegans* were tested and the results were interesting. First the N2 wild type strain was tested and the results were inconclusive. The second strain we tested were KX84 which is a *ced-3* mutant so the worms could not undergo apoptosis. The results are as follows.

N2 wild type

The N2 wild type screens were successful and many mutants were found. Figure 2 shows the breakdown of the mutations found and their percentages. Many different types of mutants were found but the main two mutants were Pvul and Egl. Of the mutants found 47.07% were Pvul and 34.14% were Egl.

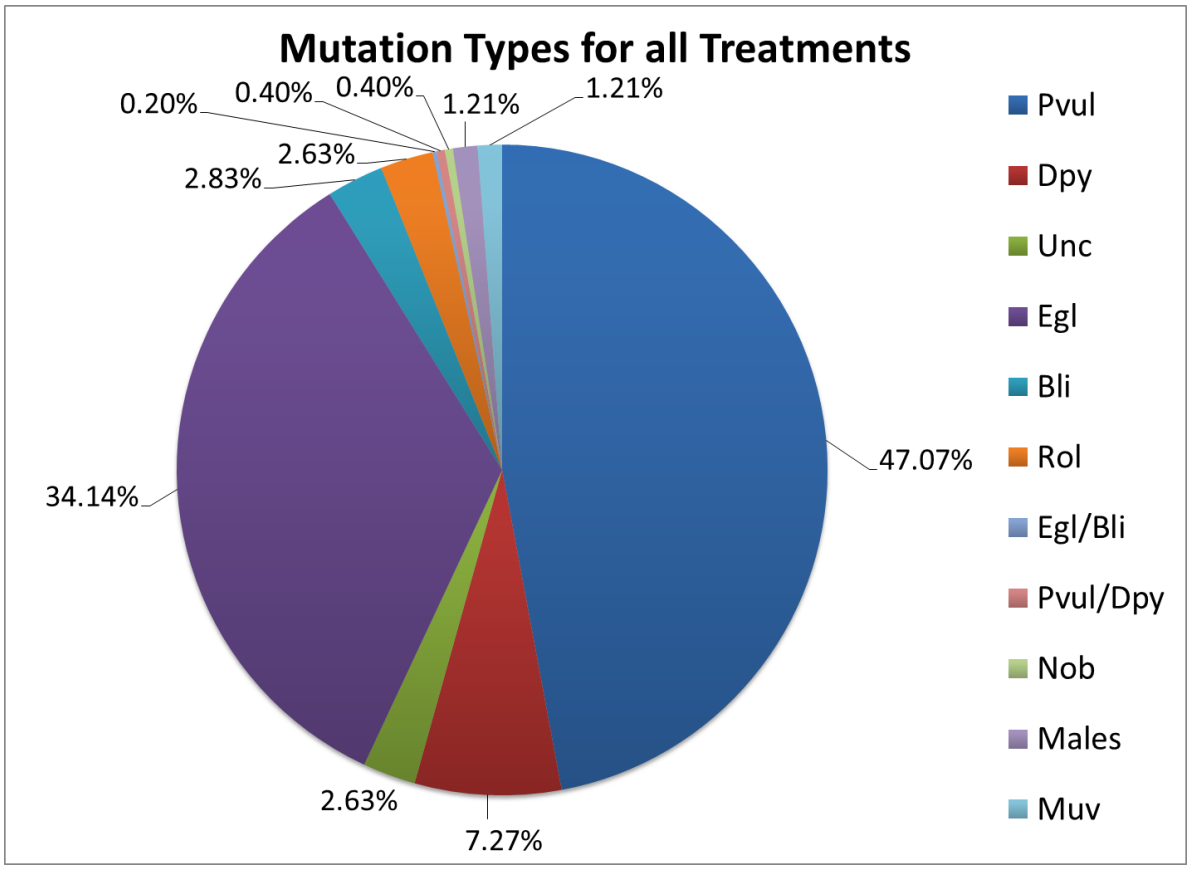

Figure 2. The N2 mutation types for all treatments. Pvul is protruding vulva. Dpy is dumpy. Unc is uncoordinated. Egl is egg laying deficiency. Bli is blister. Rol is roll. Egl/Bli and Pvul/Dpy are double mutants. Nob is no back end. Males is a plate with male offspring either a HIM or transformer mutation. Muv is multi vulva.

The N2 wild type strain results were thought-provoking because we had an outcome that we did not predict. Table 1 shows the mutations and haploid genes for each round of testing. The mutations per number of haploid genes for each treatment are as follows: EMS 1.16E-01, $NiCl_2$ 1.06E-02, EMS + $NiCl_2$ 7.25E-02, and Control (PBS) 3.75E-03. We found it unusual that the EMS + $NiCl_2$ treatment was less that EMS alone. Another way to see this is Figure 3, and it is evident that EMS + $NiCl_2$ treatment is less. We hypothesize that the N2 wild type worms were undergoing apoptosis because the EMS + $NiCl_2$ treatment was harsh on the worms. This is when we decided to try to move on to KX84 mutated strain. There was another issue of getting the different treatments to be significantly different. Table 2 shows the t-test comparisons between

the treatments. After comparing all the treatments, the EMS and EMS + $NiCl_2$ were not significantly different and neither were $NiCl_2$ and Control (PBS). We decided to test the KX84 strain to get more accurate results.

Combined Data				
	EMS	$NiCl_2$	EMS + $NiCl_2$	Control
Round 1	N= 400	N= 800	N= 400	N= 800
	M= 40	M= 5	M= 24	M= 2
Round 2	N= 400	N= 800	N= 400	N= 800
	M= 47	M= 5	M= 36	M= 3
Round 3	N= 800	N= 800	N= 800	N= 800
	M= 102	M= 10	M= 73	M= 4
Round 4	N= 800	N= 800	N= 800	
	M= 89	M= 14	M= 41	
M/N	278/2400	34/3200	174/2400	9/2400
	EMS	$NiCl_2$	EMS + $NiCl_2$	Control
	1.16E-01	1.06E-02	7.25E-02	3.75E-03

Table 1. The combined data for the N2 treatments. N is the number of haploid genes and M is the number of mutations. The mutations per number of haploid genes for each treatment are as follows: EMS 1.16E-01, $NiCl_2$ 1.06E-02, EMS + $NiCl_2$ 7.25E-02, and Control (PBS) 3.75E-03.

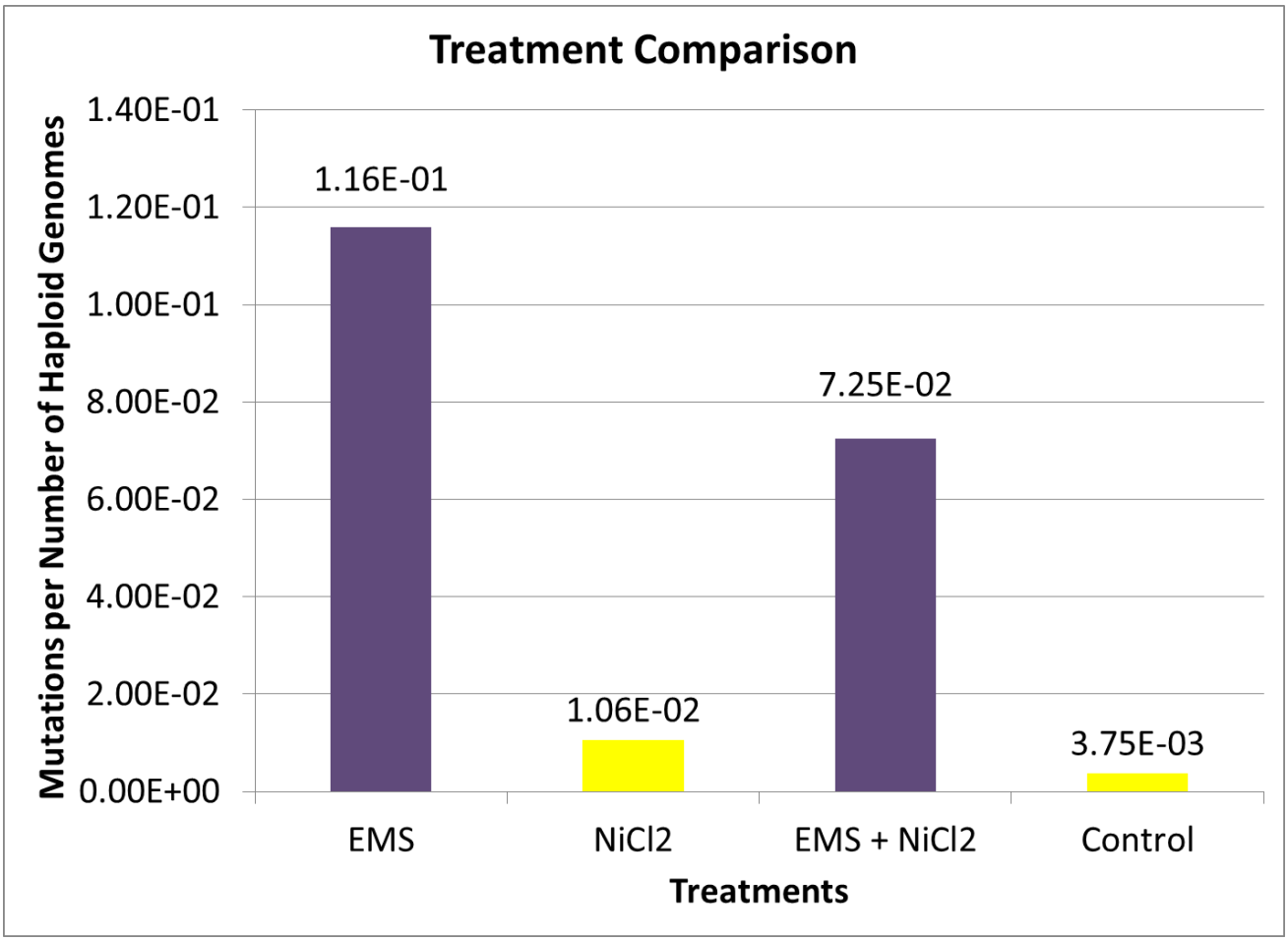

Figure 3. The combined data for the N2 treatments. N is the number of haploid genes and M is the number of mutations. The mutations per number of haploid genes for each treatment are as follows: EMS 1.16E-01, $NiCl_2$ 1.06E-02, EMS + $NiCl_2$ 7.25E-02, and Control (PBS) 3.75E-03. After comparing all the treatments, the EMS and EMS + $NiCl_2$ were not significantly different and neither were $NiCl_2$ and Control (PBS) represented by the colors.

Comparison	T-test P-Value (Two-tailed, Two-sample unequal variance)
EMS and $NiCl_2$	0.02016078
EMS and EMS+$NiCl_2$	0.50498953
EMS and Control	0.0133029
$NiCl_2$ and EMS+$NiCl_2$	0.02412335
$NiCl_2$ and Control	0.2918377
EMS+$NiCl_2$ and Control	0.01292908

Table 2. The N2 t-test comparisons. The red values show the treatment comparisons are still less than 0.05 and insignificant.

KX84 *ced-3* mutant

The KX84 strain screens were done and many mutants were found. Figure 4 shows the breakdown of the mutations found and their percentages. Many different types of mutants were found but the main two mutants were Pvul and Egl. But this time Egl was the highest percent with 68.68% and Pvul with 22.03%.

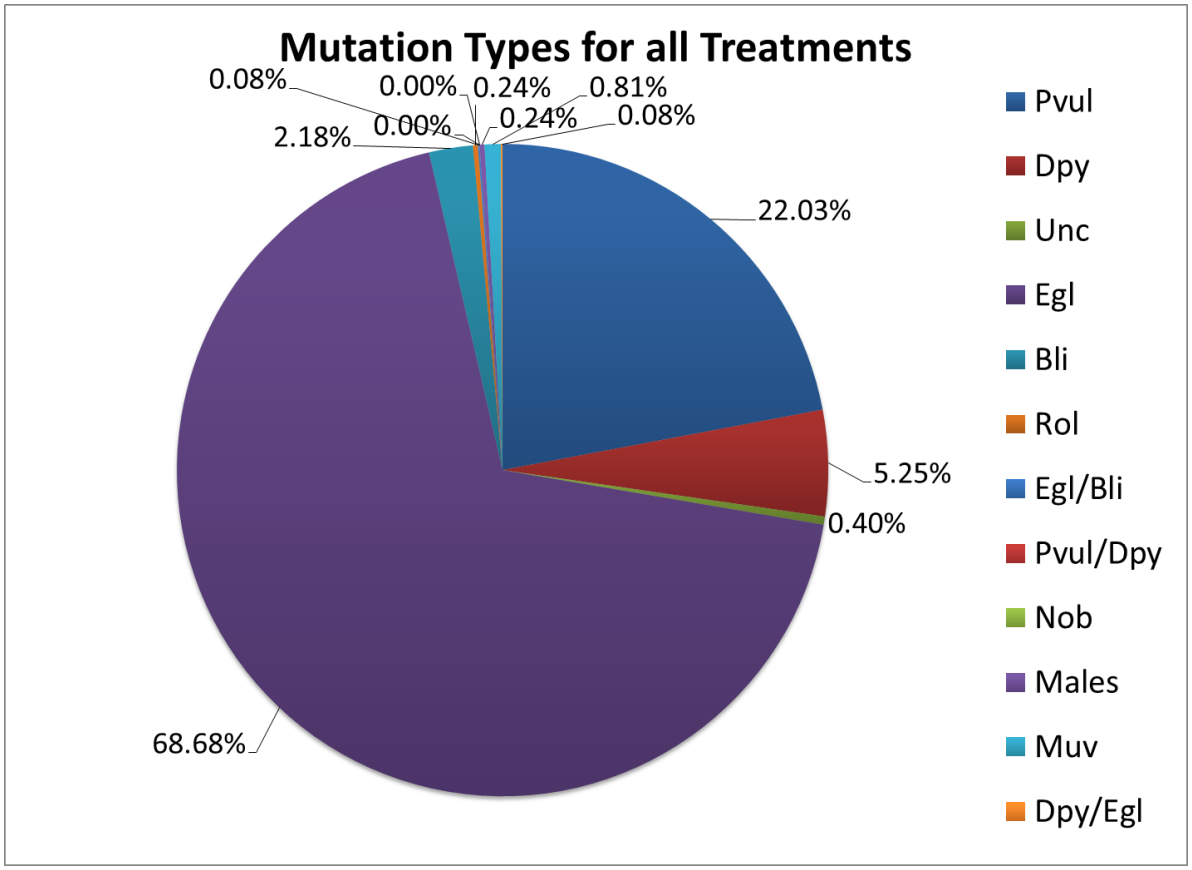

Figure 4. The KX84 mutation types for all treatments. Pvul is protruding vulva. Dpy is dumpy. Unc is uncoordinated. Egl is egg laying deficiency. Bli is blister. Rol is roll. Egl/Bli and Pvul/Dpy are double mutants. Nob is no back end. Males is a plate with male offspring either a HIM or transformer mutation. Muv is multi vulva.

The KX84 strain results were successful because we obtained more desirable results. Table 3 shows the mutations and haploid genes for each round of testing. The mutations per number of haploid genes for each treatment are as follows: EMS 1.43E-01, $NiCl_2$ 3.83E-02, EMS + $NiCl_2$ 1.93E-01, and Control (PBS) 2.28E-02. Another way to see this is Figure 5, and it is evident that EMS + $NiCl_2$ treatment is more than EMS with this strain. There was another issue of getting the different treatments to be significantly different. Table 4 shows the t-test comparisons between the treatments. After comparing all the treatments, the EMS and EMS + $NiCl_2$ were not significantly different and neither were $NiCl_2$ and Control (PBS). The N2 and KX84 testing were both successful and gave us more information and ideas for future research.

Combined Data				
	EMS	$NiCl_2$	EMS + $NiCl_2$	Control
Round 1	N= 800	N= 800	N= 800	N= 800
	M= 122	M= 27	M= 181	M= 22
Round 2	N= 800	N= 800	N= 800	N= 800
	M= 129	M= 31	M= 143	M= 13
Round 3	N= 800	N= 800	N= 800	N= 800
	M= 108	M= 34	M= 136	M= 19
Round 4	N= 800		N= 800	N= 800
	M= 99		M= 156	M= 19
M/N	458/3200	92/2400	616/3200	73/3200
	EMS	$NiCl_2$	EMS + $NiCl_2$	Control
	1.43E-01	3.83E-02	1.93E-01	2.28E-02

Table 3. The combined data for the KX84 treatments. N is the number of haploid genes and M is the number of mutations. The mutations per number of haploid genes for each treatment are as follows: EMS 1.43E-01, $NiCl_2$ 3.83E-02, EMS + $NiCl_2$ 1.93E-01, and Control (PBS) 2.28E-02.

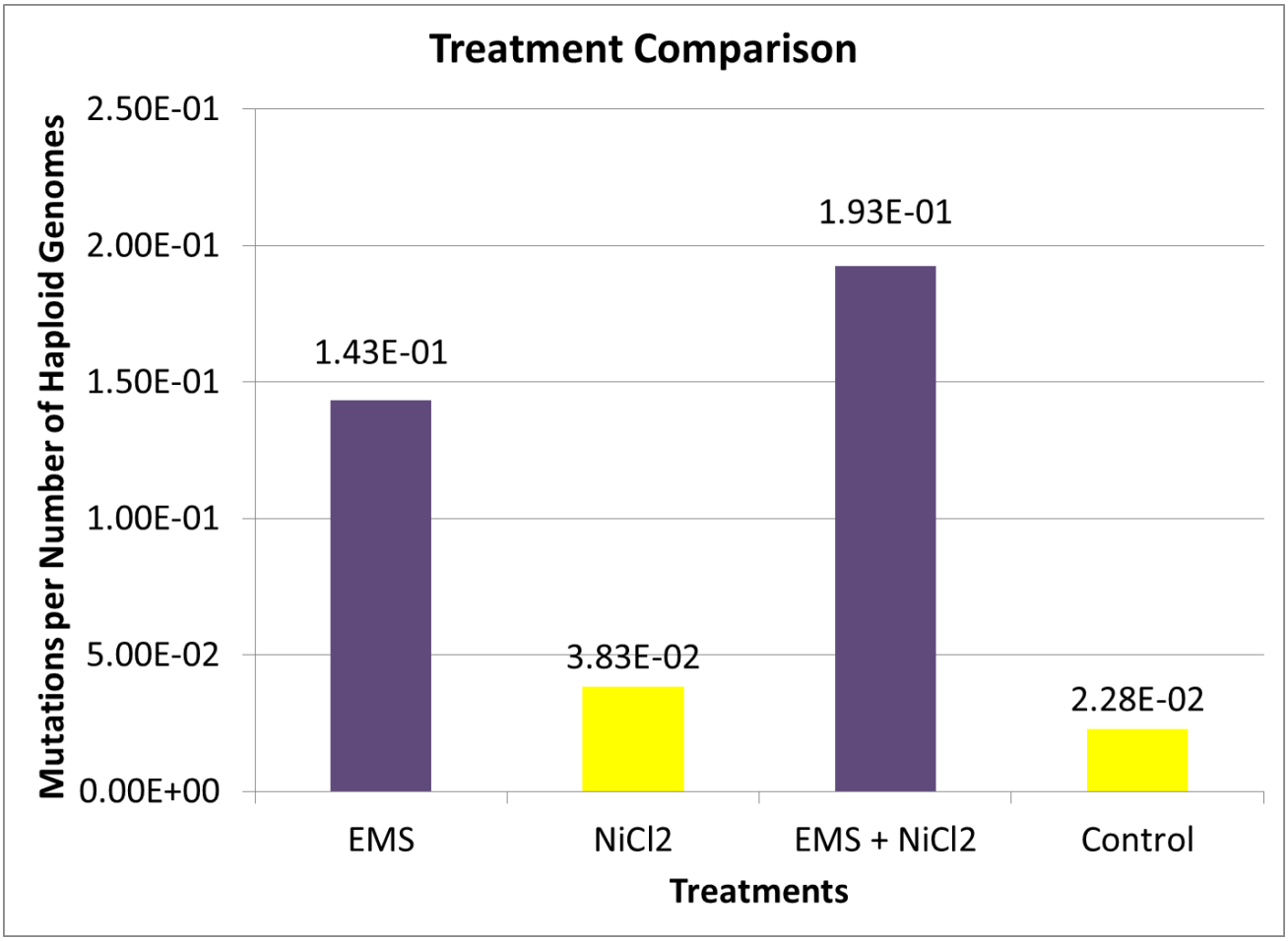

Figure 5. The combined data for the KX84 treatments. N is the number of haploid genes and M is the number of mutations. The mutations per number of haploid genes for each treatment are as follows: EMS 1.43E-01, $NiCl_2$ 3.83E-02, EMS + $NiCl_2$ 1.93E-01, and Control (PBS) 2.28E-02. After comparing all the treatments, the EMS and EMS + $NiCl_2$ were not significantly different and neither were $NiCl_2$ and Control (PBS) represented by the colors.

Comparison	**T-test P-Value (Two-tailed, Two-sample unequal variance)**
EMS and $NiCl_2$	0.04792689
EMS and EMS+$NiCl_2$	0.5318435
EMS and Control	0.01827299
$NiCl_2$ and EMS+$NiCl_2$	0.02087761
$NiCl_2$ and Control	0.46661486
EMS+$NiCl_2$ and Control	0.00941419

Table 4. The KX84 t-test comparisons. The red values show the treatment comparisons are still less than 0.05 and insignificant.

CONCLUSIONS

Nickel is an important metal that is present in nature. It comes from volcanoes and erosion naturally, but most of it comes from anthropogenic sources, such as welding and metal alloy production. It is a known to cause allergic reactions and cancer. This experiment helped shed light on nickel toxicity, its affects, and the mechanism that it uses. The N2 results were helped because we learned that the mutagens can be harsh and cause apoptosis. We also learned that nickel can cause mutations alone but less than EMS. The KX84 results improved our knowledge on the mechanism. From the graph in Figure 5, it almost looks like the EMS + $NiCl_2$ treatment is the addition of the EMS and $NiCl_2$ treatments. This is still not definitive because the EMS + $NiCl_2$ and EMS treatments and $NiCl_2$ and Control PBS comparisons were not significantly different. The data does look additive which suggests that nickel is a direct mutagen and attacks DNA itself. More runs or a different experiment needs to be done to obtain a definite answer to whether nickel is a direct or indirect mutagen. If it is direct we can see how it attacks DNA and if it is an enabler we can find how it is enabling other mutagens to harm the DNA. When we fully discover this we can start making actions to trying to decrease the amount of nickel we are exposed to.

REFERENCES

Atkinson, J. (2016). *Nickel as a mutagen and as an enabler of EMS genotoxicity in Caenorhabditis elegans.* Greenville: East Carolina University.

Brenner, S. (1974). The genetics of *Caenorhabditis elegans*. *Genetics*, 71-94.

Dubins, J. S. (1986). Nickel(II) genotoxicity: potentiation of mutagenesis of simple alkylating agents. *Mutation Research*, 187-199.

Gartner, A. B. (2008). *Germline survival and apoptosis.* Retrieved from WormBook: doi/10.1895/wormbook.1.145.1

Huffnagle, I. M., Joyner, A., Rumble, B., Hysa, S., Rudel, D., & Hvastkovs, E. G. (2013). *Dual Electrochemical and Physiological Apoptosis Assay Detection of in Vivo Generated Nickel Chloride Induced DNA Damage in Caenorhabditis elegans.*

Kasprzak, K. S., Sunderman Jr., F. W., & Salnikow, K. (2003). *Nickel carinogenesis.*

Ke, Q. D. (2006). Alterations of histone modifications and transgene silencing by nickel chloride. *Carcinogenesis*, 1481-1488.

Korthals, G. W. (1996). Short-term effects of cadmium, copper, nickel and zinc on soil nematodes from different feeding and life-history strategy groups. *Applied Soil Ecology*, 107-117.

Lu, H., Xianglin, S., Costa, M., & Huang, C. (2005). *Carcinogenic effect of nickel compounds.*

Miki, H. K. (1987). Inhibition of intercellular communication by nickel(II): antagonistic effect of magnesium. *Carcinogenesis*, 1757-1760.

Rudel, D., Douglas, C. D., Huffnagle, I. M., Besser, J. M., & Ingersoll, C. G. (2013). *Assaying Environmental Nickel Toxicity Using Model Nematodes.*